A Catalytic Mechanism for the Enzyme Benzylamine Oxidase from Pig Plasma

By C. E. TAYLOR, R. S. TAYLOR, C. RASMUSSEN and P. F. KNOWLES
Astbury Department of Biophysics, University of Leeds, Leeds LS2 9JT, U.K.

(Received 3 May 1972)

Initial-velocity and product-inhibition studies on the enzyme benzylamine oxidase from pig plasma indicate that the order of substrate addition and product release is benzylamine on, ammonia off, oxygen on, hydrogen peroxide off, benzaldehyde off. Ammonia, but not benzaldehyde, is released under strictly anaerobic conditions which provides independent evidence for this order. Benzyl alcohol is a substrate for the enzyme. A chemical mechanism consistent with all the data is proposed.

A group of amine oxidases distinct from the flavin-containing mitochondrial amino acid oxidases (Meister & Wellner, 1963) has been recognized during the past 20 years. Examples of this group have been found in mammals (Tabor *et al.*, 1954; Blaschko *et al.*, 1959; Yamada & Yasunobu, 1962; Mondovi *et al.*, 1964), plants (Mann, 1955, 1961) and micro-organisms (Yamada *et al.*, 1969). Although the substrate specificity of these enzymes differs, there is considerable evidence to suggest that they contain cupric copper (Buffoni & Blaschko, 1964; Yamada *et al.*, 1963; Mondovi *et al.*, 1967; Buffoni *et al.*, 1968) and either pyridoxal phosphate (Yamada & Yasunobu, 1963; Blaschko & Buffoni, 1966) or some carbonyl compound (Hill, 1967). One of the better-characterized enzymes in this group is the benzylamine oxidase (EC 1.4.3.4) from pig plasma (Buffoni & Blaschko, 1964), which has been crystallized in homogeneous form and shown to contain 3–4 mol of pyridoxal phosphate (Blaschko & Buffoni, 1965) and approximately three atoms of cupric copper (Buffoni & Blaschko, 1964; Buffoni *et al.*, 1968)/mol of enzyme. Little is known about the catalytic mechanism for this enzyme. The present paper is mainly concerned with the application of steady-state-kinetic methods to study the order of substrate addition to and product leaving from the enzyme.

Experimental

Materials

Sephadex G-25 and DEAE-Sephadex A-50 were obtained from Pharmacia, Uppsala, Sweden; DEAE-cellulose DE-32 (microgranular), Aristar-grade $(\text{NH}_4)_2\text{SO}_4$ (used in the enzyme purification beyond stage III) and general chemicals (all AnalaR grade) were from British Drug Houses Ltd., Poole, Dorset, U.K. Columns (1.0- and 1.5-litre fixed vol.) were purchased from Wright Scientific, London, N.W.6,

U.K. Glass chromatography columns (5 litre; 100cm long $\times$ 8.5cm diam.; fitted with a porosity 2 sinter) and a silica spectrophotometer cell (capacity 4ml; 1 cm light-path) fitted with a dual-bulb (each 4ml capacity) Thunberg head and with an Araldite-sealed brass hollow screw in the side arm, were made by Northern Scientific, York, Yorks., U.K. Argon (99.9% pure), pure O_2 , argon containing 1% H_2 and an argon- O_2 gas mixer (model 100) were from Air Products Ltd., Rotherham, Yorks., U.K. Hydroxyapatite was prepared according to Tiselius *et al.* (1956) and stored at room temperature in 1 mM-potassium phosphate buffer, pH 6.8.

Benzylamine, obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K., was converted into the hydrochloride and recrystallized three times from ethanol-water before use. Benzyl alcohol was purified from benzaldehyde by treatment with lithium aluminium hydride followed by distillation under N_2 at reduced pressure. Comparison of E_{285} with a sample taken from a freshly opened bottle of AnalaR benzaldehyde showed that the benzyl alcohol contained less than 0.006% of benzaldehyde. H_2O_2 was standardized by permanganate titration (Kolthoff & Sandell, 1952). Enzyme and substrates for the NH_3 assay were bought from C. F. Boehringer und Soehne, Mannheim, Germany.

Enzyme isolation

The procedure used was a modification of that published by Buffoni & Blaschko (1964). Only those steps which have been changed will be described fully.

Step I. Separation of pig plasma. As a routine 15 litres of plasma were obtained from a batch of 22 litres of fresh pig's blood. Subsequent steps were carried out at 4°C.

Step II. $(\text{NH}_4)_2\text{SO}_4$ precipitation. The $(\text{NH}_4)_2\text{SO}_4$ fraction precipitating between 35 and 55% saturation was dissolved in 0.01 M-potassium phosphate

buffer, pH7.0, and desalted on a Sephadex G-25 column (5 litres bed vol.), which had been previously equilibrated with the 0.01M-phosphate buffer, pH7.0.

Step III. DEAE-cellulose chromatography. By increasing the bed volume from 0.7 to 4.0 litres a whole batch (see step I) could be chromatographed in one operation.

Step IV. The desalted material from step III [desalted on a Sephadex G-25 column (1.5 litre bed vol.) instead of by dialysis] was adsorbed on a column (1.5 litres bed vol.) of DEAE-Sephadex equilibrated with 0.005M-potassium phosphate buffer, pH7.0. A linear elution gradient (4 litres of 0.2M-NaCl in the 0.005M-phosphate buffer, pH7.0, in the reservoir and 4 litres of the 0.005M-phosphate buffer, pH7.0, in the mixing chamber) was applied to the column. Fractions were analysed for enzymic activity and for ceruloplasmin, the latter by measuring the E_{605} (Curzon, 1966). Although the ceruloplasmin peaked before the amine oxidase, a complete separation of the two proteins could not be achieved.

