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THE MECHANISM OF INHIBITION OF BENZYLAMINE OXIDASE BY 3,5-DIETHOXY-4-AMINOMETHYLPYRIDINE (B_{24})

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B_{24}, 3,5-diethoxy-4-aminomethylpyridine, is a specific inhibitor of the semicarbazide-sensitive amine oxidase with high affinity for benzylamine ($BnNH_2$ · SSAO). It is a site-directed inhibitor of pig plasma benzylamine oxidase (BAO) with an affinity for the enzyme much higher than that for benzylamine. B_{24} inhibition is dependent on the molar ratio B_{24}/BAO because the inhibitor reacts mole to mole with the enzyme and benzylamine appears to be ineffective in removing the inhibitor from the adduct [EI]. B_{24} is a weak substrate of BAO and for this reason the degree of inhibition (when the molar ratio B_{24}/BAO is lower than 1) decreases with the incubation time as well as with the preincubation time. This decrease is dependent on the gradual release of free enzyme which reacts with the substrate, giving [ES] without any interfering free B_{24}. When the B_{24}/BAO molar ratio is higher than 1, the free enzyme released by the oxidative deamination of B_{24} reacts with the substrate, but the free B_{24} present competitively inhibits the formation of [ES] and the affinity of benzylamine is therefore reduced.

This is the reason why B_{24}, in the kinetic experiments in which the inhibitor is not preincubated with the enzyme, may appear to be a competitive inhibitor or a mixed inhibitor, mainly competitive. When B_{24} is preincubated with the enzyme and the initial rate of benzylamine oxidation is measured, it appears as a non-competitive inhibitor becoming a mixed one only when the B_{24}/BAO molar ratio is high and the incubation time is long.

Keywords: Semicarbazide-sensitive amine oxidase (SSAO); Benzylamine oxidase; Enzymatic inhibitors; Benzylamine

Abbreviations: B_{24} = 3,5-diethoxy-4-aminomethylpyridine; $BnNH_2$ = benzylamine; PhCHO = benzaldehyde; BAO = benzylamine oxidase; GC–MS = gas chromatography–mass spectrometry; EI = electronic impact; CI = chemical ionization.

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INTRODUCTION

3,5-Diethoxy-4-aminomethylpyridine (B_{24}) is a specific inhibitor of benzylamine oxidase (BAO) and of tissue semicarbazide-sensitive amine oxidase with high affinity for benzylamine ($BnNH_2 \cdot SSAO$).[1-5]

It is active *in vitro* and *in vivo*:[4] in the rat the drug is well absorbed by the oral route with a bioavailability of 51.5%. It has a distribution volume of 2.5–2.9 l/kg and a blood half life of 2.0 h. In rat tissue SSAO enzymes are strongly inhibited at doses of 25–50–100 mg/kg i.p.[5] *In vitro* experiments have shown that the inhibition of the pure pig plasma BAO can be fully reversed by dialysis.[4] Studies on the inhibition kinetics have concluded that it is of mixed, mainly competitive type of inhibitor.

It has been previously shown that B_{24} forms an adduct with pig plasma benzylamine oxidase (BAO) which was isolated and identified[6] and which clearly showed that B_{24} is a site-directed inhibitor of pig plasma BAO, and a very weak substrate.[6]

These observations encouraged us to further study the mechanism of inhibition of pig plasma BAO by B_{24} and this paper presents the results obtained and a possible model of the type of BAO inhibition by B_{24}.

EXPERIMENTAL PROCEDURE

Materials

7-^{14}C-Benzylamine hydrochloride (54 mCi/mmol) was purchased from Amersham (Buckinghamshire, England), benzylamine hydrochloride from Sigma (St. Louis, MO, USA), 1-pentane-sulphonic acid sodium, salt HPLC grade from Eastman Kodak (Rochester, NY, USA), methanol Lichrosorb from Merck (Darmstadt, Germany) and catalase from Boehringer Mannheim (Milan, Italy). The HPLC column Nucleosil C18 300/8/4 was purchased from Macherey-Nagel (Dueren, Germany). All other reagents were of pure analytical grade.

