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# TRIMETHYLAMINE DEHYDROGENASE FROM A METHYLOTROPHIC BACTERIUM

## I. ISOLATION AND STEADY-STATE KINETICS

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## Summary

1. The isolation of trimethylamine dehydrogenase (EC 1.5.99.7) from a restricted facultative methylotroph to electrophoretic homogeneity is described.

2. The molecular weight and subunit molecular weights were found to be 146 800 for the enzyme by sedimentation equilibrium ultracentrifugation and 70 000—80 000 for the two non-identical subunits by sodium dodecyl sulphate gel electrophoresis.

3. Initial velocity studies indicate that the enzymatic reaction proceeds by a Ping-Pong mechanism.

4. Further kinetic evidence was obtained by analysis of product inhibition patterns using the alternate substrate diethylamine and the products acetaldehyde and ethylamine as product inhibitiors, for the release of ethylamine before the addition of phenazine methosulphate and for the existence of an enzyme · two-carbon unit complex as a stable form of the enzyme.

5. Some properties of the unusual prosthetic group of trimethylamine dehydrogenase and its photodegradation product are described in preliminary form.

## Introduction

In the last decade a number of dehydrogenase enzymes which can furnish one-carbon units for utilization by either the serine or ribose phosphate path-

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ways which operate in methylotrophic microorganisms have been isolated [1]. These dehydrogenases, which include the enzymes methanol dehydrogenase [2], methylamine dehydrogenase [3] and trimethylamine dehydrogenase (EC 1.5.99.7) [4] catalyze the following reactions (PMS means phenazine methosulphate):

$$CH_3OH + PMS \xrightarrow[NH_4^+]{\text{methanol dehydrogenase}} CH_2O + PMSH_2$$

$$CH_3NH_2 + H_2O + PMS \xrightarrow[\text{dehydrogenase}]{\text{methylamine}} CH_2O + PMSH_2 + NH_3$$

$$(CH_3)_3N + H_2O + PMS \xrightarrow[\text{dehydrogenase}]{\text{trimethylamine}} (CH_3)_2NH + PMSH_2 + CH_2O$$

In all three cases one of the products is an aldehyde and much greater activity is obtained with phenazine methosulphate than with a wide range of alternate electron acceptors. The natural electron acceptor for these enzymes is unknown, but it has been suggested that it may be a flavoprotein which is linked to an electron transport chain (ref. 3, but see also ref. 5).

Uncertainty also exists as regards the nature of the coenzymes which are utilized as redox cofactors in these enzymes. In the case of methanol dehydrogenase, Anthony and Zatman [6] have suggested that the cofactor is probably a pteridine derivative and proposed a mechanism for catalysis which involves a $N^5$, $^{10}$-methylene pteridine, which is derived from oxidation of a 5-methyl pteroate by phenazine methosulphate. So far, however, no kinetic analysis which could support this mechanism has appeared in the literature. Sperl et al. [7] have suggested, on the basis of more recent evidence, that the coenzyme is a hydrated lumazine derivative. This proposal is of considerable interest, since 6,7-dimethyl-8-ribityl lumazine is the immediate biosynthetic precursor of riboflavin, and the possible role of lumazines in biological redox reactions has hitherto not been recognized.

In the case of methylamine dehydrogenase, Eady and Large [3] have suggested, on the basis of inhibitor specificity, that this enzyme may be similar to the copper and pyridoxal phosphate-containing amine oxidases. However, later work indicated the non-involvement of copper, whereas identity of the isolated coenzyme with known vitamin $B_6$ derivatives could not be confirmed [9].

Colby and Zatman [8] made the very interesting observation that trimethylamine dehydrogenase has an inhibitor specificity resembling that of the monoamine oxidases which are inhibited by certain substituted hydrazines, but available evidence indicated that the coenzyme is not a flavin (Colby, J., personal communication).

These observations made the mechanism of action of these dehydrogenases and the nature of their prosthetic groups of considerable interest.

In this paper, the steady-state kinetics of trimethylamine dehydrogenase from bacterium W3Al, a facultative methylotroph [10] with trimethylamine and diethylamine as amine substrates, as well as some observations regarding the prosthetic groups of this enzyme are reported.

## Materials and Methods

*Growth of bacterium W3A1*

We are indebted to Dr. L.A. Zatman of the University of Reading for a culture of bacterium W3A1. The culture medium used in this study consisted of the mineral base E of Owens and Keddie [11] to which filter-sterilized 6% trimethylamine hydrochloride, pH 7.0, had been added to a final concentration of 0.3% with respect to trimethylamine. The organisms were grown in 250-ml conical flasks, containing 100 ml medium, on a metabolic shaker at 30° C and were harvested by centrifugation after 20 h of growth. Bacterium W3A1 produced a considerable amount of slime during growth on trimethylamine and consequently the net weight of the organisms was not established.