Step V. Hydroxyapatite fractionation. Column chromatography of the enzyme on hydroxyapatite as described by Buffoni & Blaschko (1964) was unsatisfactory since, because of slow column flow rates, elution of the enzyme required 10 days. A batchwise hydroxyapatite procedure gave good increases in specific activity and completely separated the enzyme from ceruloplasmin. The desalted material from step IV was treated with 210ml of hydroxyapatite, 550ml of 0.006M-potassium phosphate buffer, pH6.8, added and the whole stirred for 40 min. The supernatant after centrifugation (200g for 5 min) was decanted and the hydroxyapatite extracted a further three times as above with 350ml portions of the same buffer. All the supernatants were combined, clarified by centrifugation (15 min at 22000g) and brought to 55% saturation with solid $(\text{NH}_4)_2\text{SO}_4$, the pH being adjusted to 7.0 with aq. NH_3 . The precipitate was left overnight, centrifuged and dissolved in 0.05M-potassium phosphate buffer, pH7.0. The hydroxyapatite was further extracted with

4×350ml of 0.06M-potassium phosphate buffer, pH6.8, by the same procedure described above, the supernatants were combined and brought to 55% saturation with $(\text{NH}_4)_2\text{SO}_4$. This solution, containing 14.6 units of enzyme with specific activity 0.068 unit/mg of protein, was added to the next batch of enzyme after step IV. Some ceruloplasmin still remained adsorbed to the hydroxyapatite after the 0.06M-phosphate buffer, pH6.8, elution but could readily be eluted with 0.2M-potassium phosphate buffer, pH6.8.

Step VI. Crystallization. The procedure described by Buffoni & Blaschko (1964) was followed.

The whole purification procedure is summarized in Table 1.

Methods

Protein concentrations. The 260/280nm method of Warburg & Christian (1941) was used for all kinetic experiments. Gravimetric measurement of protein showed that the true protein concentration is $0.75 \times$ the 260/280nm method value. The corrected protein concentration was used in the tests for benzaldehyde and NH_3 under anaerobic conditions.

Measurement of enzymic activity. A Unicam SP.700 (Pye Unicam, Cambridge, Cambs., U.K.) with absorbance-scale expansion and with a cell holder thermostatically controlled to $\pm 0.1^\circ\text{C}$ by a Haake Cryostat KT62 (Haake, Berlin, Germany) was used to monitor benzaldehyde production at 250nm (Tabor *et al.*, 1954). One unit of benzylamine oxidase activity is defined as the amount of enzyme which catalyses the transformation of $1 \mu\text{mol}$ of substrate/min at 25.0°C under the standard assay conditions of Tabor *et al.* (1954).

The Unicam SP.700 was also used to study difference spectra on the enzyme between 220 and 300nm and for the studies on anaerobic production of benzaldehyde and NH_3 . For the NH_3 assay, an SP.750 scale-expansion unit was attached since the ΔE was small. O_2 concentrations were measured using a Clark-type electrode (Rank Brothers, Botti-

Table 1. *Purification procedure for pig plasma benzylamine oxidase*

For details see the text.

Step	Fraction	Volume (ml)	Protein (mg)	$10^3 \times$ Specific activity (units/mg of protein)	Total activity (units)	Purification	Yield (%)
I	Plasma	15000	1125000	0.225	254	1	100
II	35–55%-saturation $(\text{NH}_4)_2\text{SO}_4$	2770	174500	0.720	125	3.2	49
III	DEAE-cellulose	440	14500	4.15	60	18.5	23.5
IV	DEAE-Sephadex	227	2880	20.3	58.5	90.0	22.5
V	Hydroxyapatite	19.2	350	110	38.5	490	15.2
VI	Crystallization	13.0	250	134	33.5	600	13.2

sham, Cambs., U.K.) incorporated into the circuit described by Estabrook (1967). The circuit was modified (Taylor, 1971) to give the $10\times$ scale expansion necessary for K_m determinations on benzyl alcohol.

General kinetic properties

I. Michaelis constants for benzylamine and O_2 .
(a) Benzylamine. The benzylamine concentration was varied over the range 0.0466–3.33 mM in air-saturated solution (0.185 mM- O_2) and O_2 -saturated solution (1.1 mM- O_2). To ensure that this latter concentration of O_2 did saturate the enzyme, the rates of reaction of an assay mixture equilibrated at atmospheric pressure for 70 s with a 50% argon- O_2 mixture and with pure O_2 were compared. The rates were identical.

(b) O_2 . The O_2 concentration was varied over the range 0.050–1.1 mM at a fixed benzylamine concentration of 3.33 mM. The experimental procedure for measuring dissolved O_2 concentration is given in the section describing initial-velocity measurements on the enzyme.

II. Dependence of rate on ionic strength. Kinetic runs were carried out by using standard assay mixtures at pH 7.4 and 37°C. The ionic strength was varied by using sodium phosphate buffers with molarity from 0.1 M to 1.0 M.

III. Effect of pH on the apparent maximum velocity and apparent Michaelis constant for benzylamine. The benzylamine concentration was varied over the range 0.112–3.33 mM at each pH (5.8, 6.6, 7.4, 8.2 and 9.0) in 0.05 M-sodium phosphate buffer at 37°C.

Initial-velocity studies

These experiments were carried out at various fixed O_2 concentrations with changing benzylamine concentrations and at various fixed benzylamine concentrations with changing O_2 concentrations, the temperature being 37°C. The assay mixture (1.70 ml of 0.1 M-sodium phosphate buffer, pH 7.4, benzylamine and water to a final volume of 2.90 ml) was contained in a spectrophotometer cell fitted with a serum cap (Subaseal Ltd., Barnsley, Yorks., U.K.). The gas was introduced either directly from the O_2 cylinder or from the argon- O_2 gas mixer through a stainless steel serum needle (Thackray Ltd., Leeds, Yorks., U.K.) for 60 s, then the enzymic reaction was initiated by the addition of enzyme (0.10 ml) from a syringe (Hamilton 705). Passage of gas through the solution was continued for a further 10 s to ensure complete mixing of enzyme and substrate, then the needles were removed and the holes in the serum cap filled with Evostik (Evode Ltd., Stafford, Staffs., U.K.). It was known from a control experiment that the assay mixture became saturated with pure O_2 in

Fig. 1. Experiment to determine the time taken to saturate a solution (vol. 3 ml) with pure O_2 at atmospheric pressure and at 37°C

For details see the text.