Methods

Enzyme and Inhibitor Preparation

Pure pig plasma BAO was prepared according to Buffoni and Blaschko.[7] B_{24} was prepared according to Bertini *et al.*[2]

Gas Chromatography–Mass Spectrometry (GC–MS)

Mass spectrometry was carried out in a Varian Saturn 4 GC–MS system comprising a Star 3400 CX gas chromatograph coupled with a Saturn 4D mass spectrometer (ionic trap). A J&W DB5 column (5% phenyl, 95% methylpolysilyloxane, 30 m × 0.25 mm, 0.25 μm) was used. Helium was the carrier gas. Injector temperature was 250°C, transfer line 270°C. The temperature program was: 50°C × 2 min, 20°C/min up to 300°C. Injections were performed in splitter mode (2 min purge-off valve). The EI (electronic impact) scan was between *m/z* 40–450; the CI (chemical ionisation) scan was between *m/z* 60–450 with gas reagent isobutane using the Varian SECI wave form.

HPLC

HPLC was carried out in a LKB HPLC 2150 used with a C18 column. The mobile phase was methanol 65% (v/v) in 0.02 M Na–pentane–sulphonate brought to pH 3.0 with acetic acid. The flow rate was 1 ml/min and peaks were recorded at 290 nm.

Enzyme Assay

^{14}C-benzylamine was used as substrate and the assay was carried out at 37°C under the following conditions: 0.067 Na–phosphate buffer pH 7.4, 0.15 ml or 0.05 ml plus 0.1 ml of enzyme solution in 0.05 M Na–K–phosphate buffer pH 7.0, 0.05 ml of catalase (43 U/ml), 0.05 ml of buffer pH 7.4 or 0.05 ml of B_{24} at different concentrations. After 10 min preincubation, 0.05 ml of ^{14}C-benzylamine at different concentrations was separately added and the reaction was followed for 20 min or longer. The reaction was stopped with 0.1 ml of 3 N hydrochloric acid and the aldehyde formed was extracted with 1 ml of ethyl acetate. The ethyl acetate extract (0.5 ml) was counted in 10 ml of Instagel in a Packard liquid scintillation spectrometer.

Aldehyde of B_{24} Determination

BAO, 6.17 nmol, was incubated at 37°C for 30 min with 1.54–3.08–6.17–12.34 nmol B_{24} in 1.1 ml total volume of 0.05 M Na–K–phosphate buffer pH 7.0.

At the end of the incubation period the reaction was stopped with 0.2 ml of 0.7% (w/v) perchloric acid. Proteins were removed by centrifugation at 12 000*g* × 40 min. One ml of the supernatant was made alkaline with 0.05 ml of 13% (w/v) NaOH and extracted with 3 ml of ethyl acetate. The

extracts were dried under nitrogen and analysed by HPLC. The B_{24}-aldehyde appeared with a retention time of 4 min. Peaks eluted from the HPLC were analysed by GC–MS.

GC–MS was also carried out directly on the ethyl acetate extracts obtained under the following conditions: 29.5 nmol of BAO were incubated for 2 h at 37°C with 56 nmol of B_{24} in 2 ml of 0.067 M Na–phosphate buffer pH 7.4. At the end of the incubation period B_{24}-aldehyde was extracted with 10 ml of ethyl acetate.

Kinetic Constants

The kinetic constants for BAO in the absence and presence of B_{24} were obtained in duplicate using the following benzylamine concentrations in the reaction mixture as described in the enzyme assay: 333–166–83.3–41.6–16.6 μM. Kinetic constants were statistically computed according to Wilkinson.[8]

Mathematical Fitting

The mathematical fitting of the results was obtained with the software "Mathematica"[10] using the NDSolve for numerical solution of the system of differential equations.

RESULTS

Changes of BAO Absorbance by B_{24}

As already described[5] the addition of B_{24} to BAO produced a strong variation in the BAO absorbance at 530 nm (Figure 1). The maximum increase in the absorbance was obtained when the B_{24}/BAO molar ratio was 1 or higher than 1. The relationship between the B_{24} concentration and the increase of absorbance permitted calculation of the M constant of the B_{24}–BAO interaction which was 64 000 ± 1510 (mean ± s.e. of 5 determinations). This constant was called k_2 and was obtained by the linear plot of 1/[I] on 1/Δ which was linear with a correlation coefficient of 0.998 ± 0.00032 (mean ± s.e. of 5 experiments) where Δ was the increase in the absorbance at 530 nm, making 1 the maximum value.

