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Microbial Metabolism of Amino Alcohols

PURIFICATION AND PROPERTIES OF COENZYME B_{12}-DEPENDENT ETHANOLAMINE AMMONIA-LYASE OF *ESCHERICHIA COLI*

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1. The 120-fold purification of ethanolamine ammonia-lyase from *Escherichia coli* extracts, to apparent homogeneity, is described. Ethanolamine, dithiothreitol, glycerol and KCl protected the apoenzyme from inactivation. 2. At the optimum pH 7.5, K_m values for ethanolamine and coenzyme B_{12} were 44μM and 0.42μM respectively. The K_m for ethanolamine was markedly affected by pH, transitions occurring at pH 7.0 and 8.35. 3. The enzyme was specific for ethanolamine as substrate, none of the 18 analogues tested being active. L-2-Aminopropan-l-ol (K_i 0.86μM), DL-1-aminopropan-2-ol (K_i 2.2μM) and DL-1,3-diaminopropan-2-ol (K_i 88.0μM) inhibited competitively. 4. Enzyme activity was inhibited, irreversibly and non-competitively, by the coenzyme analogues methyl-cobalamin (K_i 1.4nM), hydroxocobalamin (K_i 2.1nM) and cyanocobalamin (K_i 4.8nM). 5. Iodoacetamide inhibited in the absence of ethanolamine, but only slightly in its presence. *p*-Hydroxymercuribenzoate inhibited markedly even in the presence of ethanolamine. Dithiothreitol and 2-mercaptoethanol (less effectively) restored activity to the enzyme dialysed against buffer containing ethanolamine. 6. Although K^+ ions stabilized the enzyme during dialysis or storage, they were not necessary for activity. 7. Gel filtration showed the enzyme to be of high molecular weight, ultracentrifugal studies giving $s_{20,w}$ of 16.4 and an estimated mol.wt. 560400. The isoelectric point for the apoenzyme was approx. pH 5.0. Urea inhibited enzyme activity at concentrations above 1M (95% inhibition at 3M) and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis indicated protein subunits of mol.wt. 61400. 8. Immunological studies showed that the *E.coli* enzyme was closely related to those of other enterobacteria, but only distantly to that of *Clostridium* sp. A double precipitin band suggested that the apoenzyme may be made up of two protein components.

Ethanolamine ammonia-lyase (EC 4.3.1.7), until recently known only in a species of *Clostridium* (Bradbeer, 1965), has been found playing a catabolic role in strains of *Escherichia coli* and *Klebsiella aerogenes* (Scarlett & Turner, 1976). Nutritional evidence has suggested that a coenzyme B_{12}-dependent ethanolamine-deamination system operates in a number of enterobacteria (Chang & Chang, 1975; Clough *et al.*, 1975; Blackwell *et al.*, 1976).

The ethanolamine ammonia-lyase of *E. coli* is of particular interest in two respects. Its properties may be compared with those of the enzyme from the largely dissimilar *Clostridium* sp. and induction of the enzyme appears to require both the substrate and a cobalamin compound acting in concert (Blackwell *et al.*, 1977). The properties, particularly the mechanism of action, of the clostridial enzyme have been investigated extensively (Barker, 1972; Babior, 1975; Krouwer & Babior, 1977).

We report the purification of ethanolamine ammonia-lyase of *E. coli* to apparent homogeneity and describe some of its properties. Immunological evidence confirms that the enzyme is present in a variety of bacteria.

Materials and Methods

Micro-organisms and media

E. coli, the other bacteria used and a strain of *Corynebacterium*, were obtained from the National Collection of Industrial Bacteria, Aberdeen, Scotland, U.K., and are indicated by their appropriate accession numbers. *Erwinia carotovora* was obtained from the National Collection of Plant Pathogenic Bacteria, Harpenden, Herts., U.K. These bacteria were maintained aerobically on nutrient agar and cultivated on the simple synthetic media previously described (Scarlett & Turner, 1976; Blackwell *et al.*,

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Abbreviation used: SDS, sodium dodecyl sulphate.

1977). The ethanolamine-fermenting *Clostridium* sp., originally isolated by Hayward (1961), was generously given by Professor A. W. Johnson, University of Sussex, U.K. This *Clostridium* sp. received as a spore suspension, was grown anaerobically on semisynthetic medium as described by Joblin *et al.* (1976).

