Biochimica et Biophysica Acta, 321 (1973) 382–396

BBA 66969

STUDIES ON THE MECHANISM OF ETHANOLAMINE-AMMONIA LYASE USING 2-ETHOXYETHYLAMINE AS A SUBSTRATE

P. Y. LAW AND J. M. WOOD

Department of Biochemistry, School of Chemical Sciences, University of Illinois, Urbana, Ill. 61801 (U.S.A.)

(Received January 22nd, 1973)
(Revised Manuscript received May 1st, 1973)

SUMMARY

In addition to ethanolamine and propanolamine it was found that 2-ethoxyethylamine is a substrate for the corrin-dependent enzyme ethanolamine-ammonia lyase. The enzyme has the same V for 2-ethoxyethylamine as ethanolamine, but the K_m for this substrate is $4.3 \cdot 10^{-3}$ M. Acetaldehyde and ethylamine were isolated and characterized as reaction products from 2-ethoxyethylamine. When deuterium and tritium replaced hydrogens at the amino carbon of 2-ethoxyethylamine a primary isotope effect was observed for hydrogen transfer with $k_H/k^2_H = 2.2$ and $k_H/k^3_H = 4.0$. Tritium was shown to be transferred from the amino carbon of 2-ethoxyethylamine into the 5′-methylene group of 5,6-dimethylbenzimidazole B_{12} coenzyme (DMBC) during catalysis. The extent of tritium incorporation into DMBC was shown to be dependent on the ratio of ethanolamine-ammonia lyase holoenzyme to tritiated 2-ethoxyethylamine. Also, tritium was shown to be incorporated into the 5′-methylene group of DMBC from ethanolamine labelled with tritium in the amino carbon, but this exchange was shown to be non-specific.

When DMBC was photolysed under anaerobic conditions in the presence of 2-ethoxyethylamine, labelled with tritium in the amino carbon, then the methylene radical generated by photolysis specifically abstracts tritium from the amino carbon of this substrate. The nucleoside product was identified as 5′-deoxyadenosine. When similar photolysis experiments with DMBC were carried out in ethanolamine, tritiated in the amino carbon, non-specific abstraction of both hydrogen and tritium from both carbons was observed. Acetaldehyde was isolated and characterized as a product from both 2-ethoxyethylamine and ethanolamine in these photolysis experiments. The quantity of acetaldehyde formed is directly proportional to the quantity of nucleoside formed by the photolysis of DMBC. These data are consistent with those obtained from enzymatic studies indicating that substrate-dependent homolysis of the Co–C σ bond occurs as a prerequisite for hydrogen transfer in ethanolamine-ammonia lyase.

Abbreviation: DMBC, 5,6-dimethylbenzimidazole B_{12} coenzyme.

INTRODUCTION

The conversion of ethanolamine to acetaldehyde and ammonia by the corrin-dependent enzyme ethanolamine-ammonia lyase has been shown to involve intramolecular hydrogen transfer of one of the hydrogen atoms from the carbon atom bearing the hydroxyl group via the 5′-methylene group of 5,6-dimethylbenzimidazole B_{12} coenzyme (DMBC) to the adjacent carbon atom with inversion of configuration (ref. 1 and Arigoni, D., personal communication). Concomitant with transfer of hydrogen is migration of the amino group to facilitate deamination to yield acetaldehyde as the product[2]. Babior *et al.*[3–5] demonstrated that a low spin Co^{II} EPR signal and a carbon radical EPR signal appeared upon the addition of ethanolamine to ethanolamine-ammonia lyase. Law *et al.*[6] demonstrated substrate-dependent homolysis of the Co–C bond by using a spin-labelled cobinamide coenzyme in place of DMBC. 5′-Deoxyadenosine was isolated by Carty *et al.*[7] as the nucleoside intermediate in this reaction. Based on these experiments a mechanism has been proposed for intramolecular transfer of hydrogen via a 5′-methylene radical of DMBC which is generated by substrate-dependent homolysis of the Co–C σ bond[3–7]. When deuterium or tritium replace the hydrogen on the carbon atom bearing the hydroxyl group in ethanolamine, then a primary isotope effect is observed for the transfer of hydrogen via the 5′-methylene group of DMBC[8]. Furthermore, when the carbon atom bearing the hydroxyl group in ethanolamine is labelled with ^{14}C then the acetaldehyde formed was shown to be labelled in the aldehyde carbon[2]. These last two experiments confirm that the transfer of the amino group is preferred to transfer of the hydroxyl group in this deamination reaction.

In this communication we show that for the modified substrate 2-ethoxyethylamine, labelled with tritium in the amino carbon, intramolecular transfer of hydrogen occurs via the 5′-methylene group of DMBC. Furthermore, for the substrate ethanolamine, labelled with tritium in the amino carbon, exchange with the 5′-methylene group of DMBC similarly occurs. These experiments together with photolysis experiments with DMBC confirm that the 5′-methylene radical is involved in hydrogen abstraction to generate a substrate radical which can rearrange to accept hydrogen from the 5′-methyl group of 5′-deoxyadenosine generated in the enzyme.

MATERIALS

Homogeneous ethanolamine-ammonia lyase apoenzyme was a generous gift from Dr B. M. Babior. DMBC was synthesized by the method described by Law *et al.*[6]. Deuterated lithium aluminum hydride ($LiAl^2H_4$) was purchased from Alpha Inorganic Company. 2-Ethoxyethylamine was purchased from Aldrich Chemical Company. Terra Marine Bioresearch supplied the 5′-deoxyadesine. Tritiated lithium aluminum hydride ($LiAl^3H_4$) 100 Ci/mole was obtained from New England Nuclear. 2-Amino-[2-$^3H_2$]ethanol (320 Ci/mole) was obtained from Amersham–Searle. All the above chemicals were used without further purification.

METHODS

Enzyme assays

Ethanolamine-ammonia lyase enzyme activity towards 2-ethoxyethylamine

was determined by coupling the formation of acetaldehyde to yeast alcohol dehydrogenase. K_m and V values were determined for 2-ethoxyethylamine by varying the concentration of this substrate in an assay mixture containing 0.1 μmole NADH, 5 units of yeast alcohol dehydrogenase and 3.0 μg of ethanolamine-ammonia lyase apoenzyme in 1.0 ml of 0.05 M phosphate (K^+) buffer, pH 7.4. Reactions were started by adding 1.0 nmole of DMBC. The concentration of deuterated 2-ethoxyethylamine was determined by the ninhydrin method described by Gornall[9]. The concentration of tritiated 2-ethoxyethylamine was determined by its specific activity.