Fig. 2. Determination of dissolved O_2 concentration after bubbling argon- O_2 mixtures of various compositions through a solution (vol. 3 ml) at 37°C

For details see the text.

60 s (Fig. 1). The absolute O_2 concentrations corresponding to the different argon- O_2 mixtures and of air were determined polarographically. The gas was

bubbled through an assay mixture containing no enzyme at 37°C for 70s (Fig. 2). Since the O₂ content of air-saturated water at 37°C is known [*Handbook of Chemistry and Physics*, 33rd edn., 1951–1952, p. 1481; Chemical Rubber Publishing Co., Cleveland, Ohio] the O₂ concentration can be expressed in mol/litre.

Product inhibition studies

The general procedure was similar to that described for the initial-velocity studies; each product in turn was added to the assay mixture under the experimental conditions summarized in the legends to Figs. 8–19. For the experiments where saturating conditions of the fixed substrate were used, the concentrations were 3.3 mM and 1.1 mM for benzylamine and O₂ respectively. These concentrations are approximately an order of magnitude greater than the K_m values. The concentration of product inhibitor used under saturating conditions of fixed substrate was equal to or less than 10× that used under non-saturating conditions. Practical difficulties were encountered with NH₃ and with benzaldehyde. To observe inhibition with NH₃, it was necessary to compare the rate of benzaldehyde production in 1 M-ammonium phosphate buffer, pH 7.4, with the rate in 1 M-sodium phosphate buffer, pH 7.4. The conductivities of these two buffers were identical, which eliminated the possibility that the effect was due to a difference in ionic strength. For the inhibition studies with benzaldehyde, a saturated aqueous solution of benzaldehyde was prepared (a freshly opened bottle of this reagent was used since benzaldehyde is readily converted into benzoic acid on storage). The concentrations of benzaldehyde used in the experiments were calculated based on a ϵ at 250 nm of 12000 litre·mol⁻¹·cm⁻¹ (Dearden & Forbes, 1958). Under conditions of saturating benzylamine concentration (O₂ concentration changing) and saturating O₂ concentration (benzylamine concentration changing) the concentrations of benzaldehyde needed to give inhibition were too great to be measured by using the

standard assay procedure in 1 cm-light-path spectrophotometer cells because of the high E_{250} of benzaldehyde. For these experiments, therefore, the assay mixture was scaled down to give a final volume of 0.6 ml and the reaction was carried out in 0.2 cm-light-path spectrophotometer cells. The amount of O₂ dissolved in 0.6 ml of assay mixture at 37°C when O₂-argon mixtures of various compositions were bubbled for 30s through the solution was determined polarographically as described more fully for the initial-velocity studies; the results of this calibration are summarized in Table 2.

Analysis of kinetic data

Double-reciprocal plots of experimental results were obtained by using the weighted linear-regression computer procedure described by Wilkinson (1961). Secondary plots of slope against inhibitor concentration and intercept against inhibitor concentration were made and the K_i slopes and K_i intercepts determined.

Anaerobic product analysis

For clarity the full method is included with the results of these experiments.

Table 2. O₂ concentration in 0.6 ml of assay mixture after bubbling of various O₂-argon mixtures for 30s through the solution

For details see the text.

% of O ₂ in O ₂ -argon mixture	[Dissolved O ₂] (mM)
100	1.1
16.7	0.4
9.1	0.24
4.7	0.2

Fig. 3. Effect of buffer strength on pig plasma benzylamine oxidase activity

For details see the text.

Results

General kinetic properties

I. Michaelis constants for benzylamine and O₂. The apparent Michaelis constants at 37°C for benzylamine in O₂-saturated solutions is 0.48 ± 0.035 mM. The Michaelis constant at 37°C for O₂ is 0.12 ± 0.022 mM.

II. Dependence of rate on ionic strength. The rate decreased with increasing ionic strength as shown in Fig. 3.

III. Effect of pH on the maximum velocity and apparent Michaelis constant for benzylamine. Double-reciprocal plots of velocity against benzylamine concentration in air-saturated solution were made and the maximum velocity and Michaelis constants were determined at each pH value. The results are plotted in Figs. 4 and 5.

Initial-velocity studies

The effect of changing benzylamine concentration at different fixed concentrations of O₂ is shown in Fig. 6 and of changing O₂ concentration at different fixed concentrations of benzylamine in Fig. 7. The apparent Michaelis constant/apparent maximum velocity ratios are shown in Table 3.

Fig. 4. Dependence of apparent K_m value on pH

Sodium phosphate buffer (0.05 M) was used at 37°C. For further details see the text.

Product-inhibition studies

All the results were recorded as double-reciprocal plots (Figs. 8–19). The values of K_i intercept and K_i slope are shown in Table 4.

Confirmatory tests

(i) Production of benzaldehyde from benzylamine under anaerobic conditions. Argon containing 1% H₂ was catalytically deoxygenated by the method described by Swann & Bray (1972). All metal-glass joints in the apparatus were sealed with Araldite. Analysis of the gas by the method of Sweetser (1967) indicated residual O₂ of 9.0 p.p.m. which corresponds to an O₂ concentration in solution at 25°C of $0.011 \mu\text{M}$. This is three orders of magnitude less than the enzyme concentration used. The deoxygenated argon supply was coupled via a three-way tap to the side-arm of an anaerobic spectrophotometer cell fitted with a dual-bulb Thunberg head. The screwed connexion to the side-arm was made gas-tight with a rubber 'O' ring. Bulb (a) [containing 0.3 ml of benzylamine oxidase (13.1 mg/ml)] and bulb (b) [containing 2.65 ml of buffered assay solution plus $50 \mu\text{l}$ of benzylamine hydrochloride solution (0.2 M)] were frozen and made anaerobic by ten cycles of evacuation, thawing, introduction of O₂-depleted argon and refreezing. It was known from a separate experiment that benzylamine oxidase did not lose any activity during ten successive freezing and thawing cycles.