The growth of *E. coli* on media that induced ethanolamine ammonia-lyase formation was followed turbidimetrically as A_{540}^{1cm}. For high enzyme activity it was important for bacteria to be harvested at absorbance values less than 1.0, usually 0.95–1.0. Harvested bacteria, washed and resuspended in 0.02M-Tris/HCl, pH7.5, could be stored at −20°C for several weeks without loss of enzyme activity.

Assay of ethanolamine ammonia-lyase activity

A colorimetric assay similar to that described previously (Scarlett & Turner, 1976) was used as a routine for crude extracts and partly purified enzyme. Incubation mixtures contained, in a total volume of 1ml: Tris/HCl buffer, pH7.5, 100μmol; ethanolamine/HCl, 2μmol; coenzyme B_{12} (5′-deoxydecanosylcobalamin), 2nmol; enzyme. Reactions were usually started by the addition of enzyme, and incubation was at 37°C for 1–5min in dim light to minimize photolysis of the coenzyme. In some cases, reaction was started by the addition of cofactor to otherwise complete reaction mixtures. Iodoacetamide (5mM) was included in some reaction mixtures to inhibit selectively contaminating CoA-dependent acetaldehyde dehydrogenase. Reactions were stopped by the addition of 0.6ml of 0.1M-3,3-dimethylglutaric acid/NaOH buffer, pH4.0, and 0.4ml of 1% (w/v) 3-methyl-2-benzothiazolinone hydrazone reagent (Paz *et al.*, 1965; Sawicki & Sawicki, 1975). The acetaldehyde formed was measured colorimetrically as previously described (Jones & Turner, 1973), the value $\varepsilon_{670} = 6\times10^4$ litre·mol^{-1}·cm^{-1} being used. Phosphate interfered with this assay.

A spectrophotometric assay, coupling acetaldehyde formation to NADH oxidation in the presence of excess of alcohol dehydrogenase (Kaplan & Stadtman, 1968*a*), was used for partly purified and pure enzyme preparations. Reaction mixtures contained in a total volume of 1 ml: potassium phosphate buffer, pH7.5, 50μmol; ethanolamine/HCl, 10μmol; coenzyme B_{12}, 2nmol; yeast alcohol dehydrogenase, 5 units; NADH, 0.1μmol; enzyme. Reactions, started by the addition of enzyme, were carried out in a quartz cell at 37°C. Initial rates were measured by the ethanolamine-dependent oxidation of NADH at 340nm. With 0.005–0.010 units of enzyme/ml of reaction mixture, the rate of reaction was linear with time for at least 5min at 37°C. The value $\varepsilon_{340} = 6.22\times10^3$ litre·mol^{-1}·cm^{-1} for NADH (Horecker & Kornberg, 1948) was used for its measurement.

A unit of enzyme activity is defined as the amount that catalyses the formation of μmol of the product, or the transformation of 1μmol of the substrate, per min under the conditions described.

Purification of ethanolamine ammonia-lyase

Cell-free extracts of *E. coli* were prepared, treated with charcoal, fractionated with $(NH_4)_2SO_4$ and the active fraction was subjected to DEAE-cellulose chromatography essentially as described previously (Blackwell *et al.*, 1977). Minor modifications were that the ion-exchanger was equilibrated with 0.05M-Tris/HCl, pH7.5, containing 20% (w/v) glycerol, 4mM-dithiothreitol and 10mM-KCl; protein was eluted with a linear gradient of 0–0.5M-KCl in the same buffer mixture supplemented with 10mM-ethanolamine. All enzyme-containing fractions, eluted as a single peak, were pooled and concentrated by ultrafiltration. Volumes up to 50ml were concentrated by using a Millipore immersible molecular separator (Millipore Corp., Bedford, MA, U.S.A.), giving a concentration of 4–6mg of protein/ml. The concentrated enzyme was further purified by molecular exclusion chromatography on Bio-Gel A-5m agarose. A column of washed agarose beads (100–200mesh) was equilibrated with degassed 0.10M-Tris/HCl, pH7.5, containing 20% (w/v) glycerol, 4mM-dithiothreitol, 0.2M-KCl and 10mM-ethanolamine. The enzyme sample (4–8ml) was applied to a column (100cm×1.6cm) of the agarose and eluted with about 250ml of the equilibration buffer by downward flow. The hydrostatic pressure did not exceed 100cm of water and a flow rate of 7–8 ml/h was used. Further details are given in the Results section.