Isolation and characterization of enzymatic reaction products from 2-ethoxyethylamine

Acetaldehyde was characterized as its dimedone adduct and as its 2,4-dinitrophenylhydrazone. Ethylamine was characterized as its phenylthiourate derivatives.

The dimedone adduct was prepared according to the method described by Abeles *et al.*[10]. It was crystallized from ethanol–water and had a melting point of 136–140 °C (literature value, 139 °C)[10]. The 2,4-dinitrophenylhydrazone of the enzymatic reaction products from tritiated 2-ethoxyethylamine was characterized by comparing the R_F values with that from acetaldehyde in *n*-butanol–ethanol–water (7:1:2, by vol.), *tert*-amyl alcohol–ammonium hydroxide–ethanol–water (5:3:1:1, by vol.) and chloroform. Eastman 6060 silica gel thin-layer chromatographic plates were used. The tritium present in the hydrazone was determined by scintillation counting. The results of this experiment are presented in Table I.

TABLE I

CHROMATOGRAPHY OF THE 2,4-DINITROPHENYLHYDRAZONE FORMED FROM 2-ETHOXYETHYLAMINE

Solvent 1, *n*-butanol–ethanol–H_2O (7:1:2, by vol.); Solvent 2, *t*-amyl alcohol–NH_4OH–ethanol–H_2O (5:3:1:1, by vol.); Solvent 3, chloroform.

Solvent	*R_F values*		*Radioactivity (dpm)*
	Standard acetaldehyde 2,4-dinitrophenylhydrazone	*Reaction product from 2-ethoxyethylamine*	
1	0.68	0.68	190 000
2	0.71	0.70	250 000
3	0.64	0.63	130 000

The phenylthiourate derivatives of enzymatic reaction products were prepared as described by Schriner *et al.*[11]. After recrystallizing from 95% ethanol, the derivative had a melting point at 105 °C (authentic phenylthiourate derivative of ethylamine melts at 106 °C). This phenylthiourate derivative was characterized further by its NMR spectrum in [^{2}H]chloroform. The spectrum of the phenylthiourate product was compared to the phenylthiourate derivative of the substrate 2-ethoxyethylamine (Fig. 1). Integration of the triplet at 1.08 δ and the aromatic protons at 7.2 δ gives a ratio of 3 to 5. This NMR spectrum is consistent with that of the phenylthiourate derivative of ethylamine where the methyl group (triplet) has 3 protons and the aromatic ring has 5 protons.

The synthesis of deuterated 2-ethoxyethylamine

2-Ethoxyethylamine deuterated in the amino carbon was prepared by the

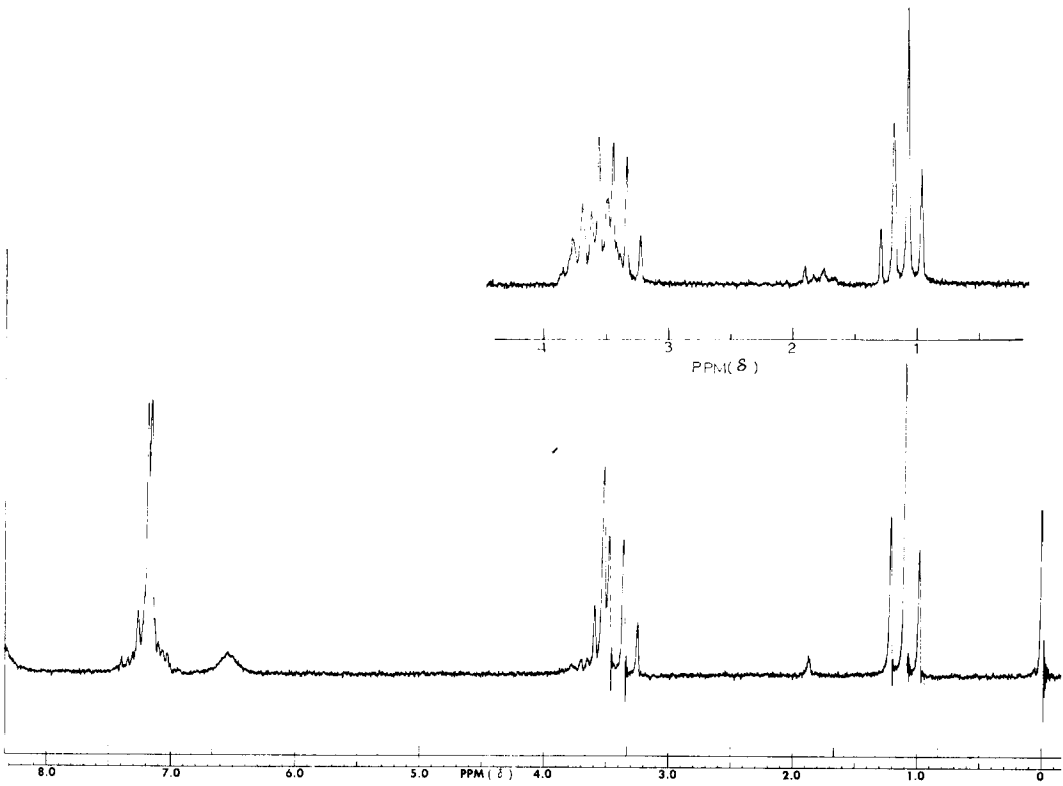


Fig. 1. H NMR spectrum in [^{2}H]chloroform of the phenylthiourate derivative isolated from the enzymatic conversion of 2-ethoxyethylamine by ethanolamine-ammonia lyase. Inset is the H NMR spectrum of the 2-ethoxyethylamine thiourate derivative.

reduction of ethoxyacetamide with $LiAl^2H_4$ in anhydrous diethyl ether. Diethyl ether was dried by distillation over $LiAlH_4$. 2 g (0.0193 mole) of ethoxyacetamide was dissolved in 200 ml of dry diethyl ether and added carefully to 1.65 g of $LiAl^2H_4$ in 150 ml of diethyl ether. This mixture was refluxed for 36 h. The residual $LiAl^2H_4$ was removed by dropwise addition of water at 4 °C, and after the reaction mixture stopped gassing, 10.0 ml of 20% (w/v) potassium tartrate was added. After filtration, the filtrate was drained over anhydrous sodium sulfate and the diethyl ether was removed by vacuum distillation. The reaction product was purified by fractional distillation at 104–107 °C. Yield 44%.