*Enzyme purification*

*Extraction.* The organisms were washed once with a volume of distilled water equal to that of the original growth medium. Upon agitation, the bulky pellet formed a viscous suspension. The volume of the cell suspension was measured and sufficient 0.5 M potassium phosphate was added to obtain a final buffer concentration of 0.1 M. Breakage of the cells was performed by sonication at 0—5°C. In order to remove most of the slime from the bacterium without concomitant loss of enzyme, the cells were sonicated at the maximum setting of the instrument, for 2—3 min periods, followed by centrifugation to collect unbroken cells. The pellets from each successive period of sonication were resuspended in progressively smaller volumes of 0.1 M potassium phosphate buffer, pH 7.0, and subjected to a further period of sonication, whereas the supernatants were retained. This procedure was continued till the cells were completely solubilized. Sonication was interrupted whenever the temperature reached 5° C. It appeared that the first 2-min period of sonication served mainly to remove slime from the organisms as judged by the greatly reduced volume of cells which was obtained upon centrifugation. The bulk of the enzyme was only released in the subsequent stages of sonication. The supernatants which contained enzyme were pooled to obtain the final crude extract, amounting to about 75 ml from 12 l of growth medium.

*$(NH_4)_2SO_4$ fractionation.* The crude extract was brought to 50% $(NH_4)_2SO_4$ saturation at 0° C by cautious addition of the solid, and the precipitate was collected by centrifugation after 2 h. The enzyme was contained in the supernatant and was subsequently precipitated by increasing the percentage saturation to 80%. After 1 h the precipitate was collected by centrifugation and redissolved in 0.05 M potassium phosphate buffer, pH 7.2.

*DEAE-Cellulose chromatography.* The active fraction from the $(NH_4)_2SO_4$ fractionation was dialyzed against 2 l of 0.05 M phosphate buffer, pH 7.2, for 16 h with one change of buffer after 8 h. The dialyzed protein was concentrated to 20 ml by ultrafiltration on an Amicon ultrafiltration apparatus using a PM-30 membrane and then applied to a DEAE-cellulose column (2 × 25 cm) which had been equilibrated with the buffer used for dialysis. The enzyme was eluted using a 1 l linear salt gradient from 0.05 M potassium phosphate, pH 7.2, to 0.5 M NaCl in the same buffer. The active fractions which were yellow in

colour were assayed for enzyme activity, pooled and then concentrated to a volume of 20 ml by ultrafiltration.

*Sephadex G-200 gel chromatography.* The concentrated enzyme from the previous step was applied to a Sephadex G-200 column (2 × 85 cm) which had been equilibrated with 0.05 M potassium phosphate buffer, pH 7.0, and eluted at a flow rate of about 15 ml/h. The active fractions were pooled and concentrated to a volume of 20 ml by ultrafiltration. Ethylene glycol which had been precooled to -20° C was slowly added to the enzyme to a final concentration of 20%. The enzyme was found to be stable for months if stored at -20° C in 20% ethylene glycol.

*Polyacrylamide gel electrophoresis.* Polyacrylamide gel electrophoresis was performed as described by Davis [12]. Sample preparation for sodium dodecyl sulphate gel electrophoresis was performed as described by Weber and Osborne [13] and the actual electrophoresis essentially as described by Laemmli [14]. After electrophoresis the proteins were fixed by leaving the gels overnight in 10% trichloroacetic acid, stained in the staining solution of Weber and Osborne [13] and finally diffusion destained in their destaining solution.

*Enzyme assays.* Enzyme assays were performed as described by Colby and Zatman [8] at pH 7.7 and 30° C. A molar extinction coefficient of 21.69 $mM^{-1} \cdot cm^{-1}$ for dichlorophenolindophenol at pH 7.7 was assumed. In the kinetic studies using trimethylamine as a substrate an Aminco-Chance dual wavelength splitbeam spectrophotometer was used at a scale expansion of 0—0.05 Å. In all other spectrophotometric work a Beckman Model 25 spectrophotometer was used at a scale expansion of 0—0.25 Å. In agreement with the findings of King [15] we found it necessary to correct for a slow non-enzymatic reaction when phenazine methosulphate was used at a concentration above about 1 mM in assay mixtures.

*Analytical procedures.* The concentration of the substituted amines was determined by Kjehldahl distillation into boric acid and back titration using standard HCl. Acetaldehyde was determined by measuring the alcohol-dehydrogenase catalyzed decrease in absorbance at 340 nm of $NADH_2$ in 0.1 M sodium pyrophosphate buffer at pH 7.7. Protein was determined by the Lowry method [16].

*Ultracentrifugation.* Sedimentation equilibrium analysis were performed according to Yphantis [17] in a Spinco Model E ultracentrifuge equipped with a scanning densitometer. The enzyme was dialyzed for 16 h against 0.1 M potassium phosphate buffer, pH 7.0. Three different protein concentrations giving final absorbance readings at 440 nm of 0.19, 0.25 and 0.30 respectively, were analyzed simultaneously using the six-channel centrepiece. The monochromator was set to the absorbance maximum of the enzyme-bound prosthetic group at 440 nm and densitometer tracings were obtained at 15 398 rev./min and also at 12 320 rev./min. Data were analyzed using the equation

$$M = \frac{2\,RT\,\mathrm{d}\ln c}{(1 - \bar{v}\rho)\omega^2\,\mathrm{d}r^2}$$

where $c$ is proportional to the densitometer pen deflection, and a value of 0.735 for the partial specific volume as calculated from the amino acid composition was used.