Bulb (b) was thawed [keeping bulb (a) frozen] and the contents decanted carefully into the spectrophotometer cell. The measured E_{250} was 0.907. Bulb (a) was then thawed, the whole cell contents thoroughly mixed and left at 25°C for 10 min. The E_{250} after mixing was 1.79. Since the E_{250} due to the enzyme had been determined to be 0.956, the calculated E_{250} after mixing, assuming no benzaldehyde liberation, is $0.956 + (0.907 \times 2.7/3.0) = 1.771$. Thus the ΔE_{250}

Fig. 5. Dependence of V_{max} on pH

Sodium phosphate buffer (0.05 M) was used at 37°C. For further details see the text.

Table 3. K_m/V_{max} ratios for different fixed substrate concentrations

For details see the text.

Fixed concentrations of O_2

$[O_2]$ (mm)	Apparent K_m (mm)	Apparent V_{max} ($\Delta E/min$)	K_m/V_{max} ratio
1.100	0.512	0.059	0.0087
0.180	0.266	0.029	0.0093
0.097	0.176	0.019	0.0091

Fixed concentrations of benzylamine

[Benzylamine] (mm)	Apparent K_m (mm)	Apparent V_{max} ($\Delta E/min$)	K_m/V_{max} ratio
3.300	0.192	0.059	0.0033
0.170	0.0476	0.013	0.0036
0.110	0.0383	0.011	0.0036
0.083	0.0326	0.0081	0.0040
0.067	0.0286	0.0072	0.0040

Table 4. Values of K_i intercept and K_i slope from inhibition studies

For details see the text.

Inhibitor	Changing substrate	Fixed substrate	K_i intercept (mm)	K_i slope (mm)
NH ₃	Benzylamine	O ₂ non-saturating	2300	1100
NH ₃	Benzylamine	O ₂ saturating	*	*
NH ₃	O ₂	Benzylamine non-saturating	—	650
NH ₃	O ₂	Benzylamine saturating	*	*
H ₂ O ₂	Benzylamine	O ₂ non-saturating	0.77	—
H ₂ O ₂	Benzylamine	O ₂ saturating	0.18	—
H ₂ O ₂	O ₂	Benzylamine non-saturating	2.6	2.1
H ₂ O ₂	O ₂	Benzylamine saturating	24	24
Benzaldehyde	Benzylamine	O ₂ non-saturating	—	0.14
Benzaldehyde	Benzylamine	O ₂ saturating	—	0.7
Benzaldehyde	O ₂	Benzylamine non-saturating	0.12	—
Benzaldehyde	O ₂	Benzylamine saturating	—	—

* Curved reciprocal plots.

after mixing is 0.019. This corresponds to $0.019/12000 = 1.58 (\pm 0.16) \mu M$ -benzaldehyde. The concentration of enzyme is $6.7 \mu M$, assuming a molecular weight of 195000 (Buffoni & Blaschko, 1964). Thus the number of mol of benzaldehyde produced/mol of enzyme is 0.24 (assuming one active site/enzyme molecule) and 0.12 (assuming two active sites/enzyme molecule). Given that the small ΔE_{250} recorded could result from either slight leakage of O_2 into the system causing partial enzyme turnover or slight concentration of the solutions by the evacuation procedure, it seems reasonable to conclude that no benzaldehyde is produced anaerobically.

(ii) Production of NH_3 from benzylamine under anaerobic conditions. The method used was based on

that of Levitzki (1970) in which NH_3 liberated by the amine oxidase reaction is linked to NADH oxidation through the glutamate dehydrogenase system. Conditions are chosen such that the initial velocity is proportional to the NH_4^+ concentration. Even after extensive dialysis, it was found that benzylamine oxidase contained endogenous NH_4^+ [from the $(NH_4)_2SO_4$ used for storage]. The procedure now given measures the increase in NH_4^+ production after substrate addition. The same spectrophotometer cell as in the test for anaerobic production of benzaldehyde was used.

In both experiments (1) and (2) bulb (a) contained 0.20 ml of NADH (2.4 mm in 1% $NaHCO_3$), 0.15 ml of α -oxoglutarate (1.0 M, pH 7.6), 0.90 ml of potassium

Fig. 6. Initial-velocity study at different fixed O_2 concentrations with benzylamine concentration changing

For details see the text. O_2 concentrations: $\square$, 0.097 mm; ∇ , 0.18 mm; $\circ$, 1.1 mm.

Fig. 7. Initial-velocity study at different fixed benzylamine concentrations with oxygen concentration changing

For details see the text. Benzylamine concentrations: $\triangle$, 200 mm; $\square$, 20 mm; $\blacksquare$, 10 mm; ∇ , 6 mm; $\triangledown$, 5 mm; $\circ$, 4 mm; $\bullet$, 3.3 mm.

phosphate buffer (0.4M, pH7.6) and 0.60ml of glutamate dehydrogenase (0.5mg/ml, pH7.6). In experiment (1) 0.35 ml and in experiment (2) 0.70 ml of benzylamine oxidase (13.1mg/ml) was used and water was added to give a final volume of 2.95 ml in each case. Bulb (b) contained 0.05ml of benzylamine (0.2M).

The solutions were made anaerobic by ten cycles of freezing, evacuation, thawing and introduction of O_2 -depleted argon and then the contents of bulb (a)

Fig. 8. Product-inhibition study with benzylamine concentration changing, O_2 concentration non-saturating and NH_3 inhibiting

For details see the text. Buffers were: $\circ$, 1.0M-ammonium phosphate, pH7.4; $\bullet$, 1.0M-sodium phosphate, pH7.4.

Fig. 9. Product-inhibition study with benzylamine concentration changing, O_2 concentration saturating and NH_3 inhibiting

For details see the text. Buffers were: $\bullet$, 1.0M-ammonium phosphate, pH7.4; $\circ$, 1.0M-sodium phosphate, pH7.4.