The phenylthiourate derivative of deuterated ethoxyethylamine was prepared, and this derivative gave the following elemental analysis. Theoretical: C, 58.40; H, 6.19; N, 12.39; S, 14.15%. Found: C, 57.70; H, 6.12; N, 12.27; S, 14.60%. The structure of the deuterated 2-ethoxyethylamine was confirmed by NMR (Fig. 2). When ethoxyacetamide is reduced with $LiAlH_4$ and 2-ethoxyethylamine is isolated, then a triplet is present at $\delta = 2.88$ (Fig. 2b). Furthermore, the simple quartet of ethoxyacetamide at $\delta = 3.5$ (Fig. 2a) gives a very complex splitting pattern in 2-ethoxyethylamine (Fig. 2b). When ethoxyacetamide is reduced with $LiAl^2H_4$, the triplet at $\delta = 2.85$ is lost (Fig. 2c), and the signal at $\delta = 3.56$ is resolved into an

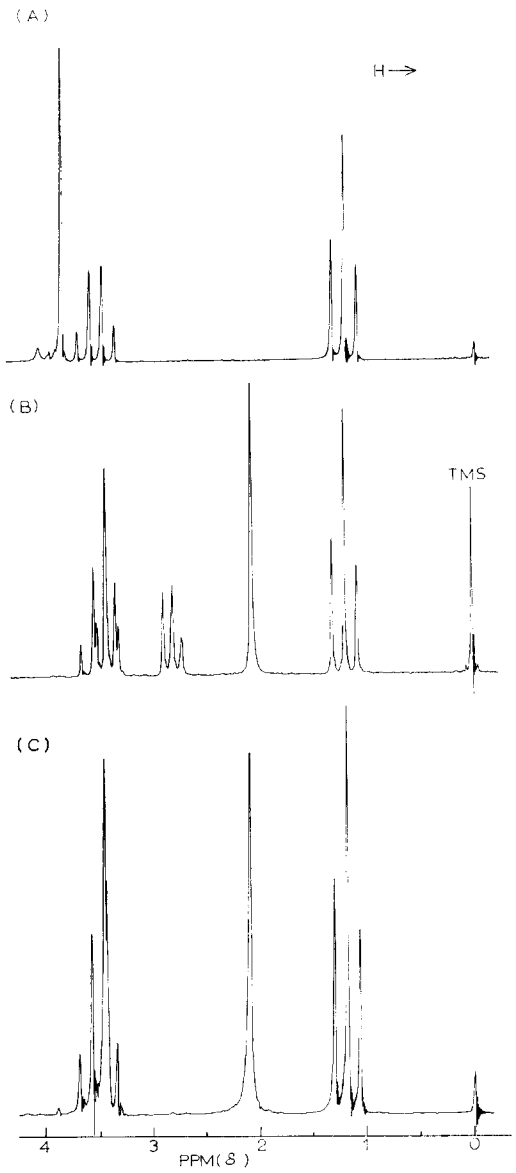


Fig. 2. H NMR spectra of (a) ethoxyacetamide, (b) 2-ethoxyethylamine, and (c) 2-ethoxy-[1-$^2H_2$]ethylamine in [^{2}H]chloroform.

overlapping quartet and a singlet. This proves that the signal at $\delta = 2.88$ is due to the protons on the amino carbon, and that the signal at $\delta = 3.55$ is due to the protons on the carbon bearing the ethoxy group together with methylene protons of the ethoxy group itself. The singlet at $\delta = 3.47$ is due to the methylene protons of the carbon bearing the ethoxy group, and the quartet at $\delta = 3.55$ is due to the methylene group of the ethoxy group itself split by the protons of the adjacent methyl

group. The signal at $\delta = 2.18$ represents the protons on the amino group and is found in both 2-ethoxyethylamine and deuterated 2-ethoxyethylamine (Figs 2b and 2c).

The synthesis of tritiated 2-ethoxyethylamine

2-Ethoxyethylamine (tritiated in the amino carbon) was prepared by the reduction of ethoxyacetamide with $LiAl^3H_4$ using an identical procedure to that described for the synthesis of deuterated 2-ethoxyethylamine. The tritiated product was checked for purity by chromatography in *n*-butanol–acetic acid–water (4:1:5, by vol.) on Eastman 6064 cellulose thin-layer chromatographic plates. Tritiated 2-ethoxyethylamine and control 2-ethoxyethylamine were detected by spraying with ninhydrin ($R_F = 0.59$). For this synthesis the specific activity was determined as 50 Ci/M, and the yield from the synthesis was 63%. 0.12 g of ethoxyacetamide was reduced with 38 mg of $LiAl^3H_4$ (100 Ci/M) to obtain the above yield and specific activity.