Protein in cell-free extracts was measured by a biuret procedure (Turner, 1966) or, in more dilute solutions (25–500μg/ml), by the method of Lowry *et al.* (1951). Protein in column fractions was estimated by the method of Warburg & Christian (1941) and checked, after concentration, by that of Lowry *et al.* (1951). Bovine serum albumin [Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K.] was used as the standard for protein assays.

Disc gel electrophoresis

Enzyme preparations were examined by polyacrylamide-gel electrophoresis by the general procedure of Ornstein & Davis (1964). Separating gels of 7.5% were used at a running pH of 8.9. Up to 100μl of sample (10–100μg of protein) was applied together with Bromophenol Blue tracking dye. After electrophoresis, protein bands were stained with Coomassie Brilliant Blue R250 by standard procedures.

Ultracentrifugal analysis of purified ethanolamine ammonia-lyase

Sedimentation studies were done with a Beckman model E analytical ultracentrifuge equipped with an AnD series rotor using standard procedures. The enzyme sample, used at 1 mg of protein/ml, was dialysed against potassium phosphate buffer, pH 7, *I* 0.10, containing 0.1 mM-ethanolamine and 1 mM-dithiothreitol, before ultracentrifuging. The sedimentation coefficient was determined by velocity centrifugation at 60000 rev./min in a double-sector cell with charcoal-filled centrepiece, at 10°C. The minimum molecular weight was calculated, making standard assumptions about the hydration, density and shape of the protein.

SDS/polyacrylamide-gel electrophoresis

Enzyme subunit size was investigated after denaturation with SDS by the general procedure of Weber *et al.* (1972). Pure ethanolamine ammonia-lyase (5–100 μg) was treated at 100°C with 1% SDS and 1% 2-mercaptoethanol in 0.1 M-sodium phosphate buffer, pH 7, for 2 min. After cooling, the sample was dialysed against 0.01 M-sodium phosphate buffer, pH 7.2, containing 0.1% SDS and 0.1% 2-mercaptoethanol. Dialysed samples (3–10 μg of protein) mixed with Bromophenol Blue tracking dye, were applied to 7.5% polyacrylamide separating gels containing 0.1% SDS, and run at pH 7. Electrophoretic mobilities were compared with those of similarly treated standard protein. These, supplied for the purpose (BDH Chemicals, Poole, Dorset, U.K.), were of accurately known molecular weights in the range 14300–71500.

Determination of isoelectric point for purified enzymes

The pI of ethanolamine ammonia-lyase was determined by isoelectric focusing using a flat bed LKB 2117 Multiphor electrophoresis apparatus (LKB-Produkter, ABS-16125 Bromma, Sweden). Ultradex polydextran gel (LKB) was swollen in the appropriate Ampholine solution, providing a pH gradient of 4–8. Electrofocusing was carried out and protein, enzyme activity and pH were measured in thin sections of gel, by standard procedures according to the manufacturers instructions (LKB).

Preparation of antiserum to ethanolamine ammonia-lyase and immunological testing of bacterial extracts

A total of 900 μg of apoenzyme purified from *E. coli* was inoculated into a Dutch White rabbit according to the following schedule. An initial 300 μg injected intramuscularly was followed after 1 and 2 weeks by 120 μg intramuscular injections and after 3, 9 and 10 weeks by 120 μg intravenous injections. Test bleeds were taken after 4 and 11 weeks. For the intramuscular injections the protein sample (1–1.5 ml) was emulsified with an equal volume of Freund's complete adjuvant (Freund, 1947) and inoculated at multiple sites. Intravenous injection was into a peripheral ear vein. The animal was bled into dry glass tubes, the blood left to clot and serum obtained by centrifuging at 500 *g* for 15 min at 0°C within 2 h. The serum was stored at −20°C for up to 5 months.

Immunodiffusion tests were carried out by the double-diffusion technique (Ouchterlony, 1949), with 1.5% agar plates. Undiluted whole rabbit antiserum (30 μl) was placed in a central 5 mm diameter well and bacterial test samples (30 μl; 10 mg of protein/ml) were placed in six similar peripheral wells placed 12 mm apart (centre to centre). Diffusion occurred over 24–48 h at room temperature, and plates were examined throughout this period using dark background illumination. Results were recorded photographically.