Fig. 10. Product-inhibition study with O_2 concentration changing, benzylamine concentration non-saturating and NH_3 inhibiting

For details see the text. Buffers were: ●, 1.0M-ammonium phosphate, pH7.4; ○, 1.0M-sodium phosphate, pH7.4.

Fig. 11. Product-inhibition study with O_2 concentration changing, benzylamine concentration saturating and NH_3 inhibiting

For details see the text. Buffers were: ●, 1.0M-ammonium phosphate, pH7.4; ○, 1.0M-sodium phosphate, pH7.4.

Fig. 12. Product-inhibition study with benzylamine concentration changing, O_2 concentration non-saturating and H_2O_2 inhibiting

For details see the text. H_2O_2 concentrations: □, 1.38mM; Δ, 0.55mM; ○, zero (no inhibitor).

were decanted carefully into the spectrophotometer cell [keeping bulb (b) frozen] and the cell allowed to equilibrate to $25^\circ C$ (5 min) before measuring the rate of NADH disappearance at 340nm. This rate was uniform over a period of 5 min. (It was known from control experiments that freezing and thawing did not denature either the glutamate dehydrogenase or benzylamine oxidase.) The benzylamine in bulb (b) was thawed and the whole cell contents thoroughly mixed (3 min) before again measuring the rate of NADH disappearance. As in the control this rate was uniform over 5 min. At the end of the experiment, the cell contents were opened to the air and mixed. An increased rate of NH_3 production corresponding to turnover of the benzylamine oxidase was observed.

In experiment (1) the $\Delta E_{340}/\text{min}$ before adding benzylamine was 0.002 and that after adding benzylamine was 0.006. Thus the $\Delta E_{340}/\text{min}$ due to NH_3 produced by the anaerobic reaction between benzylamine oxidase and benzylamine was 0.004.

From the standard curve (Fig. 20) $\Delta E_{340}/\text{min}$ of 0.004 corresponds to $0.011 \pm 0.001 \mu\text{mol}$ of NH_3/ml . The benzylamine oxidase concentration in the assay was $0.0079 \mu\text{mol}/\text{ml}$. The results obtained from experiment (2) showed that the NH_3 released is

Fig. 13. Product-inhibition study with benzylamine concentration changing, O_2 concentration saturating and H_2O_2 inhibiting

For details see the text. H_2O_2 concentrations: ▲, 1.38 mM; ■, 0.55 mM; ●, zero (no inhibitor).

Fig. 14. Product-inhibition study with O_2 concentration changing, benzylamine concentration non-saturating and H_2O_2 inhibiting

For details see the text. H_2O_2 concentrations: ●, 1.4 mM; ○, zero (no inhibitor).

Vol. 130

Fig. 15. Product-inhibition study with O_2 concentration changing, benzylamine concentration saturating and H_2O_2 inhibiting

For details see the text. H_2O_2 concentrations: □, 13.8 mM; ▲, 5.5 mM; ○, zero (no inhibitor).

Fig. 16. Product-inhibition study with benzylamine concentration changing, O_2 concentration non-saturating and benzaldehyde inhibiting

For details see the text. Benzaldehyde concentrations: ■, 0.14 mM; △, 0.072 mM; ●, zero (no inhibitor).

Fig. 17. Product-inhibition study with benzylamine concentration changing, O_2 concentration saturating and benzaldehyde inhibiting

For details see the text. Benzaldehyde concentrations: ○, 0.57 mM; ▲, 0.71 mM; ●, zero (no inhibitor).

dependent on the benzylamine oxidase concentration (Fig. 21). It is concluded that NH_3 in excess of 1 mol/mol of enzyme is released from benzylamine under anaerobic conditions.

(iii) *Benzyl alcohol as a substrate for benzylamine oxidase.* Owing to the high initial E_{250} of benzyl alcohol, this experiment was carried out by using an O_2 electrode. The K_m at $37^\circ C$ in air-saturated buffer was 10 mM (Fig. 22).

Discussion

General kinetic properties

Effect of ionic strength. It is noteworthy that whereas the initial velocity decreases with increasing ionic strength for pig plasma benzylamine oxidase, the initial velocity increases with increasing ionic strength up to a maximum of approx. 0.5 M for the beef plasma enzyme (Yamada & Yasunobu, 1962).

Effect of pH. The dependence of the K_m value for benzylamine on pH for the pig plasma enzyme is of a similar form to that of the rabbit plasma enzyme (McEwan, 1965). The variation of V_{max} with pH is somewhat different in that the V_{max} for the rabbit enzyme reaches a constant value at pH 8.0 (McEwan, 1965) whereas above pH 8.2 the V_{max} for the pig enzyme decreases. A similar analysis of kinetic data

Fig. 18. Product-inhibition study with O_2 concentration changing, benzylamine concentration non-saturating and benzaldehyde inhibiting

For details see the text. Benzaldehyde concentrations: ▲, 0.098 mM; Δ, 0.049 mM; ●, 0.019 mM; ○, zero (no inhibitor).

Fig. 19. Product-inhibition study with O_2 concentration changing, benzylamine concentration saturating and benzaldehyde inhibiting

For details see the text. Benzaldehyde concentrations: ○, 0.45 mM; ●, zero (no inhibitor).

Fig. 20. Standard curve for NH_3 concentration determination at 25°C

For details see the text.

Fig. 21. Dependence of NH_3 liberated from benzylamine by benzylamine oxidase on the enzyme concentration

For details see the text. The bars represent limits of errors.