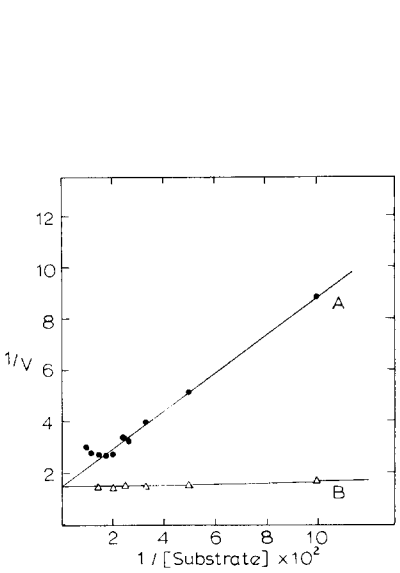
Tritium transfer to DMBC

Tritium incorporation into DMBC for both ethanolamine (tritiated in the amino carbon) and 2-ethoxyethylamine (tritiated in the amino carbon) was studied at different ethanolamine-ammonia lyase to substrate ratios. Since ethanolamine-ammonia lyase has two active sites, the ratio of active sites to substrate was considered at all times. In the case of tritiated ethanolamine, different concentrations of substrate were added to 0.8 mg of ethanolamine-ammonia lyase apoenzyme and 1.0 mg of DMBC in 0.3 ml 0.05 M phosphate (K^+) buffer at pH 7.4. Different concentrations of tritiated 2-ethoxyethylamine were added to 1.6 mg of apoenzyme and 1.0 mg DMBC in 0.3 ml 0.05 M phosphate (K^+) buffer pH 7.4. Such reaction mixtures were incubated at 4 °C in the dark for 1 h. Then the DMBC was reisolated from 20 μl of reaction mixture together with residual substrate, and these two compounds were separated by paper electrophoresis in 0.025 M sodium acetate buffer at pH 4.1 at a potential of 350 V for 2 h. Under these conditions DMBC migrates approximately 1.0 cm, whereas unreacted substrate migrates approximately 11.5 cm towards the anode. DMBC was located by its red color, and substrate was detected by spraying with ninhydrin. DMBC spots were eluted from the paper with distilled water into scintillation vials and tritium incorporation into DMBC was determined by scintillation counting in a Beckman LS-133 scintillation spectrometer using the toluene and ethanol scintillation fluid described by Ziegler *et al.*[12] and the procedure modified by Drysdale[13].

RESULTS

2-Ethoxyethylamine as a substrate for ethanolamine-ammonia lyase

Lineweaver–Burk plots for the substrates ethanolamine and 2-ethoxyethylamine are compared in Fig. 3. 2-Ethoxyethylamine has the same V as ethanolamine but a much larger K_m ($4.3 \cdot 10^{-3}$ M) compared to the K_m for ethanolamine ($2.9 \cdot 10^{-5}$ M)[14]. It is expected that the replacement of the hydroxyl substituent with an ethoxy substituent should weaken substrate binding to the enzyme. Furthermore, significant substrate inhibition is observed with 2-ethoxyethylamine at substrate concentrations greater than 8.0 μmoles. A similar V value for both ethanolamine and 2-ethoxy-



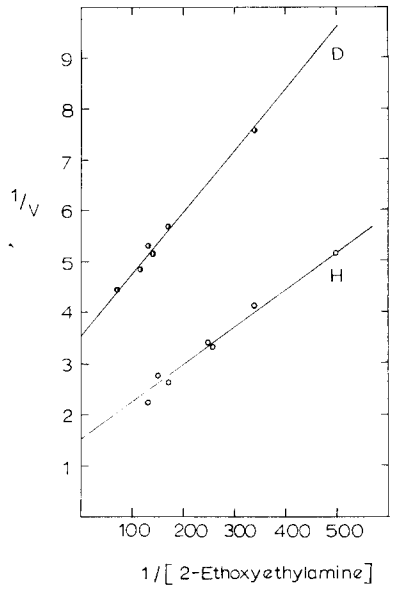


Fig. 3. Lineweaver–Burk plots of ethanolamine ammonia-lyase using (a) 2-ethoxyethylamine (●—●), and (b) ethanolamine (△ - - △) as substrate, respectively. For assaying mixtures, see text. The reactions were started by adding 1.0 nmole of DMBC. The rates of the reactions were observed by measuring absorbance at 340 nm with respect to time.

Fig. 4. Lineweaver–Burk plots of ethanolamine-ammonia lyase using (a) 2-ethanolamine (○—○), and (b) 2-ethoxy-[1-$^2H_2$]ethylamine (◑—◑) as substrates, respectively. Assay mixtures and conditions are the same as those described in Fig. 3.

ethylamine indicates the possibility of the formation of a similar enzyme–substrate complex with each substrate.

Isotope effects

A deuterium isotope effect was determined spectrophotometrically by comparing the activity of ethanolamine-ammonia lyase for 2-ethoxyethylamine and 2-ethoxyethylamine (deuterated in the amino carbon) (Fig. 4). For each substrate a different V was observed and an isotope effect of $k_H/k^2_H = 2.2$ was determined. With 2-ethoxyethylamine (tritiated in the amino carbon) a tritium isotope effect was determined by measuring the %H and %^{3}H transfer by the enzyme with time. The %H transferred was determined spectrophotometrically, and the %^{3}H transferred was determined by using the relationship:

$$S^3_H = 1 - \frac{2(P^3_H - 0.5\ P_H)}{100}$$

where P^3_H and P_H are percentages of ^{3}H and H transferred[8]. S^3_H refers to that amount of the 2-ethoxyethylamine (tritiated in the amino carbon) for which tritium is transferred instead of hydrogen, and represents the fraction of molecules which are unchanged at the end of the reaction. From these data the isotope effect for tritium can be calculated from the formula:

$$\gamma \ln S^3_H = \ln S_H$$

TABLE II

TRITIUM ISOTOPE EFFECT IN THE REACTION OF ETHANOLAMINE DEAMINASE USING 2-ETHOXY-[1-$^3H_2$]ETHYLAMINE AS SUBSTRATE

Reaction mixtures contained 10 μmoles of 2-ethoxyethylamine, 38 nmoles of 2-ethoxyethylamine (tritiated in the amino carbon, specific activity 50 Ci/mole), 0.1 μmole of NADH, 5.0 units of alcohol dehydrogenase, 8.3 nmoles of DMBC, 12 μg of ethanolamine-ammonia lyase, and 50 μmoles of potassium phosphate buffer (pH 7.4) in a total volume of 1.05 ml. Reactions were run at room temperature in the dark and were stopped by photolysis for 30 s with a projector (350 W bulb) under aerobic conditions.