Chemicals

Unless stated otherwise, all chemicals were the purest available from BDH Chemicals, Poole, Dorset, U.K., or Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K. [2-^{14}C]Ethan-1-ol-2-amine was from The Radiochemical Centre, Amersham, Bucks., U.K. L-2-Aminopropan-1-ol, L-2-aminobutan-1-ol and 3-methyl-2-benzothiazolinone hydrazone hydrochloride were from Aldrich Chemical Co., Gillingham, Dorset, U.K. DL-1-Aminopropan-2-ol was from Ralph N. Emanuel, Wembley, Middx., U.K. Yeast and horse liver alcohol dehydrogenases were from Boehringer Mannheim, Lewes, Sussex, U.K.

Results

Properties of ethanolamine ammonia-lyase in crude extracts

Enzyme activity in extracts of bacteria, disrupted in 0.02 M-Tris/HCl, pH 7.5, using an Aminco French pressure cell (Scarlett & Turner, 1976) was stable for several weeks at −20°C. Extracts lost 25–50% activity when stored overnight at 0–4°C in the dark. Storage under illumination, known to decompose coenzyme B_{12} to give an inhibitory analogue (Hogenkamp *et al.*, 1962), led to greater activity losses. Crude extracts were treated as a routine with activated charcoal (Blackwell *et al.*, 1977), to ensure long-term enzyme stability. Frozen samples were thawed slowly and only when required. Enzyme activity in crude extracts was assayed colorimetrically (see the Materials and Methods section).

At the pH optimum of 7.5, the K_m values for ethanolamine and coenzyme B_{12} (5′-deoxyadenosylcobalamin) were 0.2mM and 0.08 μM respectively. Inhibitory analogues of coenzyme B_{12} were hydroxo-, methyl- and cyano-cobalamin, each of which inhibited by approx. 30% when tested at 0.1 μM with coenzyme B_{12} at 2 μM. In addition to the 16 ethanolamine analogues tested previously and found to inhibit the ammonia-lyase (Blackwell *et al.*, 1977), L-2-aminopropan-l-ol and L-2-aminobutan-1-ol were potent inhibitors, decreasing activity by 88 and 63% respectively when tested at the same concentration as ethanolamine (2mM). The analogues inhibited the growth of *E. coli* on glycerol + ethanolamine + vitamin B_{12}, but not on glycerol + $(NH_4)_2SO_4$ media. Neither analogue supported growth when supplied as the sole source of nitrogen.

Purification of ethanolamine ammonia-lyase

A partial purification of the enzyme from crude extracts of *E. coli* has been described (Blackwell *et al.*, 1977). A summary of the steps involved is given in the Materials and Methods section and in Table 1. All preparations of the apoenzyme, after charcoal treatment, contained ethanolamine, dithiothreitol, glycerol and K^+ ions as stabilizing agents. The incubation of any apoenzyme preparation with coenzyme B_{12} in the absence of ethanolamine led to rapid inactivation.

Pooled fractions from DEAE-cellulose chromatography were concentrated by ultrafiltration and subjected to molecular-exclusion chromatography on a column of large-pore-size agarose beads (fractionation range 10000–5000000 daltons for globular proteins). The results are shown in Fig. 1. Whereas gel filtration on Bio-Gel A-1.5m resulted in the elution of ethanolamine ammonia-lyase almost immediately after the void volume, with only about 3-fold purification, the use of Bio-Gel A-5m (Fig. 1) resulted in good separation of the enzyme from other major peaks of protein with about 6-fold purification and 50–60% recovery at this stage. The KCl and dithiothreitol content of the buffer were important for good separation and recovery of apoenzyme during gel filtration. Overall, the enzyme was purified about 120-fold from crude cell-free extracts. The results of a typical purification are given in Table 1.