$$[E + I] \xrightarrow{k_2} [EI]. \quad (1)$$

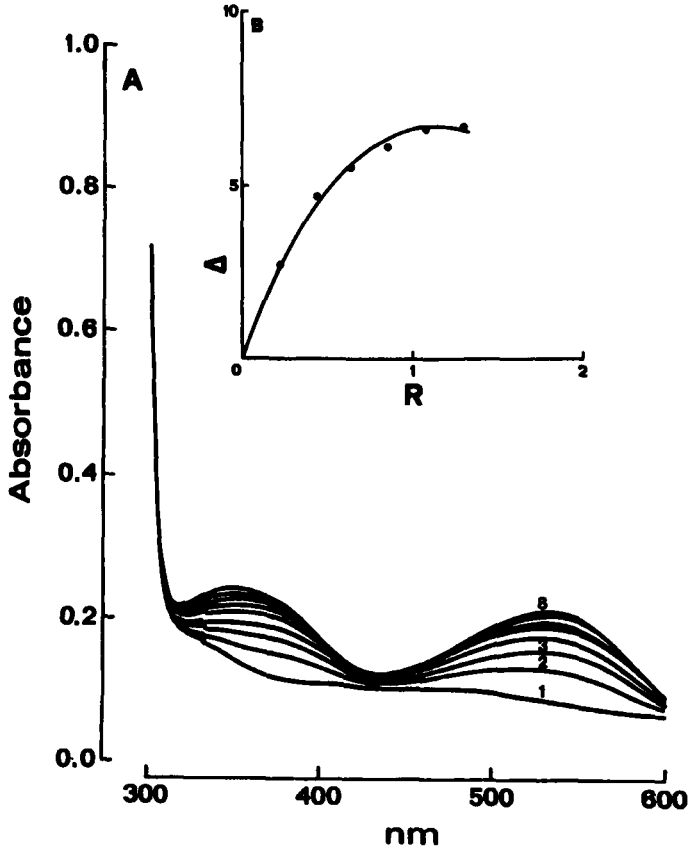


FIGURE 1 Variation in the visible spectrum of pig plasma benzylamine oxidase by the addition of different amounts of B_{24}. To 0.9 ml of 19.1 μM BAO in 0.05 M Na–K–phosphate buffer pH 7.0, 20 μl of the same buffer containing 3.72 nmol of B_{24} were added every 10 min at 37°C and the spectrum was recorded 5 min after every addition. (A) Spectrum of BAO and its progressive modification following B_{24} additions: (1) native enzyme; (2)–(8) progressive variation of the spectrum with the progressive addition of B_{24}. (B) Increase in the absorbance at 530 nm with the increase of the B_{24}/BAO molar ratio: Δ = increase of the absorbance, $R = B_{24}$/BAO molar ratio.

The equation does not consider k_{-2}, nevertheless the complex [EI] is slowly transformed into [EI*].[6] The decrease in the [EI] absorbance at 530 nm showed that the logarithm of the absorbance, in agreement with the following equation, was linear over time for 2 h (correlation coefficient 0.973), then the absorbance reached a constant value

$$[EI*] \rightarrow [EI]e^{-kt}. \qquad (2)$$

Production of B_{24}-Aldehyde

B_{24} is gradually deaminated by pig plasma BAO. The formed aldehyde was detected in HPLC as described in the methods and identified in GC–MS. Figure 2 shows the amount of aldehyde produced at different B_{24} concentrations and Figure 3 shows the GC–MS identification of this aldehyde.

From these results it was possible to calculate the constant for the rate of oxidation of B_{24} under the described conditions which was $0.00082 \pm 0.0001\ M^{-1}s^{-1}$ (mean ± s.e. of 3 determinations). This rate constant was called k_3.

Under the same conditions the rate of benzylamine oxidation calculated from the V_{max} reported in Table I was $0.204 \pm 0.007\ M^{-1}s^{-1}$ (mean ± s.e. of 5 determinations) and was called k_4. The same kinetic experiments allowed calculation of the molar constant of benzylamine–BAO interaction (k_1) 3182 ± 205 (mean ± s.e. of 5 experiments).