Incubation time (min)	*Hydrogen Transferred*		*Tritium isotope effect*
	%H	*%^3H*	
3	6.9	4.4	3.9
5	11.5	7.6	3.3
7	14.4	8.98	4.0
10*	21.9	13.8	4.3
20**	11.5	4.6	5.0

* 16.6 nmoles of DMBC added to the reaction mixture.
** Reaction mixture incubated at 4 °C.

where S_H is the amount of protonated substrate remaining when the reaction is stopped, and γ is the kinetic isotope effect[15]. The results of these experiments are shown in Table II. From these data the average $k_H/k^3_H = 4.0$. This isotope effect is independent of the concentration of DMBC added to reaction mixtures, but dependent on the temperature of incubation. At 4 °C k_H/k^3_H increases to 5.0 which is indicative of a primary isotope effect for tritium. The theoretical tritium isotope effect was determined as $k_H/k^3_H = 3.5$ by applying the Swain relationship[16].

$$K^3_H/K_H = 1.11\ (K^2_H/K_H)$$

Tritium incorporation into DMBC

Tritium incorporation into DMBC was measured at various ethanolamine-ammonia lyase to substrate ratios using both 2-ethoxyethylamine (tritiated in the amino carbon) and ethanolamine (tritiated in the amino carbon). For both substrates there is an increase in the quantity of tritium incorporated into DMBC with an increase in the substrate to enzyme ratio (Fig. 5). Incorporation of tritium is independent of the substrate to DMBC ratio (Fig. 5b).

For tritium incorporation, both tritiated substrates exhibit saturation effects with DMBC bound to ethanolamine-ammonia lyase. For tritiated ethanolamine (spec. act. = 320 Ci/mole), saturation occurs at $2.75 \cdot 10^6$ dpm; for tritiated 2-ethoxyethylamine (spec. act. = 50 Ci/mole), saturation occurs at $6.6 \cdot 10^5$ dpm. Since two molecules of DMBC are bound to each molecule of apoenzyme[17], then if tritium incorporation is dependent on the amount of DMBC bound, the maximum theoretical incorporation of tritium is 4 per molecule of apoenzyme or 2 per active site. From the amount of apoenzyme used in each tritium incorporation experiment (1.54 nmoles in tritiated ethanolamine experiments and 3.1 nmoles in the tritiated ethoxyethylamine experiments), the maximum theoretical incorporation can be calculated. For tritiated ethanolamine, this value would be $2.5 \cdot 10^6$ dpm; and for tritiated 2-ethoxyethylamine this would be $6.2 \cdot 10^5$ dpm. These theoretical values are close to the experimental results indicating and confirming that 2 DMBC molecules bind to

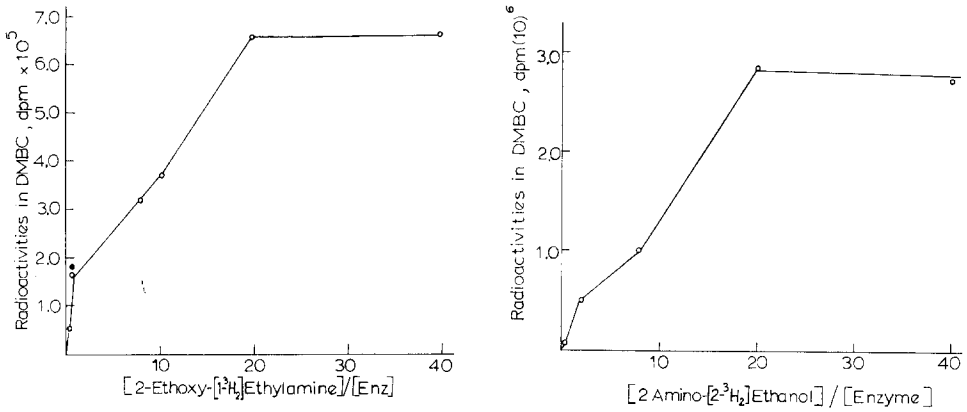


Fig. 5. (a) Incorporation of tritium into DMBC from 2-amino-[2-$^3H_2$]ethanol at different substrate to active site ratios. The incubation mixtures contained 0.8 mg apoenzyme, 1 mg DMBC and various amount of tritiated ethanolamine in 0.3 ml of phosphate (K^+) buffer, pH 7.4. The mixtures were incubated for 1 h in the dark at 4 °C. DMBC was isolated by electrophoresis (see text). (b) Incorporation of tritium into DMBC from 2-ethoxy-[1-$^3H_2$]ethylamine at different substrate to active site ratios. 1.6 mg of apoenzyme was used in the reaction mixtures. (●) indicates 2 mg of DMBC was in reaction mixtures and (○) indicates 1 mg of DMBC in reaction mixtures.

ethanolamine-ammonia lyase apoenzyme and that both DMBC molecules function independently in catalysis.

The position of tritium incorporation into DMBC from 2-ethoxyethylamine was confirmed by photolysis and separation of the photolysed products on a Sephadex G-25 gel filtration column. The reaction mixture set up for the transfer of tritium into DMBC (Methods) from 2-ethoxyethylamine (tritiated in the amino carbon) was taken and 0.2 ml was lyophilized to remove low-boiling components. The resulting powder was dissolved in 0.02 ml of 0.05 M phosphate (K^+) buffer pH 7.4 and applied to a Sephadex G-25 column (1 cm × 40 cm). Components in the reaction products were separated in the dark on this column which operated with a flow rate of 0.5 ml/min; and 0.3-ml fractions were collected. Each fraction was diluted with 0.5 ml of 0.05 M phosphate (K^+) buffer, pH 7.4. Protein was determined by the method of Lowry *et al.*[18]. DMBC concentration was determined at 500 nm, and tritium incorporation was determined by the scintillation counting procedure, outlined in Methods. Fig. 6 shows that the majority of tritium incorporation is associated with the DMBC profile and not with the protein profile. Toraya *et al.*[19] reported the dissociation of DMBC from the holoenzyme dioldehydrase in the absence of K^+. But in this case, the dissociation of the DMBC from the ethanolamine-ammonia lyase holoenzyme is due to the denaturation of the protein as indicated by the loss of activity after lyophilization.