Disc gel electrophoresis was used to monitor enzyme purification at each stage (see the Materials and Methods section) and clearly showed the removal of inactive proteins. The initial fractions of the peak of activity shown in Fig. 1 (fractions 20–23 inclusive), constituting about one-third of the enzyme, typically showed only one protein component. These fractions were combined and used in most of the work de-

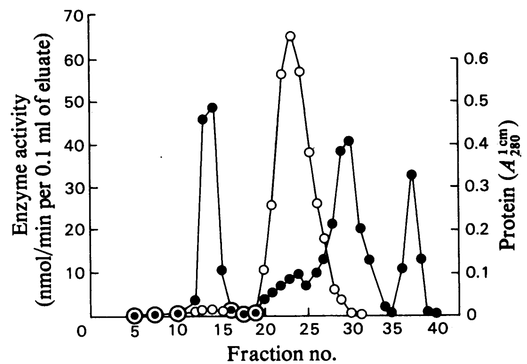

Fig. 1. *Gel filtration of partially purified ethanolamine ammonia-lyase apoenzyme from E. coli*
A sample of enzyme partially purified by $(NH_4)_2SO_4$ fractionation and DEAE-cellulose chromatography, containing 34mg of protein, was concentrated and applied to a column of Bio-Gel A-5m agarose as described in the Materials and Methods section. Protein was eluted with degassed 0.10M-Tris/HCl buffer, pH7.5, containing glycerol (2M), KCl (0.2M), ethanolamine (10mM) and dithiothreitol (1mM). Fractions (about 5ml) were collected and enzyme activity (○) was measured spectrophotometrically (see the Materials and Methods section). Protein (●) was detected spectrophotometrically, as A_{280}^{1cm}.

scribed below. Peak fractions eluted later, contaminated with lower-molecular-weight proteins, were pooled and rechromatographed on Bio-Gel A-1.5m. Whereas the enzyme was eluted soon after the void volume, most contaminating proteins were retained by the gel. Enzyme partially purified by this procedure was used when a homogeneous preparation was not required, e.g. in preliminary experiments.

Properties of purified ethanolamine ammonia-lyase

Enzyme activity was measured by the spectrophotometric method (see the Materials and Methods section). Routine tests showed that NADH oxidation was dependent on the presence of both ethanolamine ammonia-lyase and its substrate. The incubation of enzyme with cofactor in the absence of ethanolamine resulted in its rapid, complete and irreversible inactivation. Reactions were always started by the addition of enzyme or coenzyme B_{12} to otherwise complete reaction mixtures. Pure apoenzyme preparations exhibited extremely low residual activity in the absence of added coenzyme, less than 2% of the activity found in its presence.

Stability of the pure enzyme. The enzyme in Tris/HCl buffer supplemented with glycerol, dithiothreitol, KCl and ethanolamine (see the Materials and

Table 1. *Purification of ethanolamine ammonia-lyase apoenzyme from E. coli*

The enzyme was purified by a modification of the procedure of Blackwell *et al.* (1977), and its activity was assayed colorimetrically, as described in the Materials and Methods section. The first 30–40% of the enzyme eluted from Bio-Gel A-5m agarose, as an apparently homogeneous protein, was of somewhat higher specific activity than that of the combined fractions showing peak activity indicated below. A unit of enzyme activity is defined as the amount that catalyses the formation of 1 μmol of acetaldehyde/min, under the standard assay conditions.

Fraction	Total enzyme activity (units)	Specific activity (units/mg of protein)
Crude extract	235.5	0.12
Charcoal-treated extract	235.5	0.12
$(NH_4)_2SO_4$ precipitate (30% satn.)	141.3	0.48
DEAE-cellulose eluate (combined peak fractions)	49.9	2.29
Bio-Gel A-5m agarose eluate (combined peak fractions)	28.0	13.9

Table 2. *Stability of purified ethanolamine ammonia-lyase during dialysis*

Samples of purified apoenzyme were dialysed at 0–4°C against 200 vol of 0.10M-Tris/HCl buffer, pH 7.5, supplemented with dithiothreitol (1 mM), ethanolamine (10 mM), KCl (0.2 M) and glycerol (2 M) as indicated below. Samples were removed at the times indicated and enzyme activities measured under standard conditions. Ethanolamine and KCl were added as appropriate and reactions started by the addition of coenzyme B_{12}. The results are expressed as the percentage of the activity originally present which remained after dialysis.