## Results

*Enzyme purification*

It was found that the enzyme could be purified with a yield of approx. 50% by employing the three-step purification outlined in Materials and Methods. The final preparation had a specific activity of 1.07 which agrees quite well with that of the trimethylamine dehydrogenase which was purified by Colby and Zatman [8] from bacterium 4B 6. Data obtained during a typical purification is summarized in Table I and a representative DEAE-cellulose chromatogram is shown in Fig. 1. The enzyme chromatographed essentially as a single peak on Sephadex G-200 gel chromatographs. The purified enzyme migrated as a single band on polyacrylamide gel electrophoretograms.

*Molecular weight of the purified enzyme*

Log $c$ vs. $r^2$ plots of the data from sedimentation equilibrium were linear indicating the absence of association dissociation pehnomena in the purified enzyme (Fig. 2), and a molecular weight of 146 800 was calculated. In sodium dodecyl sulphate gel electrophoretograms the purified enzyme migrated as two closely spaced bands of molecular weight approx. 70 000—80 000. These results are, therefore, similar to the results which were obtained by Colby and Zatman [18] for the enzyme from bacterium 4B 6.

*Kinetic studies on trimethylamine dehydrogenase*

*Initial velocity studies.* Initial velocity patterns showing the dependence of reaction velocity on the concentration of one substrate at different fixed levels of the second substrate are presented in Figs. 3 and 4 for the oxidation of trimethylamine and diethylamine, respectively. The initial velocity patterns consist basically of a series of parallel straight lines. The parallel patterns are, however, complicated by substrate inhibition, by phenazine methosulphate in the case of trimethylamine oxidation and by both substrates in the case of diethylamine oxidation. It was pointed out by Cleland [19] that substrate inhibition may be a valuable aid in deciding whether the observation of parallel lines in

TABLE I

PURIFICATION OF TRIMETHYLAMINE DEHYDROGENASE FROM BACTERIUM W3A1

Trimethylamine dehydrogenase activities were determined at pH 7.7 by using the spectrophotometric method of Colby and Zatman [8]. One unit of enzyme activity = 1 $\mu$mol · $min^{-1}$ · $mg^{-1}$.

| | Volume (ml) | Total activity (units) | Total protein (mg) | Specific activity (units/mg) |
|---|---|---|---|---|
| Crude extract | 75 | 37.3 | 660 | 0.0565 |
| $(NH_4)_2SO_4$ fraction | 35 | 41.3 | 188 | 0.22 |
| DEAE-cellulose eluate * | 16 | 26.6 | 27.8 | 0.96 |
| Sephadex G-200 eluate *, ** | 30 | 21.8 | 20.4 | 1.07 |

* Assayed after concentration by ultrafiltration.

** In 20% ethylene glycol.

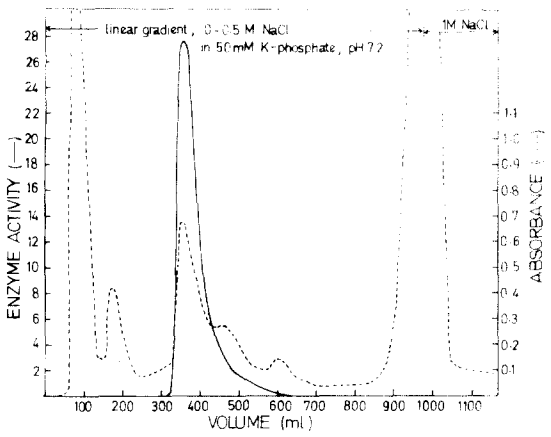



**Fig. 1. DEAE-cellulose chromatography of the 50—80% $(NH_4)_2SO_4$ precipitate. Flow rate, 25 ml/h. Column dimensions, 2 X 25 cm. Activity is expressed as change in absorbance at 600 nm per min per ml of enzyme solution.**

initial velocity patterns is due to Ping Pong (double displacement) type kinetics or to a low value of the constant term in the denominator of the rate equation for a sequential mechanism. Replots of the intercepts from Figs. 3 and 4 indicate that in both cases the substrate inhibition by phenazine methosulphate may be overcome by saturation with the variable amine substrates (Fig. 5). It is also evident that saturation with phenazine methosulphate abolishes the substrate inhibition by diethylamine. The initial velocity patterns for the oxidation of both amine substrates are therefore consistent with a Ping Pong mechanism in which substrate inhibition may be ascribed to combination of the inhibiting substrate with the wrong stable enzyme form.

It is of interest that intercept replots (Fig. 5) does not yield the same $K_b$ value for phenazine methosulphate as the electron acceptor in diethylamine as opposed to trimethylamine oxidation. This observation may be interpreted in one of two possible ways. In the case of the enzyme rhodanese Jarabak and

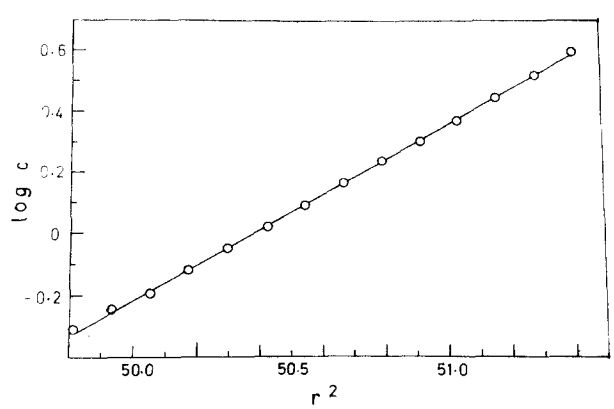



**Fig. 2. Log $c$ vs. $r^2$ plots of data from a sedimentation equilibrium experiment conducted at 20°C and at 12 320 rev./min.**