When an identical column was run with the reaction mixture after aerobic photolysis, then the tritium incorporated did not coincide with the corrinoid elution profile, but the radioactivity profile did appear in the same fractions as an adenosine (C-8 tritiated) marker[20]. Since the aerobic photolysis product of DMBC is adenosine-5′-aldehyde[21], then these data show that the tritium is incorporated into the nucleoside moiety. It can be demonstrated that the tritium is incorporated into the 5′-

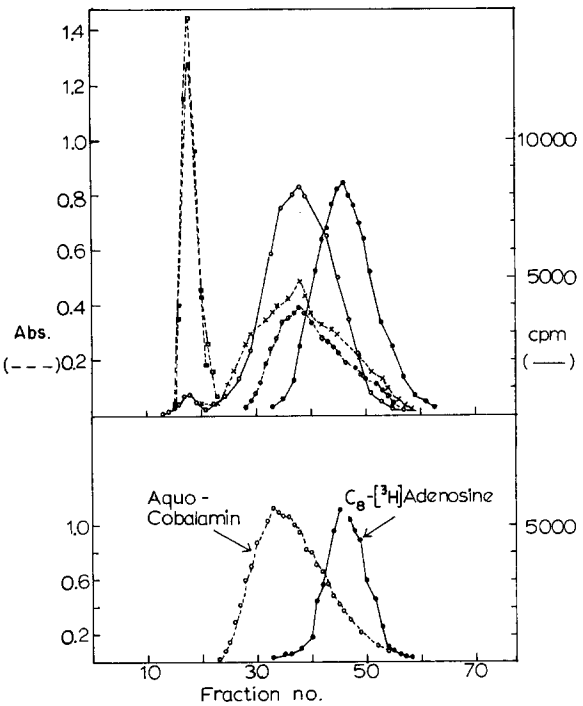


Fig. 6. Elution profile for reaction products from Sephadex G-25. Protein concentrations were determined by the method of Lowry *et al.*[18] before photolysis (□—□) and after photolysis (■—■). B_{12} concentrations were measured by absorbance at 500 nm before photolysis (×—×) and after photolysis (◑—◑). Tritium incorporation was measured before photolysis (○—○) and after photolysis (●—●). Lower elution profile are those of the standards aquocobalamin and adenosine (tritiated at C-8).

methylene carbon of the adenosyl moiety by permanganate oxidation of the adenosine-5′-aldehyde to adenosine-5′-carboxylic acid[22]. When this oxidation was carried out with the adenosine-5′-aldehyde isolated from the tritium incorporation experiments, and the solvent was removed by lyophilization, it was determined that only 3.5 ± 0.2% of the original radioactivity associated with DMBC remained with the residue. This experiment demonstrates that tritium is incorporated into the 5′-methylene carbon of the adenosyl moiety. When C-2 tritiated ethanolamine was used as an alternative to tritiated 2-ethoxyethylamine, identical elution profiles were observed.

In the case of 2-ethoxyethylamine, if the transfer of hydrogen from the substrate to the 5′-methylene group of DMBC is from the amino carbon directly then it would be expected that 33.3% of the tritium should be incorporated into DMBC when the substrate to active site ratio is 1:1. After homolytic cleavage of the Co–C bond then the abstraction of one tritium from the amino carbon of tritiated 2-ethoxyethylamine by the 5′-methylene radical generated by homolysis will give a methyl group at the 5′-methylene of 5′-deoxyadenosine which contains one tritium. Therefore, only-half of the tritium from substrate is transferred to form 5′-deoxyadenosine, and only one-third of the tritium in the 5′-methyl group could be transferred back to the product theoretically. Experimentally, at the substrate to active

site ratio of 1:1, 26.5 ± 3.5% incorporation of tritium was observed. A value lower than the theoretical 33.3% was obtained, and this could be due to the fact that at certain active sites, the catalytic cycle occurred more than once. In such a case, the probability for the tritium incorporated into the 5′-deoxyadenosine being transferred back to the product is higher. This tritium incorporation cannot be due to exchange from the methyl group of the reaction product acetaldehyde[6,7], since only one-third of the tritium on the acetaldehyde methyl group could be transferred to DMBC when the ratio of acetaldehyde to active sites is 1:1. In this case, a maximum of 22.2% tritium could be incorporated into DMBC theoretically.

When 2-amino-[2-$^3H_2$]ethanol was used as a substrate then tritium is incorporated into the 5′-methylene carbon of the adenosyl moiety of DMBC. This experimental result is not in accordance with that previously obtained by Babior[8] which shows that tritium exchange with DMBC involves tritium at the carbon atom bearing the hydroxyl group. This incorporation can be explained either on the basis of exchange of tritium from the product acetaldehyde[6,7] or on the basis of a lack of specificity for hydrogen abstraction by the 5′-methylene radical generated by the addition of ethanolamine to ethanolamine-ammonia lyase holoenzyme. At the 1:1 substrate to active site ratio, the amount of tritium incorporated into DMBC never exceeded 10%. Together with the consideration that abstraction of hydrogen is more kinetically favorable than the abstraction of tritium, this low value of tritium incorporation into DMBC at a 1:1 substrate to active site ratio eliminates the possibility of direct transfer of tritium from the amino carbon of ethanolamine to DMBC.

Photolysis experiments

When DMBC is photolysed under anaerobic conditions then the 5′-methylene radical generated by homolysis of the Co–C bond reacts with C-8 of adenosine to give 8,5′-cyclic adenosine[21]. When this same reaction is conducted in the presence of oxygen, then the 5′-methylene radical reacts with oxygen to give adenosine 5′-aldehyde[21]. The following experiments were designed to test whether the 5′-methylene radical generated by photolysis under anaerobic conditions was long lived enough to react with substrate molecules ethanolamine and 2-ethoxyethylamine, respectively, by similar mechanisms to those observed with ethanolamine-ammonia lyase.

2-Ethoxyethylamine (tritiated in the amino carbon) was dissolved to a concentration of 10^{-2} M in 1.0 ml of 0.05 M phosphate (K^+) buffer, pH 7.4. To this solution 0.2 ml of a solution of 10 mg/ml DMBC was added with mixing, and the solution was divided into two equal 0.6-ml aliquots. One aliquot was photolysed under strictly anaerobic conditions and the second sample was photolysed in the presence of oxygen. Photolysis was conducted for 1 h with a 200-W tungsten filament source placed 20 cm from each sample. After this time 10 μl of each sample was spotted on 6 cm × 30 cm Whatman No. 1 paper for electrophoresis in 0.05 M phosphate buffer at pH 7.0 for 1 h at a potential of 350 V. Electrophoresis in this system separated unreacted 2-ethoxyethylamine from the nucleoside and aquocobalamin. The aquocobalamin and nucleoside were eluted from the paper and counted for tritium incorporation. An identical experiment with ethanolamine (tritiated in the amino carbon) was conducted also, and the results for these experiments are presented in Table III.