Addition(s)	Residual activity (% of original activity after dialysis)		
Dialysis time (h) ...	24	48	120
None	3.1	0	0
Ethanolamine	38.5	11.7	0
Ethanolamine + dithiothreitol	100.5	92.0	36.8
Ethanolamine + dithiothreitol + glycerol	107.2	64.3	59.5
Ethanolamine + dithiothreitol + glycerol + KCl	95.3	100.0	100.0
Dithiothreitol + glycerol + KCl	84.6	95.3	42.9
Ethanolamine + glycerol + KCl	63.1	66.7	25.0
Ethanolamine + dithiothreitol + KCl	83.7	92.0	20.1

Methods section) was stable for several months at −20°C. Dialysis against the buffer mixture lacking one or more of the stabilizing agents showed that all were necessary for long-term stability of the pure enzyme (Table 2). Although the pure enzyme gave only one band on gel electrophoresis, prolonged storage or freezing and thawing resulted in the appearance of a new band of higher-molecular-weight protein.

Effects of pH on enzyme activity. Under the standard conditions of assay, a broad pH optimum was found between pH 7 and 8. Maximum activity was observed at pH 7.5 in phosphate or Tris/HCl buffers. A number of buffers, including diethanolamine/HCl, borate/NaOH and glycine/NaOH, appeared to inhibit activity.

Effects of ethanolamine concentration on activity. Double-reciprocal plots of substrate concentration against reaction velocity (Lineweaver & Burk, 1934) measured under standard conditions using ethanolamine-free enzyme, gave a K_m value for ethanolamine of only 0.044 mM. When the effect of pH on the K_m was studied, a marked fall from 0.3 mM at pH 5.8 to approx. 0.03 mM at pH 7.0 was observed. Between pH 7.0 and 8.4 there was little change in the K_m value, but a further decrease occurred at higher pH values. A plot of pK_m versus pH (Fig. 2) shows points of inflection at pH 7.0 and 8.3.

Effects of coenzyme B_{12} concentration on activity. The effect of coenzyme concentration was tested over the range 0.033–5.00 μM. At low concentrations, below 0.2 μM, a significant lag was noted after the addition of cofactor before NADH oxidation commenced. The lag, of up to 1 min duration, decreased with increasing coenzyme concn. and was insignificant above the K_m of 0.42 μM obtained from double-reciprocal plots (Lineweaver & Burk, 1934).

Substrate and inhibitory activity of ethanolamine analogues. None of the analogues tested was active as a substrate on the basis of the coupled spectrophotometric assay. Horse liver alcohol dehydrogenase was used in these assays because of its broad substrate activity, with ethanolamine analogues at 10 mM, and control experiments showed that no analogue inhibited acetaldehyde-dependent NADH oxidation. The most inhibitory analogues tested were L-2-aminopropan-1-ol (72%), DL-2-aminopropan-1-ol (52%), DL-1-aminopropan-2-ol (30%) and DL-1,3-diaminopropan-2-ol (17%), which inhibited to the extent shown when both ethanolamine and analogue were 10 mM. Detailed studies showed that inhibition was competitive with respect to substrate in each case, the K_i values being 0.86 μM, 2.2 μM, 88.0 μM and 1.1 mM respectively. 2-Aminobutan-1-ol was not tested in these experiments.

Inhibitory effects of coenzyme B_{12} analogues. Hydroxo-, methyl- and cyano-cobalamin inhibited

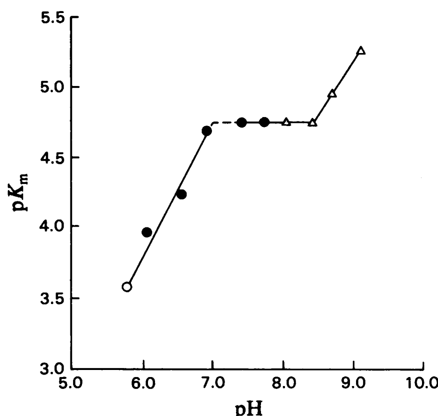

Fig. 2. *Effect of pH on the binding of substrate by ethanolamine ammonia-lyase*

The K_m for ethanolamine was determined at each pH value by the graphical procedure of Lineweaver & Burk (1934). The buffers used were 0.1 M-potassium citrate (○), 0.1 M-potassium phosphate (●) and 0.1 M-Tris/HCl (△).

completely at 0.1 mM when preincubated with enzyme for 15 min. Coenzyme B_{12} (5′-deoxyadenosylcobalamin) was added at 24 μM to start the reaction. Cyanocobalamin was slightly less inhibitory than the other analogues at lower concentrations. When analogues were tested over the concentration range 0.1 nM–0.1 mM, with coenzyme B_{12} in the range 0.033–5.0 μM, each was found to act non-competitively, with K_i values of 1.4 nM for methylcobalamin, 2.1 nM for hydroxocobalamin and 4.8 nM for cyanocobalamin. Dialysis did not restore enzyme activity, inhibition appearing to be irreversible.