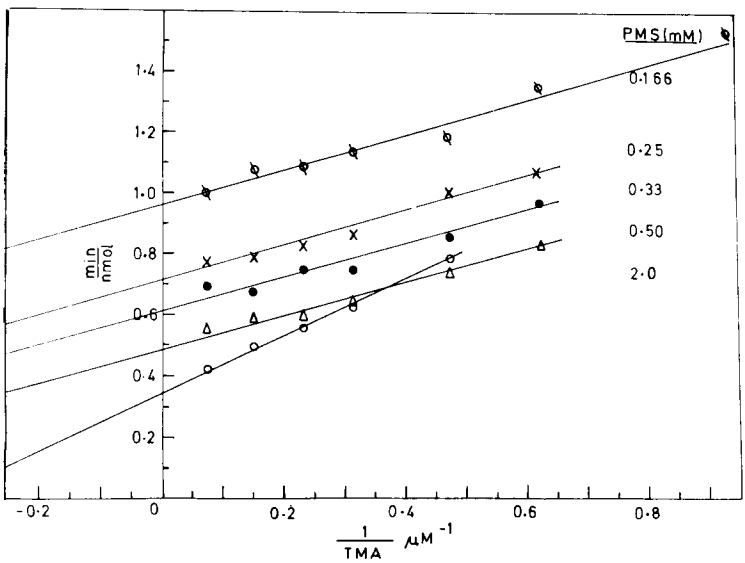



Fig. 3. Initial velocity pattern showing the dependence of reaction rate on trimethylamine concentration at various fixed levels of phenazine methosulphate. TMA, trimethylamine.

Westley [20] have shown that such a phenomenon could be ascribed to conformation dissimilarites between the stable forms of the enzyme which are obtained upon the release of the products from two alternate substrates. The difference in the $K_b$ values for phenazine methosulphate which is obtained in the oxidation of the two alternate amine substrates may therefore reflect a case of "enzyme memory" in which the stable form of the enzyme with which phenazine methosulphate combines is conformationally different in the two cases. How-

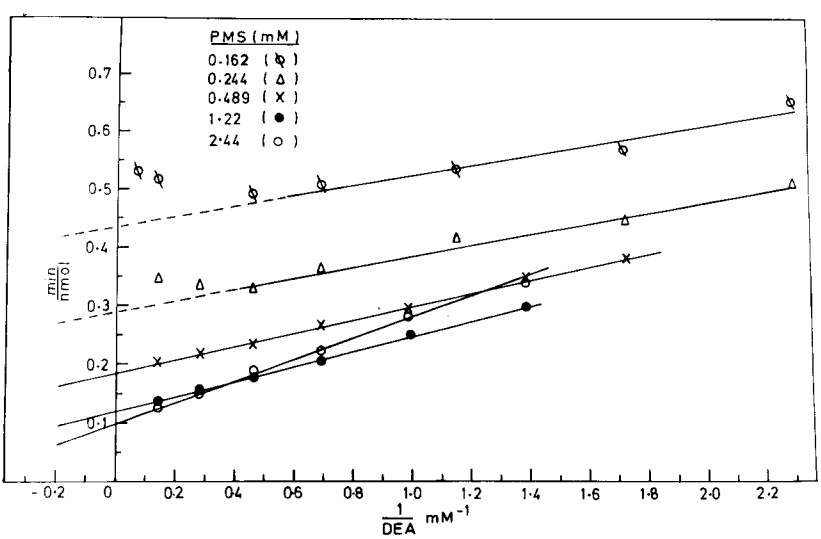



Fig. 4. Initial velocity pattern showing the dependence of reaction rate on diethylamine concentration at various fixed levels of phenazine methosulphate (PMS). DEA; diethylamine.

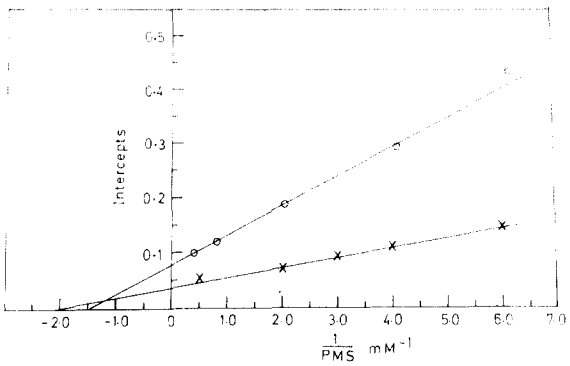



Fig. 5. Secondary replots of the y-axis intercepts from Fig. 3 (X———X) and Fig. 4 (○———○). For purposes of comparison the intercepts from Fig. 3 where multiplied by a constant factor representing the ratio of the enzyme concentrations which were used in Fig. 4 to that used in Fig. 3.

ever, if only one of the products of amine oxidation is released to generate the second stable form of the enzyme, the latter will exist as a dimethylamine, or one carbon unit, enzyme complex if trimethylamine was the amine substrate, and as an ethylamine, or two carbon unit, enzyme complex if diethylamine was the amine substrate. It is evident that the stable enzyme form with which phenazine methosulphate combines will be in the latter case different when alternate amine substrates are being oxidized. The latter possibility was supported by product inhibition studies.