Fig. 22. K_m value determination for benzyl alcohol in air at 37°C

For details see the text.

to that applied by McEwan (1965) for the rabbit enzyme was used in the present study. For the rabbit enzyme, it is assumed that the $[\text{H}^+]$ dependence of K_m is associated with the forward rate constant for enzyme-substrate complex formation and not from subsequent reactions of this complex. The reactants for the rabbit enzyme are assumed to be the protonated amine substrate and an unprotonated site on the enzyme. McEwan (1965) gives an equation:

$$K_m = \bar{K}_m [1 + \text{antilog}(\text{p}K_a^e - \text{pH}) + \text{antilog}(\text{pH} - \text{p}K_a^s)] \quad (1)$$

where $\bar{K}_m$ refers to the protonated amine and $\text{p}K_a^e$ and $\text{p}K_a^s$ are the $\text{p}K$ values for the enzyme and substrate respectively. The values of $\text{p}K_a^e$ and $\text{p}K_a^s$ may be obtained from a plot of K_m against pH . It can be shown that when the K_m value is equal to twice the value of the minimum K_m , then $\text{pH} = \text{p}K_a^s$.

From Fig. 4 values of $\text{p}K_a^e = 6.5$ and $\text{p}K_a^s = 9.0$ may be determined for the pig plasma enzyme. If these values are substituted into equation (1) a constant value of $\bar{K}_m$ with changing pH value results. Since the acid-dissociation constant for benzylamine is 9.37 (Carothers *et al.*, 1927), it may be that the higher pH -dissociation constant is associated with the enzyme rather than the substrate.

Some conclusions can be drawn from the variation of V_{max} with pH (Fig. 5). The complete rate equation consistent with the product inhibition data for the pig plasma enzyme yields

$$K_m = \frac{(k_2 + k_3)k_7 k_9}{k_1(k_3 k_7 + k_3 k_9 + k_7 k_9)}$$

$$V_{\text{max}} = \frac{k_3 k_7 k_9 E_0}{k_3 k_7 + k_3 k_9 + k_7 k_9}$$

Both K_m and V_{max} equations contain terms in k_3 , k_7 and k_9 ; the expression for V_{max} does not, however, contain k_1 , the only rate constant associated with a $[H^+]$ -dependent step in McEwan's (1965) treatment. For the pig plasma enzyme at least and probably for other serum amine oxidases we suggest that it is not valid to assign the $[H^+]$ dependence of the K_m value to a specific rate constant (k_1) and to assign the $[H^+]$ dependence of V_{max} to a completely different reaction step. Probably several of the rate constants in the expressions for K_m and V_{max} are associated with $[H^+]$ -dependent reactions.

Initial velocity studies

From Figs. 6 and 7 it can be seen that the double-reciprocal plots form a family of parallel lines. This suggests that the enzyme operates via a Ping Pong mechanism where release of the first product precedes addition of the second substrate and the enzyme exists in two or more stable forms (Cleland, 1967). Spectral evidence (Buffoni & Blaschko, 1964) indicates that there are indeed different enzyme forms in the presence and the absence of benzylamine and that the enzyme-benzylamine complex is stable. Addi-

tional support for the Ping Pong mechanism comes from the constant ratio of apparent K_m value to apparent V_{max} , as the fixed substrate concentration is changed (Cleland, 1963a). See Table 3.

Product inhibition studies

Each of the products of the reaction was examined as an inhibitor of the initial-velocity dependence on the concentration of each substrate. All possible mechanisms were tested for consistency with the types of inhibition obtained. The only Ping Pong mechanism consistent with the data was:

This is mechanism no. 18 of Cleland (1963a) in reverse, and may be called a Uni Uni Uni Bi Ping Pong mechanism. The complete steady-state rate equation is:

$$\begin{aligned}
 v = \frac{V_1 \left([A][B] - \frac{[P][Q][R]}{K_{eq}} \right)}{K_b [A] + K_a [B] + [AB] + \left(\frac{K_{ia} K_b}{K_{ip}} \text{ or } \frac{V_1 K_q K_{ir}}{V_2 K_{eq}} \right) [P] + \frac{K_b}{K_{ip}} [A][P]} \\
 + \frac{V_1 K_{ip} K_r [A][Q]}{V_2 K_{eq} K_{ia}} + \frac{K_a}{K_{ir}} [B][R] + \frac{V_1}{V_2 K_{eq}} (K_r [P][Q] + K_q [P][R] + K_p [Q][R] + [P][Q][R]) \\
 + \frac{V_1 K_{ip} K_r}{V_2 K_{eq} K_{ia} K_{ib}} [A][B][Q] + \frac{V_1 K_r}{V_2 K_{eq} K_{ia}} [A][P][Q] + \frac{V_1 K_p}{V_2 K_{eq} K_{ib}} [B][Q][R]
 \end{aligned}$$

expressed in the form of Cleland's (1963a) constants. Where

$$\begin{aligned}
 V_1 &= \frac{k_3 k_7 k_9 E_0}{k_3 k_7 + k_3 k_9 + k_7 k_9} \\
 V_2 &= \frac{k_2 k_6 E_0}{k_2 + k_6} \\
 K_a &= \frac{(k_2 + k_3) k_7 k_9}{k_1 (k_3 k_7 + k_3 k_9 + k_7 k_9)} \\
 K_b &= \frac{k_3 (k_6 + k_7) k_9}{k_5 (k_3 k_7 + k_3 k_9 + k_7 k_9)} \\
 K_p &= \frac{(k_2 + k_3) k_6}{(k_2 + k_6) k_4}, \quad K_q = \frac{k_2 (k_6 + k_7)}{(k_2 + k_6) k_8}, \quad K_r = \frac{k_2 k_6}{(k_2 + k_6) k_{10}} \\
 K_{ia} &= \frac{k_2}{k_1}, \quad K_{ib} = \frac{k_6}{k_5}, \quad K_{ip} = \frac{k_3}{k_4}, \quad K_{iq} = \frac{k_7}{k_8}, \quad \text{or} \quad \frac{(k_6 + k_7) k_9}{k_6 k_8} \\
 \text{or} \quad &\frac{k_3 k_7 + k_3 k_9 + k_7 k_9}{k_3 k_8}, \quad K_{ir} = \frac{k_9}{k_{10}} \quad \text{and} \quad K_{eq} = \frac{k_1 k_3 k_5 k_7 k_9}{k_2 k_4 k_6 k_8 k_{10}}
 \end{aligned}$$

From simplified forms of this rate equation under the various experimental conditions or from the application of Cleland's rules (Cleland, 1963*b*) the pattern of inhibition was predicted and this is compared with the experimentally determined pattern (Table 5). The agreement is complete for non-saturating conditions and most saturating conditions. The only discrepancies are those under saturating conditions with NH_3 as the product inhibitor when curved reciprocal plots were obtained. An explanation for these discrepancies might be that the assay medium necessary for the NH_3 inhibition studies was 1.0M-ammonium phosphate buffer compared with 0.1M for all other inhibition studies; in 0.1M-ammonium phosphate buffer the inhibition was so slight that the type could not be determined. In view of the observed dependence of the enzyme activity on the ionic strength of the medium (Fig. 3), a different form of the enzyme might be involved. Support for this suggestion comes from a difference spectrum of the enzyme in 0.1M-sodium phosphate buffer and in 1.0M-sodium phosphate buffer where clear indications of a conformational dependence on ionic strength was observed.