Oxidation of Benzylamine in the Presence of B_{24}

Previous experiments had shown that the degree of B_{24} inhibition decreased with the preincubation time only when the concentration of B_{24}

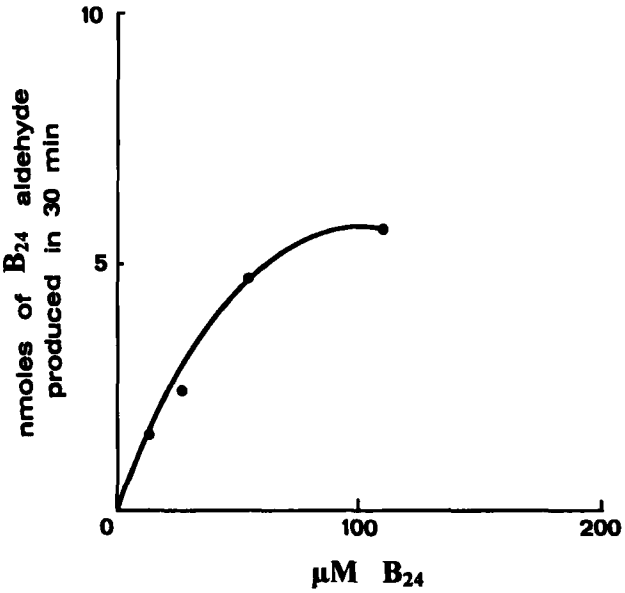


FIGURE 2 Aldehyde produced by the oxidative deamination of B_{24} catalysed by pig plasma benzylamine oxidase. 6.17 nmol of BAO were incubated for 30 min at 37°C with 1.54–3.08–6.17–12.34 nmol of B_{24} in a total volume of 1.1 ml of 0.05 M Na–K–phosphate buffer pH 7.0. The aldehyde formed was extracted as described in the methods and assayed by HPLC.

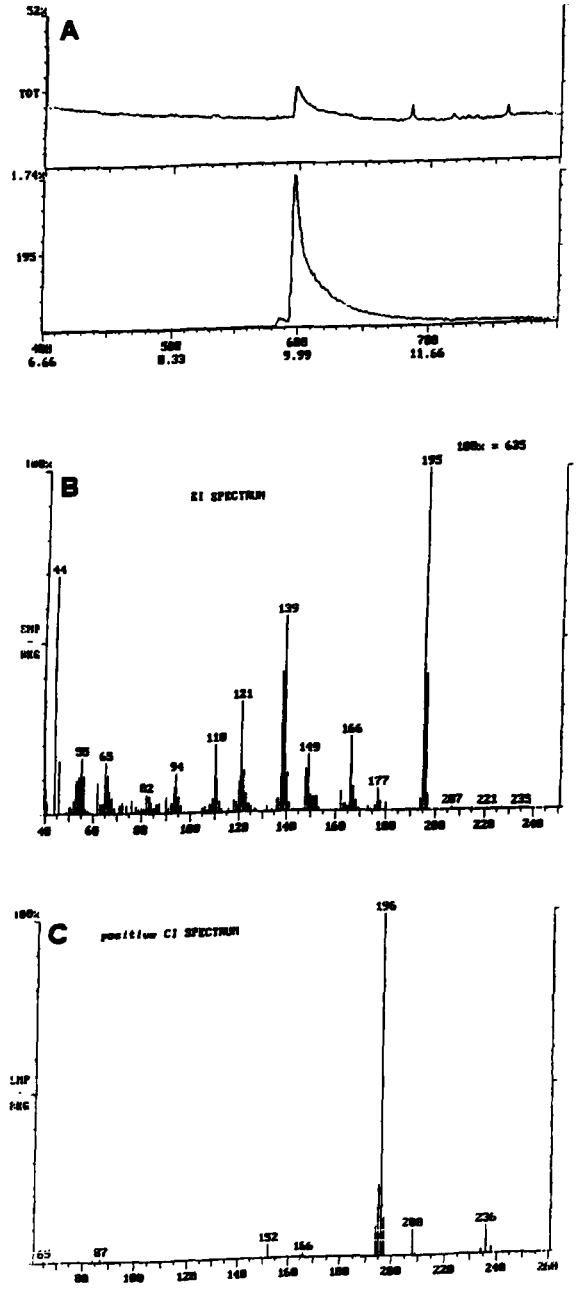


FIGURE 3 Identification by GC–MS of the aldehyde formed in the oxidation of B_{24} catalysed by pig plasma benzylamine oxidase. (A) Total ion chromatogram and ion chromatogram at m/z 195. (B) EI spectrum of the peak. (C) CI spectrum of the peak.