From the concentration of DMBC used in the above reactions if we assumed

TABLE III

TRITIUM INCORPORATION INTO THE NUCLEOSIDE FORMED BY THE ANAEROBIC PHOTOLYSIS OF DMBC IN THE PRESENCE OF SUBSTRATES

Substrates tritiated in the amino carbon	*Total tritium incorporated into the nucleoside (dpm)*		*B–A (dpm)*
	(A) Aerobic photolysis	*(B) Anaerobic photolysis*	
Ethanolamine	$6.04 \cdot 10^6$	$8.9 \cdot 10^6$	$2.8 \cdot 10^6$
2-Ethoxyethylamine	$9.7 \cdot 10^7$	$1.27 \cdot 10^8$	$3.0 \cdot 10^7$

that the 5′-methylene radical generated by homolysis will abstract hydrogen from each respective substrate, then the theoretical tritium incorporation into the nucleoside can be calculated. For 2-ethoxyethylamine (tritiated in the amino carbon) theoretically $2.8 \cdot 10^7$ dpm would be incorporated if tritium is selectively removed from the amino carbon. Experimentally it was shown that $3.0 \cdot 10^7$ dpm was incorporated into the nucleoside from tritiated 2-ethoxyethylamine (Table III). For ethanolamine (tritiated in the amino carbon) the theoretical incorporation was calculated as $1.13 \cdot 10^7$ dpm, and experimentally 24.8% of this value was found (*i.e.* $2.8 \cdot 10^6$ dpm).

The identity of the nucleoside was determined by thin-layer chromatography. The nucleoside was separated from the corrinoid and unreacted tritiated substrate by passing the lyophilized reaction mixture down a 3 cm × 20 cm Sephadex G-10 column. Unreacted substrate formed a tight complex with aquocobalamin as indicated by the association of the major radioactivity peak with the corrinoid peak. The nucleoside was located by measuring absorbance at 260 nm. Fractions containing the nucleoside were pooled and lyophilized. The residue was redissolved in 95% ethanol and spotted on Eastman 6064 cellulose chromatogram sheets together

TABLE IV

IDENTIFICATION OF THE NUCLEOSIDE FORMED FROM THE ANAEROBIC PHOTOLYSIS OF DMBC IN THE PRESENCE OF SUBSTRATES

Solvent 1, *n*-butanol–acetic acid–H_2O (4:1:5, by vol., upper layer); Solvent 2, *n*-butanol–H_2O (43:7, by vol.); Solvent 3, 90% ethanol–1 M NH_4Cl (14:6, by vol.).

Solvent	R_F	*Radioactivity incorporated into the nucleoside*			
		2-Amino-[2-$^3H_2$]ethanol		*2-Ethoxy-[1-$^3H_2$]ethylamine*	
		dpm	*% Recovery*	*dpm*	*% Recovery*
1	0.69	92 320	93.8	28 050	88.9
2	0.56	156 680	93.0	37 360	63.2
3	0.71	104 930	90.4	11 750	92.9

with a standard solution of 5′-deoxyadenosine. Chromatograms were developed in solvents described by Babior[23]. Spots corresponding to 5′-deoxyadenosine were cut out and the radioactivity determined. As shown in Table IV, with one exception, over 90% of the radioactivity added was isolated from the spots co-chromatographing with 5′-deoxyadenosine.

From the two photolysis reactions acetaldehyde was characterized as a reaction

TABLE V

2,4-DINITROPHENYLHYDRAZONE DERIVATIVE ISOLATED FROM THE REACTION PRODUCTS OF THE PHOTOLYSIS EXPERIMENTS

Solvent 1, *n*-butanol–ethanol–H_2O (7:1:2, by vol.); Solvent 2, *tert*-amyl alcohol–NH_3–ethanol–H_2O (5:3:1:1, by vol.).

Solvent	*Acetaldehyde 2,4-dinitrophenylhydrazone (R_F)*	*Photolysis products (R_F)*	
		Ethanolamine	*2-Ethoxyethylamine*
1	0.63	0.63	0.64
2	0.69	0.68	0.67

product, both by isolating its 2,4-dinitrophenylhydrazone derivative, and by studying the reaction between the photolysis reaction products and yeast alcohol dehydrogenase (Table V). For assays with yeast alcohol dehydrogenase, the total reaction volume was 1.0 ml, which contained 5 units of alcohol dehydrogenase, 0.1 μmole of NADH, and variable quantities of reaction products from photolysis experiments. The rate of the alcohol dehydrogenase reaction was dependent on the volume of the photolysis reaction products added and on the amount of DMBC used in the photolysis experiments. The quantity of acetaldehyde produced was dependent on the concentration DMBC photolysed. When 0.1 ml from a 1.2-ml photolysis reaction mixture was added to 50 units of yeast alcohol dehydrogenase with 0.1 μmole of NADH in 1.0 ml of phosphate buffer, then the absorbance at 340 nm decreased by 0.551 ± 0.047 unit. Using the molar extinction coefficient of NADH at 340 nm ($6.22 \cdot 10^3$ $M^{-1} \cdot cm^{-1}$), the total amount of acetaldehyde produced was determined to be 1.06 ± 0.09 nmoles. If there is a 1:1 correlation between the nucleoside formed from the photolysis of DMBC and the acetaldehyde produced, the maximum theoretical value for acetaldehyde produced from 2 mg of DMBC is 1.26 nmoles. These photolysis experiments show that the 5′-methylene radical generated by photolysis of DMBC specifically abstracts hydrogen from the amino carbon of 2-ethoxyethylamine, but abstracts hydrogen from both carbon atoms of ethanolamine with a preference for those hydrogens on the carbon atom bearing the hydroxyl group.

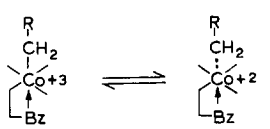
DISCUSSION

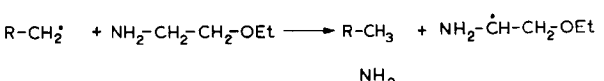
A comparative study between hydrogen transfer from the substrates ethanolamine and 2-ethoxyethylamine via the 5′-methylene radical generated from DMBC in both ethanolamine-ammonia lyase and in non-enzymatic photolysis experiments has allowed conclusions to be drawn pertaining to the mechanism of ethanolamine-ammonia lyase.