*Product inhibition studies.* Both ethylamine and dimethylamine were non-competitive inhibitors with respect to their respective amine substrates at a fixed unsaturating concentration of phenazine methosulphate (Figs. 6 and 7). In both cases intercept and slope replots (Figs. 8a and 8b) are linear indicating that these products do not form dead end inhibitor complexes with the enzyme. Colby and Zatman [8] reported dimethylamine to be a poor alternate substrate of the enzyme from bacterium 4B 6. This observation could not be substantiated in the case of the trimethylamine dehydrogenase from bacterium

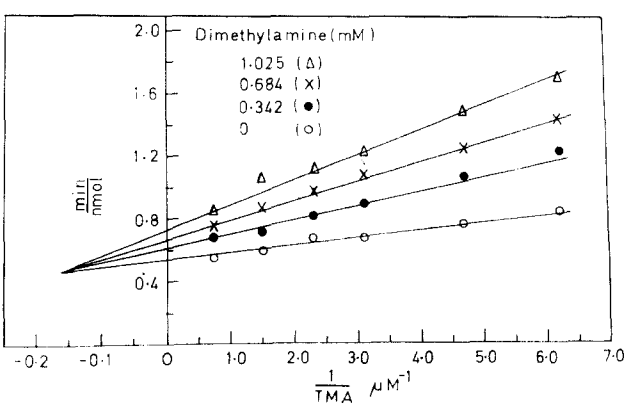



Fig. 6. Product inhibition by dimethylamine *DMA) with trimethylamine as variable substrate. Phenazine methosulphate concentration, 0.50 mM.

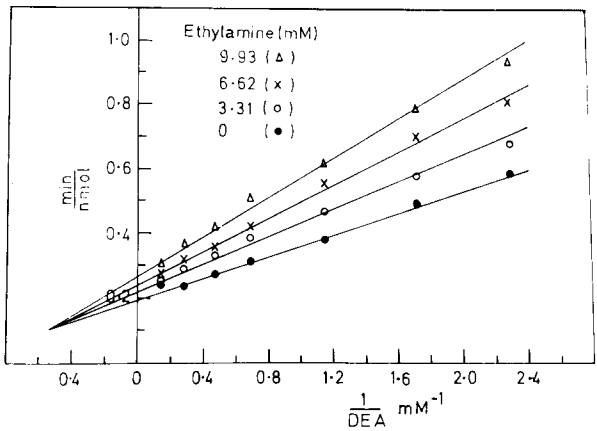



Fig. 7. Product inhibition by ethylamine with diethylamine as variable substrate. Phenazine methosulphate concentration, 0.469 mM.

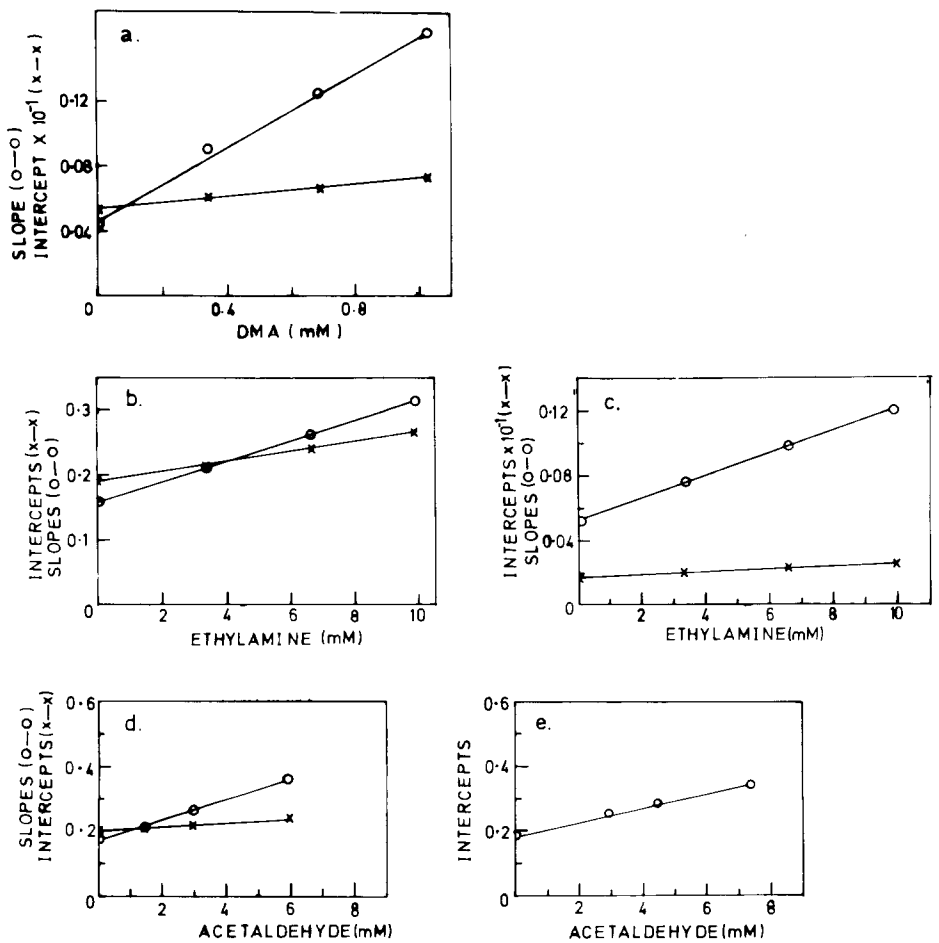



Fig. 8. Slope and intercept replots of the data from: a, Fig. 6; b, Fig. 7; c, Fig. 9; d, Fig. 10; e, Fig. 11.