TABLE I Kinetic constants for pig plasma benzylamine oxidase (BAO) in the presence and absence of B_{24} after different incubation times

Time (min)	B_{24}					
	0		0.0078 μm		0.0156 μm	
	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}
20	53.2 ± 3.2	61.7 ± 1.2	41.3 ± 2.7	34.8 ± 0.7	55.6 ± 9.9	16.1 ± 1.0
40	54.9 ± 1.2	67.4 ± 2.2	48.1 ± 2.3	44.2 ± 0.7	32.6 ± 4.5	20.3 ± 0.7
60	57.4 ± 7.3	62.0 ± 2.6	52.2 ± 6.8	48.6 ± 2.0	45.4 ± 3.1	30.4 ± 0.6
120	63.3 ± 5.5	54.3 ± 1.6	58.4 ± 4.3	49.0 ± 1.2	52.8 ± 2.0	41.4 ± 0.5

K_m (μM), V_{max} (nmoles/mg of protein/min). Values are mean ± s.e. obtained with the method of Wilkinson.[8] Experiments were carried out in duplicate at 37°C with a BAO concentration 0.0156 μM under the conditions described in the methods. 0.0078–0.0156 μM B_{24} was preincubated for 10 min. The differences between K_m obtained at various incubation times are not statistically significant (Student's *t*-test, $P > 0.05$). The difference between V_{max} obtained at various incubation times in the absence of B_{24} are not statistically significant, but are statistically significant in the presence of B_{24} ($P < 0.05$).

was lower than that of BAO. It has also been observed that the IC_{50} M of B_{24} is related to the enzyme concentration corresponding to a B_{24}/BAO molar ratio of 0.5, when the initial rate of benzylamine oxidation was tested by adding the substrate after a few minutes of preincubation.

As reported in Table I the K_m did not significantly change with the incubation time but V_{max} gradually increased.

The increase in the rate of benzylamine oxidation was observed in 5 experiments in which B_{24} at different concentrations (molar ratio B_{24}/enzyme 0.5–1.0–2.0) was preincubated with the enzyme for 10 min before the addition of substrate. Table II shows the mean values and in Figure 4 an example is reported. The IC_{50} M of B_{24} also increases with the incubation time as shown in Figure 5.

Proposed Model of BAO Inhibition by B_{24}

B_{24} is a substrate of BAO. Hence it will compete with benzylamine for oxidation:

$$E + B_{24} \rightarrow EB_{24} \rightarrow EH_2B_{24}\text{-aldehyde} \xrightarrow{O_2} E + H_2O_2 + B_{24}\text{-aldehyde}$$

$$E + BnNH_2 \rightarrow EBnNH_2 \rightarrow EH_2PhCHO \xrightarrow{O_2} E + H_2O_2 + PhCHO.$$

However this system would result in the K_m and IC_{50} for B_{24} being elevated in the presence of $BnNH_2$, whereas this is observed only if both substrates are added together. When EB_{24} (EI) is formed $BnNH_2$ apparently is unable to displace B_{24} from the enzyme. This minimum mechanism is

TABLE II Oxidation of benzylamine by pig plasma benzylamine oxidase (BAO) in the presence of different B_{24} concentrations

Time (min)	*Benzaldehyde produced (P) μM concentration*			
	Control	*B_{24}/E*		
		0.5	1.0	2.0
20	1.68 ± 0.03	0.87 ± 0.05	0.55 ± 0.03	0.25 ± 0.007
40	3.45 ± 0.17	2.22 ± 0.10	1.42 ± 0.05	0.67 ± 0.029
60	4.83 ± 0.45	3.95 ± 1.92	2.34 ± 0.10	0.98 ± 0.15
80	6.12 ± 0.43	5.03 ± 0.10	3.82 ± 0.26	1.48 ± 0.05
120	10.45 ± 0.95	8.39 ± 0.47	6.59 ± 0.14	3.29 ± 0.20
180	13.79 ± 0.99	12.45 ± 0.65	11.58 ± 0.84	7.62 ± 0.10

Means ± s.e. of 5 experiments carried out in duplicate. Experimental conditions are described under methods. Enzyme concentration = 0.0078 μM. B_{24}/E = molar ratio.