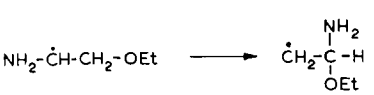
In the photolysis experiments reported here the 5′-methylene radical is expected to abstract hydrogen by a mechanism which is both kinetically and thermodynamically favorable. In this context, it has been shown that the hydrogens on both carbon atoms of tritiated ethanolamine are abstracted with some preference for those hydrogens at the carbon atom bearing the hydroxyl group, and preference for these hydrogen atoms is probably due to kinetic considerations. It is to be expected that if tritium replaces hydrogen on the amino carbon of ethanolamine, then the

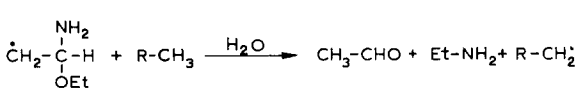
5′-methylene radical will favor hydrogen abstraction from the other carbon atom, and this explains why only 24.8% tritium was incorporated into the nucleoside. However, when the hydroxyl substituent of ethanolamine is replaced by an ethoxyl group, then hydrogen abstraction by the 5′-methylene radical is preferred at the amino carbon, because the radical generated at the amino carbon is thermodynamically more stable than that which would be generated at the other carbon. Previous results[24] indicated in the presence of alcohol or compounds which are readily attacked by radicals, anaerobic photolysis of methylcobalamin was greatly enhanced. This was explained by the abstraction of the α-hydrogens in alcohols by the methyl radical generated by photolysis. Similar considerations can be made with the anaerobic photolysis of DMBC in the presence of substrates. The methylene radical will abstract the hydrogen which is most available. There is little difference in the electronegativity of the two carbons of ethanolamine; thus non-specific abstraction of the hydrogens occurs in the photolysis experiments. Non-specific abstraction does not occur in the enzymatic deamination of ethanolamine. This could be due to the orientation of the substrate molecule in the active site which does not allow the abstraction of hydrogen from the amino carbon.

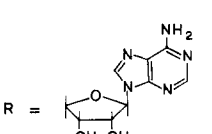
With ethanolamine-ammonia lyase we have demonstrated a primary isotope effect for hydrogen transfer to DMBC for one of the hydrogens at the amino carbon of 2-ethoxyethylamine. Babior[7] has demonstrated a primary isotope effect for one of the hydrogens at the hydroxyl carbon of ethanolamine, and we have shown that hydrogens at the amino carbon of ethanolamine are transferred to DMBC indirectly. These data are in accord with our observations in photolysis experiments with these substrates. Furthermore, for both substrates, acetaldehyde has been shown to be produced in enzymatic and in photolysis experiments. The amino-ethyl ether formed from the rearrangement catalysed by the 5′-methylene radical formed from DMBC is expected to be unstable in a protonic environment[25]. A general mechanism for the











reaction catalysed by the 5'-methylene radical formed from DMBC in photolysis experiments or in ethanolamine-ammonia lyase is presented in Scheme 1.

ACKNOWLEDGEMENT

We thank Dr B. M. Babior for his gift of homogeneous ethanolamine-ammonia lyase apoenzyme.

This research was supported by a grant from the U.S. Public Health Service AM 12599.

REFERENCES

1 Babior, B. M. (1968) *Biochim. Biophys. Acta* 167, 456–458
2 Kaplan, B. H. and Stadtman, E. R. (1968) *J. Biol. Chem.* 243, 1787–1793
3 Babior, B. M. and Gould, D. C. (1969) *Biochem. Biophys. Res. Commun.* 34, 441–447
4 Babior, B. M., Moss, T. H. and Gould, D. C. (1972) *Fed. Proc.* 31, 493
5 Babior, B. M., Moss, T. H. and Gould, D. C. (1972) *J. Biol. Chem.* 247, 4389–4392
6 Law, P. Y., Brown, D. G., Lien, E. L., Babior, B. M. and Wood, J. M. (1971) *Biochemistry* 10, 3428–3435
7 Carty, T. J., Babior, B. M. and Abeles, R. H. (1971) *J. Biol. Chem.* 246, 6313–6317
8 Babior, B. M. (1969) *J. Biol. Chem.* 244, 449–456
9 Gornall, A. G., Bardawill, C. J. and David, M. M. (1949) *J. Biol. Chem.* 177, 751–766
10 Abeles, R. H. and Lee, H. A. (1963) *J. Biol. Chem.* 238, 2367–2373
11 Schriner, R. L., Fuson, R. C. and Curtin, D. Y. (1965) *The Systematic Identification of Organic Compounds*, pp. 249–261, John Wiley and Son, New York
12 Ziegler, C. A., Chleck, D. J. and Brinkerhoff, J. (1957) *Anal. Chem.* 29, 1774–1776
13 Drysdale, G. N. (1959) *J. Biol. Chem.* 234, 2399–2403
14 Bradbeer, C. (1965 *J. Biol. Chem.* 240, 4675–4681
15 Melander, L. (1960) *Isotope Effect on Reaction Rate*, p. 24, Ronald Press, New York
16 Swain, C. G., Stivers, E. C., Shaad, E. J. and Renever, J. F. (1958) *J. Am. Chem. Soc.* 80, 5885–5893
17 Babior, B. M. and Li, T. K. (1969) *Biochemistry* 8, 154–160
18 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* 193, 265–275
19 Toraya, T., Sugimoto, Y., Tamao, Y. and Shimizu, S. (1971) *Biochemistry* 10, 3475–3484
20 Shelton, K. R. and Clark, Jr, J. M. (1967) *Biochemistry* 6, 2735–2739
21 Hogenkamp, H. P. C. (1964) *Ann. N.Y. Acad. Sci.* 112, 551–564
22 Frey, P. A., Essenberg, M. K. and Abeles, R. H. (1967) *J. Biol. Chem.* 242, 5369–5377
23 Babior, B. M. (1970) *J. Biol. Chem.* 245, 6125–6133
24 Yamada, R., Shimizu, S. and Fukui, S. (1966) *Biochim. Biophys. Acta* 124, 195–197
25 Steward, T. D. and Bradley, W. E. (1932) *J. Am. Chem. Soc.* 54, 4172–4183