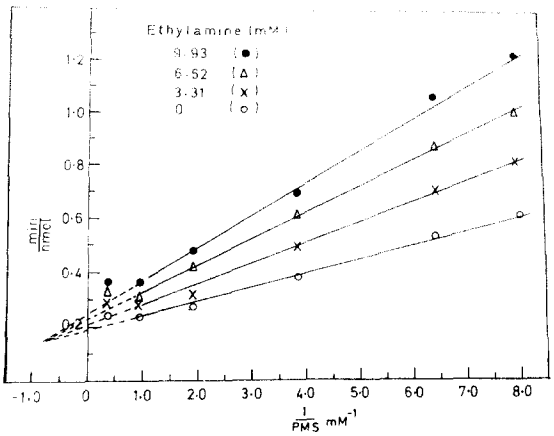



**Fig. 9 Product inhibition by ethylamine with phenazine methosulphate (PMS) as variable substrate. Diethylamine concentration, 1.44 mM.**

W3A1. Instead it appeared that both ethylamine and dimethylamine solutions obtained from commercial sources contained almost negligible amounts of contaminants which would serve as substrates of the enzyme to give a small extent of reduction of the terminal electron acceptor dichloroplenolindophenol. This problem could be overcome by a brief preincubation of the complete reaction mixture containing the amine product inhibitor and enzyme before starting the reaction by addition of the amine substrate.

Because of the low $K_a$ value for trimethylamine and the limitations in accuracy which this imposes, further studies were limited to product inhibition of diethylamine oxidation. Ethylamine also behaved as a non-competitive inhibitor when phenazine methosulphate was the variable substrate at a fixed unsaturating concentration of diethylamine (Fig. 9) and again intercept and slope replots are linear (Fig. 8c). Acetaldehyde was a non-competitive inhibitor with respect to diethylamine (Fig. 10) but an uncompetitive inhibitor when phenazine methosulphate was used as the variable substrate (Fig. 11). In both cases the second substrate was used at an unsaturating concentration and intercept

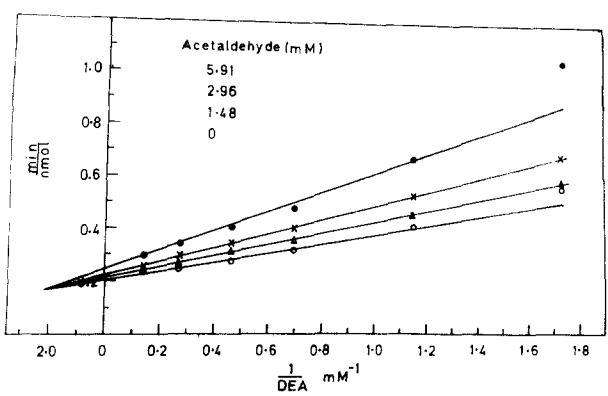



**Fig. 10. Product by alcetaldehyde with dimethylamine was variable substrate. Phenazine methosulphate concentration, 0.50 mM.**

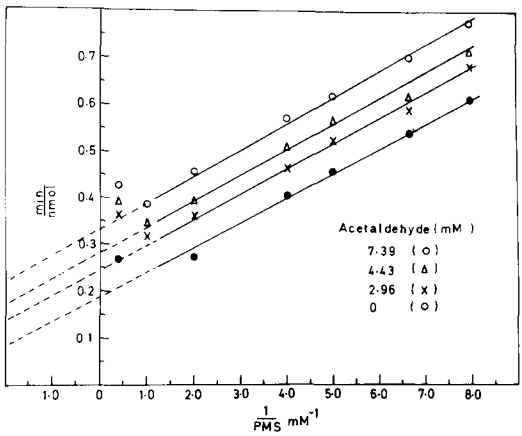
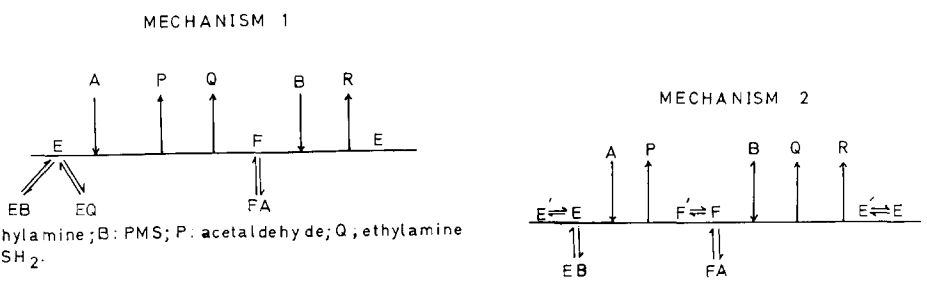



Fig. 11. Product inhibition by acetaldehyde with phenazine methosulphate (PMS) as variable substrate. Diethylamine concentration, 1.44 mM.

and slope replots shown in Figs. 8d and 8e are linear. These results indicate that there must be a point of product release between the points of addition of phenazine methosulphate and release of acetaldehyde.