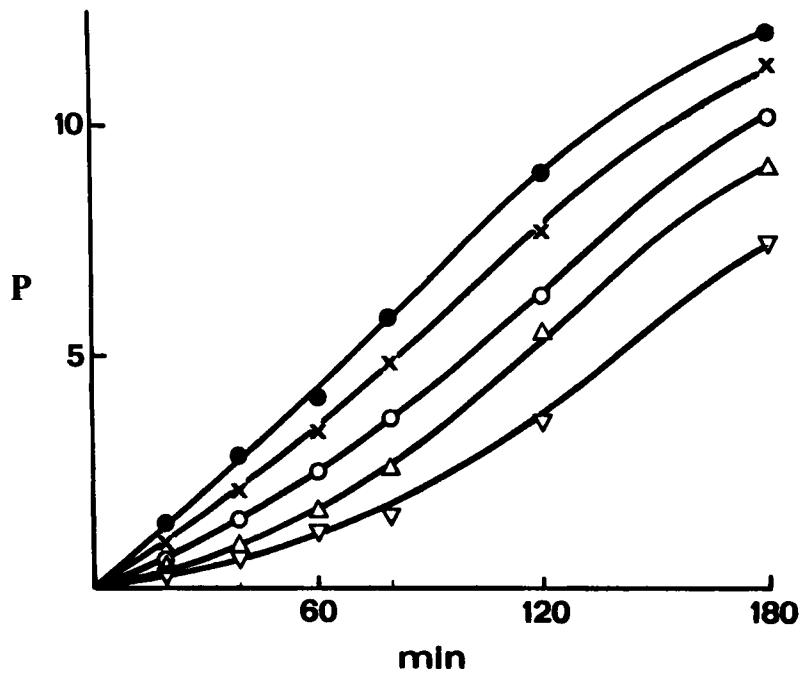


FIGURE 4 The progressive decrease in the B_{24} inhibition of pig plasma benzylamine oxidase with incubation time. The experiments were carried out using ^{14}C-benzylamine as described in methods. The concentration of benzylamine was 330 μM, BAO 0.0078 μM and B_{24} as follows (● = 0; × = 0.0039; ○ = 0.0078; △ = 0.0115; ▽ = 0.0156 μM). B_{24} was pre-incubated for 10 min at 37°C and the reaction stopped at different incubation times. Each point is the mean of 2 determinations. *P* is the benzaldehyde produced in μM concentration.

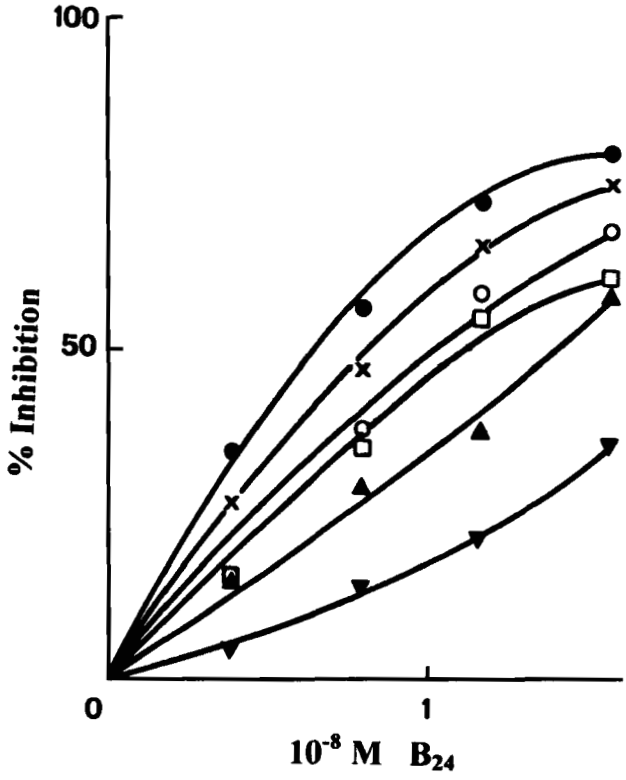
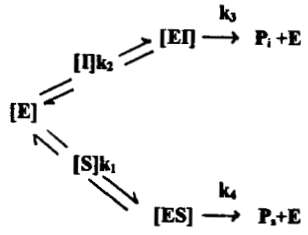


FIGURE 5 Variation of the IC_{50} M of B_{24} for pig plasma benzylamine oxidase with incubation time. The experiments were carried out using ^{14}C-benzylamine as described in the methods. The concentration of benzylamine was 330 μM, BAO 0.0078 μM, and B_{24} 0.0039–0.0078–0.0115–0.0156 μM. B_{24} was preincubated for 10 min at 37°C and the reaction stopped at different incubation times. Each point is the mean of 2 determinations. ● = 20 min; × = 40 min; ○ = 60 min; □ = 80 min; ▲ = 120 min; ▼ = 180 min.

shown below.