Calculation of kinetic constants

From replots of the slopes and intercepts of the product-inhibition results, various kinetic constants can be derived. These derivations are discussed by Cleland (1970), and details for pig plasma benzylamine oxidase are given by Taylor (1971). Table 6 lists the values of K_a , K_b , K_{ib} , K_{ip} and K_{ir} and the data from which these constants were obtained. A value for K_{iq} cannot be derived since it is not necessary to define K_{iq} to express the complete rate equation in terms of kinetic constants. The numerical values obtained by different methods for K_{ip} and K_{ir} are sufficiently close to provide supporting evidence for the mechanism proposed.

Comparison of data for the benzylamine oxidases from beef and pig plasma

Oi *et al.* (1970) carried out a steady-state kinetic analysis of beef plasma benzylamine oxidase. Both the pig and beef enzymes show a family of parallel lines when double-reciprocal plots are made from the initial-velocity results, thus indicating that the two enzymes act through a Ping Pong mechanism.

Table 5. Predicted and observed product-inhibition patterns

		For details see the text.			
Variable substrate	Product	Predicted inhibition Fixed substrate		Observed inhibition Fixed substrate	
		O_2 non-saturating	O_2 saturating	O_2 non-saturating	O_2 saturating
Benzylamine	NH_3	Non-competitive	None	Non-competitive	*
	H_2O_2	Uncompetitive	Uncompetitive	Uncompetitive	Uncompetitive
	Benzaldehyde	Competitive	Competitive	Competitive	Competitive
O_2		Benzylamine non-saturating	Benzylamine saturating	Benzylamine non-saturating	Benzylamine saturating
	NH_3	Competitive	Competitive	Competitive	*
	H_2O_2	Non-competitive	Non-competitive	Non-competitive	Non-competitive
	Benzaldehyde	Uncompetitive	None	Uncompetitive	None

* Curved reciprocal plots.

Table 6. Values for derived kinetic constants

For details see the text.		
Kinetic constants	Numerical value (M)	Results used for derivation
K_a	5.0×10^{-4}	Initial-velocity studies
K_b	1.8×10^{-4}	Initial-velocity studies
K_{ib}	1.3×10^{-4}	H_2O_2 against O_2 when benzylamine is non-saturating
K_{ip}	1.8	NH_3 against benzylamine when O_2 is non-saturating
	0.67	NH_3 against O_2 when benzylamine is non-saturating
K_{ir}	1.4×10^{-4}	Benzaldehyde against benzylamine when O_2 is non-saturating
	0.9×10^{-4}	Benzaldehyde against O_2 when benzylamine is non-saturating

For the beef enzyme, the product-inhibition results suggest that benzaldehyde is the first product to be released; this is supported by the finding that approximately 1 mol of benzaldehyde/mol of enzyme

is liberated anaerobically (Reed & Swindell, 1969; Oi *et al.*, 1970). The mechanism proposed by Oi *et al.* (1970) for the beef enzyme requires random order of H_2O_2 and NH_3 release following O_2 addition. To

Scheme 1. *Postulated chemical mechanism for pig plasma benzylamine oxidase*

Pyridoxal phosphate is abbreviated as PyrCHO.

explain the observed non-competitive inhibition of O_2 by benzaldehyde, a further isomerization of the enzyme has to be included in the mechanism.

The kinetic results presented in the present paper suggest that for the pig plasma enzyme, NH_3 is the first product to be released after benzylamine binding. O_2 is next bound and ordered release of H_2O_2 , then benzaldehyde follows. The detection of NH_3 , but not benzaldehyde, under highly anaerobic conditions supports this order. A tentative chemical mechanism consistent with the kinetic and anaerobic product evidence, together with the ability of benzyl alcohol to act as a substrate, can be proposed (Scheme 1). Features of this mechanism are as follows:

(i) Benzylamine is bound at a hydrophobic site.
 (ii) Attack by the lone-pair electrons of the amine on the carbonyl grouping of bound pyridoxal occurs giving the carbinolamine (II).
 (iii,a). The C-N bond of benzylamine is ruptured. An enzyme-bound nucleophile (B^-) is suggested to assist this novel reaction which results in a pyridoxine as one fragment and enzyme-bound benzyl alcohol as the other (compound IV).

(iii,b) Intermediate (II) could be converted into the Schiff base (III) by a reaction not on the catalytic pathway. The formation of a Schiff base between substrate and enzyme has been reported (Buffoni, 1966).

(iv,a) Intermediate (IV) under anaerobic conditions is converted into compound (VI) with release of NH_3 . The pyridoxal phosphate regenerated in complex (VI) could perhaps bind a further molecule of benzylamine, converting it into a carbinolamine. This would be incapable of further reaction owing to the proximity of the bound benzyl alcohol. The observed bleaching by benzylamine of pyridoxal in the enzyme (Buffoni & Blaschko, 1964) could thereby be rationalized.

(iv,b) Intermediate (IV) in the presence of O_2 loses a hydride ion to yield H_2O_2 . A nucleophilic grouping on the enzyme (X^-), for example persulphide (Edmons et al., 1972) and/or the cupric sites present in the enzyme, might participate in this step.