Since the initial velocity pattern of the enzyme is indicative of a Ping Pong mechanism it seems necessary to account for these observations within the framework of such a mechanism. The fact that no competitive product inhibition pattern was found using either diethylamine or phenazine methosulphate as substrate creates a problem of interpretation. If ethylamine were the first and only product to be released before the addition of phenazine methosulphate one would have expected competitive inhibition by ethylamine when phenazine methosulphate is the variable substrate, or, if both ethylamine and acetaldehyde were released before the addition of phenazine methosulphate, uncompetitive inhibition by the one product and competitive inhibition by the other with phenazine methosulphate as the variable substrate.

There are, however, two possible mechanisms which can qualitatively account for the product inhibition patterns which were observed (Scheme I). In




SCHEME I

mechanism 1 it is assumed that the aldehyde and subsequently the amine product is released before the addition of phenazine methosulphate. The non-competitive inhibition pattern of ethylamine with either phenazine methosulphate

or diethylamine as variable substrate is accounted for by assuming that ethylamine can in addition form a dead end inhibitor complex by competing with diethylamine for the same stable form of the enzyme. In mechanism 2, the product inhibition patterns are accounted for by assuming isomerization of both stable enzyme forms.

For mechanism 1 the reaction velocity in the presence of ethylamine as product inhibitor as derived by King's method is:

$$v = \frac{v_1[AB]}{K_a[B] + K_b[A] + [AB] + \frac{K_b[AQ]}{K_{iq}} + \frac{K_a[BQ]}{K_{iiq}}}$$

where $K_{iiq}$ is the inhibition constant for dead end inhibition Q. It can readily be shown that the negative $x$-axis coordinate of the point intersection of the lines in the product inhibition pattern with ethylamine (Q) as inhibitor and phenazine methosulphate (B) as variable substrate equals

$$\frac{K_{iq}}{K_b} \frac{K_a}{K_{iiq}} \frac{1}{[A]}$$

and hence depends on the level of A. At high concentrations of A the product inhibition pattern must become competitive and the lines intersect on the $y$-axis, whereas at very low A levels the point of intersection moves from the origin on the $x$-axis to approach negative infinity, thus giving an apparently uncompetitive inhibition pattern. The inhibition by ethylamine with phenazine methosulphate as variable substrate was therefore studied at different fixed levels of diethylamine from that used in Fig. 10. It was found that the $x$-coordinate of the point of intersection of the lines in the double reciprocal plots remained, within experimental error, similar to the value of 0.75 in Fig. 10. Thus at a diethylamine concentration of 2.88 mM, representing twice the concentration used in Fig. 10 the point of intersection gave an $x$-axis coordinate of 0.70 and at 0.30 mM diethylamine the $x$-axis coordinate was 1.1., whereas the rate equation for mechanism 1 predicts a shift in the $x$-axis coordinate of about 10-fold under these conditions.

These results are therefore inconsistent with mechanism 1, but not inconsistent with mechanism 2 of scheme I.

*The chromophores of trimethylamine dehydrogenase*

At present the nature of the coenzyme of trimethylamine dehydrogenase is unknown. Studies which are currently in progress and which will be reported in greater detail at a later stage have indicated that the chromophore in the enzyme from bacterium W3A1 behave in a manner similar to that from the enzyme of bacterium 4B6 (Colby, J., personal communication). Our observation thus far may be summarized as follows: purified trimethylamine dehydrogenase absorbs in the visible region with $\lambda_{max}$ at 440 nm (Fig. 12, curve a). Addition of substrate bleaches the absorbance at 440 nm with the concomitant appearance of increased absorbance at 360 nm (curve b). Upon denaturation of the enzyme with 5% trichloroacetic acid, acid-labile iron and sulphur are liberated.

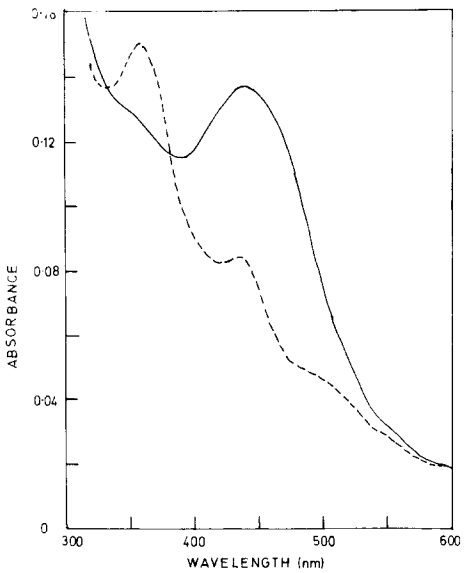



Fig. 12. Spectrum of trimethylamine dehydrogenase in 0.05 M potassium phosphate buffer (———), after the addition of diethylamine hydrochloride to give a final concentration of 1.73 mM diethylamine (- - - - -). Protein concentration approx. 0.7 mg/ml.