(3)

where P_i is the aldehyde of B_{24} and P_s the benzaldehyde and the constants k_1 and k_2 are association constants (k_{-1} and k_{-2} considered very small).

Mathematical fitting of the results reported in Table II were quite good using the following constants: k_1 3182, k_2 64 000, k_3 0.000636 $M^{-1}s^{-1}$, k_4 0.204 $M^{-1}s^{-1}$ when the molar ratio B_{24}/BAO was lower than 1. When the B_{24}/BAO molar ratio was 2 the best fitting was obtained by taking in account the competition between the 2 substrates according to the usual equation[9] (Figure 6):

$$k_1/(1 + [I]/K_i) \quad \text{(where } K_i \text{ was } 7 \times 10^{-10}\,M\text{)}.$$

The constants needed for a good fitting were those experimentally obtained with the exception of k_3 which was reduced to a lower value than that obtained by the direct measurement of B_{24}-aldehyde. This might suggest that in the presence of benzylamine the rate of B_{24} oxidation is reduced. This is in agreement with the experimental observation that the

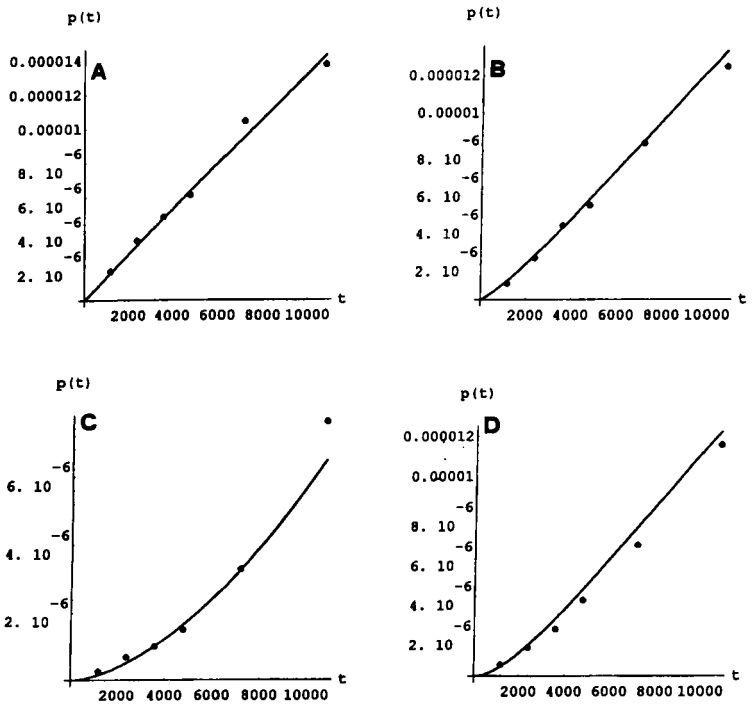


FIGURE 6 Computer fitting of the experimental results reported in Table II. Conditions used; BAO 0.0078 μM; B_{24} 0.0156–0.0078–0.0039 μM; $BnNH_2$ 333 μM. The following constants were used: k_1 3182; k_2 64 000; k_3 0.000636 $M^{-1}s^{-1}$; k_4 0.204 $M^{-1}s^{-1}$; K_i 7×10^{-10} M. When B_{24}/E molar ratio was 2, K_1 was: $K_1/(1 + [I]/K_i)$. The points are the experimental values for: A = enzyme alone; B = B_{24}/E 0.5 molar ratio; C = B_{24}/E 2 molar ratio; D = B_{24}/E 1 molar ratio. $t = s$, P = benzaldehyde produced (M).

rate of the decrease of the absorbance at 530 nm is reduced in the presence of benzylamine. The constant k of Eq. (2) was reduced to 0.00011 from 0.000638 when the enzyme concentration was 15 μM, the ratio B_{24}/E 1.55 and the concentration of $BnNH_2$ 10^{-2} M.