(v) Benzaldehyde is released from the enzyme.

The kinetic results for the beef and pig plasma enzymes would suggest different catalytic mechanisms. This is perhaps not surprising when one also considers the differences in substrate specificity (Yamada & Yasunobu, 1962; Buffoni, 1966), dependence on ionic strength (Yamada & Yasunobu, 1962) and on pH (Yasunobu & Yamada, 1962) for the two enzymes. However, the difference might be experimental. Oi et al. (1970), in their studies on the ox plasma enzyme, did not observe any changes in the product-inhibition pattern from non-saturating to saturating conditions. This could, in our opinion, be due to their failure to achieve completely saturating fixed substrate concentrations.

The detection of benzaldehyde under anaerobic conditions with the ox plasma enzyme (Reed & Swindell, 1969; Oi et al., 1970) is also subject to criticism. Neither group gave a value for the residual O_2 in the N_2 used. Moreover, the technique adopted by these authors of flowing O_2 -free N_2 over a solution of the enzyme is less likely to produce truly anaerobic conditions than the technique used in the present investigation. Finally the enzyme concentrations used in their tests were too low to yield unambiguous results.

The evidence accumulated for the overall mechanism of the pig plasma enzyme is essentially consistent. For the ox plasma enzyme, more careful kinetic studies and assays for anaerobic production of benzaldehyde and NH_3 are necessary. When these results are available, a conclusion may be drawn on whether the two enzymes function by different mechanisms.

We gratefully acknowledge discussions with Professor F. Buffoni, Professor W. W. Cleland and Dr. D. G. Herries. We thank Dr. L. Hart for computing the kinetic data and Mr. S. C. Charlton for developing part of the improved enzyme-purification procedure. This investigation was supported by Medical Research Council grants G 967/272/B and G 969/435/B.

References

- Blaschko, H. & Buffoni, F. (1965) *Proc. Roy. Soc. Ser. B* **163**, 45
- Blaschko, H., Friedman, P. J., Hawes, R. & Nilsson, K. (1959) *J. Physiol. (London)* **145**, 384
- Buffoni, F. (1966) *Proc. Int. Symp. Chem. Biol. Aspects Pyridoxal Catal.* 2nd. 363
- Buffoni, F. & Blaschko, H. (1964) *Proc. Roy. Soc. Ser. B* **161**, 153
- Buffoni, F., Della Corte, L. & Knowles, P. F. (1968) *Biochem. J.* **106**, 575
- Carothers, W. H., Bickford, C. F. & Hurwitz, G. J. (1927) *J. Amer. Chem. Soc.* **49**, 2908
- Cleland, W. W. (1963a) *Biochim. Biophys. Acta* **67**, 104
- Cleland, W. W. (1963b) *Biochim. Biophys. Acta* **67**, 188
- Cleland, W. W. (1967) *Annu. Rev. Biochem.* **36**, 77
- Cleland, W. W. (1970) *Enzymes*, 3rd. edn., **2**, 1
- Curzon, G. (1966) *Biochem. J.* **100**, 295
- Dearden, J. C. & Forbes, W. F. (1958) *Can. J. Chem.* **36**, 1362
- Edmons, D., Massey, V., Palmer, G., Beacham, L. M. & Elion, G. B. (1972) *J. Biol. Chem.* **247**, 1597
- Estabrook, R. W. (1967) *Methods Enzymol.* **10**, 41
- Hill, J. M. (1967) *Biochem. J.* **104**, 1048
- Kolthoff, I. M. & Sandell, E. B. (1952) *Textbook of Quantitative Inorganic Analysis*, 3rd. edn., p. 574, MacMillan Publishing Co., New York
- Levitzki, A. (1970) *Anal. Biochem.* **33**, 335
- Mann, P. J. G. (1955) *Biochem. J.* **59**, 609
- Mann, P. J. G. (1961) *Biochem. J.* **79**, 623
- McEwan, C. M. (1965) *J. Biol. Chem.* **240**, 2011
- Meister, A. & Wellner, D. (1963) *Enzymes*, 2nd. edn., **7**, 609

- Mondovi, B., Rotilio, G., Finazzi-Agro, A. & Scioscia-Santoro, A. (1964) *Biochem. J.* **91**, 408
- Mondovi, B., Rotilio, G., Costa, M. T., Finazzi-Agro, A., Chiancone, E., Hansen, R. E. & Beinert, H. (1967) *J. Biol. Chem.* **242**, 1160
- Oi, S., Inamasu, M. & Yasunobu, K. T. (1970) *Biochemistry* **9**, 3378
- Reed, D. J. & Swindell, R. (1969) *Fed. Proc. Fed. Amer. Soc. Exp. Biol.* **28**, 891
- Swann, J. C. & Bray, R. C. (1972) *Eur. J. Biochem.* **26**, 407
- Sweetser, P. B. (1967) *Anal. Chem.* **39**, 979
- Tabor, C. W., Tabor, H. & Rosenthal, S. M. (1954) *J. Biol. Chem.* **208**, 654
- Taylor, C. (1971) Ph.D. Thesis, University of Leeds
- Tiselius, A., Hjerten, S. & Levin, O. (1956) *Arch. Biochem. Biophys.* **65**, 132
- Warburg, O. & Christian, W. (1941) *Biochem. Z.* **310**, 384
- Wilkinson, G. N. (1961) *Biochem. J.* **80**, 324
- Yamada, H. & Yasunobu, K. T. (1962) *J. Biol. Chem.* **237**, 1511
- Yamada, H. & Yasunobu, K. T. (1963) *J. Biol. Chem.* **238**, 2669
- Yamada, H., Yasunobu, K. T., Yamano, T. & Mason, H. S. (1963) *Nature (London)* **198**, 1092
- Yamada, H., Adachi, O. & Yamano, T. (1969) *Biochim. Biophys. Acta* **194**, 751
- Yasunobu, K. T. & Yamada, H. (1962) *Proc. Symp. Chem. Biol. Aspects Pyridoxal Catal.* 453