The pellet from trichloroacetic acid precipitation, however, is bright yellow and further washing procedures does not liberate more acid-labile iron. The yellow chromophore is not released by heat denaturation in the presence of trichloroacetic acid, but may be solubilized by proteolytic digestion indicating covalent attachment to the enzyme. The absorbance spectrum of the liberated chromophore shows an absorbance maximum at 435 nm and a shoulder at about 370 nm. The chromophore is only weakly fluorescent, but upon exposure to light (even in the fluorimeter) gives rise to a fluorescent photodegradation product with excitation maximum at 394 nm and emission maximum at about 500 nm. The fluorescence disappears in a time-dependent manner upon addition of dithionite or sodium sulphite-bisulphite and also in alkali at pH > 10. Neutralization of alkali-bleached chromophore regenerates about 70% of the original fluorescence.

At present no adequate explanation for these observations can be offered. However, it is noteworthy that some pteridines such as lumazine derivatives also display complex equilibria upon titration with alkali [21.22] which could possibly account for the time-dependent fluorescence changes at high pH values, and that the fluorescence of the photodegradation product and the effect of dithionite and sulphite thereupon is similar to the behaviour of such pteridines.

## Conclusion

The trimethylamine dehydrogenase of bacterium W3A1 and bacterium 4B 6 appear to be similar enzymes as judged by their elution behaviour on DEAE-cellulose chromatography, specific activity, molecular weight and electropho-

resis on sodium dodecyl sulphate/polyacrylamine gels. Colby and Zatman [8] studied the action of a wide range of inhibitors on trimethylamine dehydrogenase from bacterium 4B 6 and found that this enzyme corresponds in its inhibitor specificity to the monoamine oxidases from animal tissues. In particular the characteristics of inhibition by substituted hydrazines was similar to that of the monoamine oxidases in that inhibition seemed to be dependent upon the presence of the electron acceptor. In this connection it was recently demonstrated by Patek and Hellerman [23] and Chuang et al. [24] that the irreversible inhibition of monoanime oxidases by substituted hydrazines or pargyline is due to the formation of an adduct between the inhibitor and the flavo-coenzyme. The phenomological similarity between the inhibition of trimethylamine dehydrogenase and monoamine oxidase by substituted hydrazines is therefore of considerable interest since present evidence suggests that the covalently bound prosthetic group of trimethylamine dehydrogenase is different from any known covalently bound flavin.

The data presented in this paper indicates that the trimethylamine dehydrogenase from bacterium W3A1 behaves kinetically in a manner similar to that reported for flavin-containing monoamine oxidases. It was shown by Oi et al. [25] and by Tipton [26] for the liver and pig brain monoamine oxidases that these reactions proceed by a double displacement mechanism. Both these authors have also presented some evidence indicating that both products, ammonium and aldehyde are released before the addition of oxygen. However, since alternate pathways of product release may operate it is not certain what mechanistic significance can be attached to interpretations of kinetic data which suggest that both products are released before the addition of electron acceptor to the enzyme. Thus Koster and Veeger [27] have shown that a double displacement mechanism operates as a very minor pathway during the oxidation of D-alanine by D-amino acid oxidase. Similarly Porter et al. [28] have presented evidence indicating that the release of aldehyde before the addition of oxygen is not an obligatory step in the oxidation of nitroethane by D-amino acid oxidase, whereas it was shown by Lockridge et al. [29] that the reduced lactate oxidase · pyruvate complex can either slowly dissociate into reduced enzyme and pyruvate or react more rapidly with oxygen. Because of the possible existence of alternate pathways it is also not possible to infer from the mere release of a product in the absence of electron acceptors that such a product release constitutes part of the predominant pathway during catalysis of an enzyme, unless it can be shown to proceed at a catalytically significant rate. Mechanistically it seems of more importance which of the products is released first. For instance, in the mechanism proposed by Porter et al. [28] for the oxidation of nitroethane by D-amino acid oxidase, nitrite should be released before the aldehyde from a covalent adduct formed between N-5 of the flavin and C-1 of nitroethane. In the case of monoamine oxidase the position is not clear. Several studies have appeared in the literature on the product inhibition patterns of various monoamine oxidases. However, inhibition by ammonia and $H_2O_2$ is only observed at high concentrations of these substances, and does not appear to be due merely to product inhibition involving a reversal of a partial reaction sequence [30,31]. However, it was proposed by Yasunobu and Oi [32] that at elevated pH ammonium is released before the aldehyde, whereas the competi-

tive inhibition of *p*-hydroxybenzaldehyde with oxygen as variable substrate may be interpreted as indicative of a similar reaction sequence in pig brain monoamine oxidase [26].

The mechanism which we have proposed as consistent with the kinetic data for trimethylamine dehydrogenase indicates the participation of a two carbon unit · enzyme complex during the catalytic cycle of this enzyme. The proposed mechanism raises an interesting question concerning the site of attachment of the two carbon unit to the enzyme, which together with the inhibition of trimethylamine dehydrogenase by monoamine oxidase inhibitors makes the nature of the coenzyme of this enzyme and its possible involvement in these reactions a most intriguing problem.

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