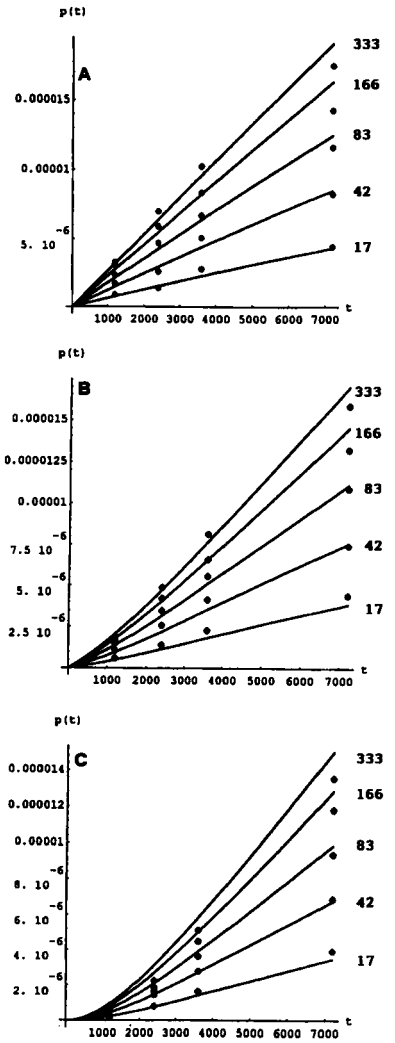


FIGURE 7 Computer fitting of the production of benzaldehyde at different $BnNH_2$ concentrations (kinetic constants reported in Table I). Conditions used: BAO 0.0156 μM; B_{24} 0.0156–0.0078 μM. Numbers on the figures indicate the $BnNH_2$ concentration (μM). The following constants were used: k_1 3182; k_2 64 000; k_3 0.000636 $M^{-1}s^{-1}$; k_4 0.204 $M^{-1}s^{-1}$. The points are the experimental values for: A = enzyme alone; B = B_{24}/E 0.5 molar ratio; C = B_{24}/E 1 molar ratio. $t = s$, P = benzaldehyde produced (M).

The analysis of benzaldehyde production at different $BnNH_2$ concentrations suggests a weak competition of $BnNH_2$. For the EI* that is probably the enzyme–B_{24} complex which is able to give the products. On the other hand the influence of $BnNH_2$ at concentrations of 17–333 μM is modest because quite a good fitting was obtained using the same constants for each $BnNH_2$ concentration (Figure 7).

The small deviation of the experimental values from the theoretical line which is observed at 7200 s and at the higher $BnNH_2$ concentrations might be an expression either of products inhibition, although catalase was used in the assay, or of an alteration of the enzyme due to the long incubation time.

DISCUSSION

These experiments show that B_{24} is a weak substrate of the pure pig plasma BAO. It is gradually deaminated to give its aldehyde which was isolated and identified in GC–MS. The rate of B_{24} oxidation is very slow and was measured in 3 experiments in which B_{24} at different concentrations was incubated with a large amount of enzyme in order to provide sufficient formed aldehyde for HPLC assay.

The formation of an adduct between B_{24} and BAO has been confirmed with the contemporaneous change in the enzyme absorbance at 530 nm as already described.[6] The formed adduct appears to be very stable.

In fact the M IC_{50} of B_{24}, when preincubated with the enzyme for a short time, is the expression of a B_{24}/E ratio of 0.5: B_{24} reacts mole to mole with the enzyme.

The initial rate of benzylamine oxidation in the presence of B_{24} is practically an expression of the amount of free enzyme. The free enzyme increases with the time of incubation because some enzyme is released by B_{24} oxidation, thus increasing the oxidation rate of $BnNH_2$. Initially $BnNH_2$ is oxidised only when the molar ratio B_{24}/BAO is lower than 1. When the molar ratio B_{24}/BAO is higher than 1, the enzyme is totally inhibited, but gradually the inhibition reverts and benzylamine is oxidised at a rate that is proportional to the freely formed enzyme but reduced by the presence of free B_{24} which is competing for the active site.

These experiments therefore show that B_{24} is a mixed-competitive site directed inhibitor which may appear as a pure non-competitive inhibitor when preincubated with the enzyme at concentrations lower than those of the enzyme. In addition they show that the preincubation time, reducing

the B_{24} concentration, may affect the inhibiting activity of low concentrations of B_{24}.

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