Effect of thiol reagents on activity. Dialysis of the enzyme against supplemented buffer mixtures lacking dithiothreitol led to rapid loss of activity (Table 2). Thus dialysis against 0.1 M-Tris/HCl, pH 7.5, containing 10 mM-ethanolamine alone led to complete loss of activity within 24 h. Incubation of such inactivated preparations with 10 mM-dithiothreitol, for 15 min at 37°C, restored 90% of the activity lost. 2-Mercaptoethanol at 10 mM was less effective under the same conditions restoring only about 50% of the activity.

Iodoacetamide, which did not inhibit the alcohol dehydrogenase of the coupled assay system, was tested by preincubating with dialysed enzyme for 10 min at 0°C at 5 and 10 mM with and without 10 mM-ethanolamine. Activity, started by the addition of cofactor, was markedly inhibited in the absence of ethanolamine, but only slightly in its presence. *p*-Hydroxymercuribenzoate was a potent inhibitor, causing 50% inhibition at 0.1 μM without preincubation with the dialysed enzyme. Enzyme activity was measured colorimetrically after reactions were started by the addition of enzyme to otherwise complete mixtures containing the inhibitor.

Search for activators or effectors of enzyme activity. EDTA, preincubated with the enzyme for 15 min at 10 mM, had no detectable effect on activity. No stimulation of activity occurred when Mg^{2+} or Mn^{2+} ions were present at 5 and 10 mM. Univalent cations had little or no effect on activity. When K^{+} ions were rigorously excluded from the enzyme preparation and other reaction-mixture components (Tris/HCl buffer was used), full activity was observed. The addition of K^{+} at 10 mM had no stimulatory effect and Na^{+} and Li^{+} did not inhibit. The possible effectors AMP, ADP and ATP, tested over the range 0–10 mM, had no effect at saturating and rate-limiting concentrations of ethanolamine (10 and 0.02 mM respectively). These adenosine phosphates had no effect on enzyme activity in crude extracts.

Molecular properties of ethanolamine ammonia-lyase. Behaviour of the enzyme during gel filtration suggested a high molecular weight. Examination of the pure enzyme in the analytical ultracentrifuge (see the Materials and Methods section) gave an average value for the sedimentation coefficient ($s_{29,w}$) of 16.4 S. Making standard assumptions, a minimum mol.wt. of 560400 was calculated for the enzyme. SDS/polyacrylamide-gel electrophoresis of the purified enzyme, under standard conditions (see the Material and Methods section), indicated one band of protein at pH 7. The average molecular weight of this protein, estimated by using standard protein markers during electrophoresis, was 61400. An oligomeric structure for the active enzyme was also suggested by the finding that urea, tested over the range 0–5.0 M, inhibited enzyme activity at concentrations above 1 M, maximally at 3 M (95% inhibition). Urea at these concentrations did not interfere with the colorimetric assay used. The isoelectric point for the enzyme was approx. pH 5.0 by isoelectric focusing (see the Materials and Methods section). Poor recovery of enzyme activity in this experiment suggested that the enzyme was unstable at the isoelectric point.

Immunological studies of ethanolamine ammonia-lyase from E. coli and other bacteria

Rabbit antiserum was obtained by inoculation with the pure enzyme from *E. coli* and reactions between serum antibody and enzyme preparations were detected by the Ouchterlony double-diffusion procedure (see the Materials and Methods section). The antiserum was specific for the parent antigen, no cross-reacting material being found in extracts of *E. coli* grown on non-inducing media.

Borth crude extracts and the enzyme purified from *E. coli* gave twin precipitin lines against the antiserum. The inner line was sharp and distinct, whereas the outer line was faint and diffuse. Extracts of several other enterobacteria, grown on media known to induce the appearance of ammonia-lyase activity, cross-reacted with the antiserum to the *E. coli* enzyme and also gave twin precipitin lines. Such bacteria included *E. coli* K12 (N.C.I.B. 9484), *Klebsiella aerogenes* (N.C.I.B. 8267) and *Salmonella typhimurium* (N.C.I.B. 10248). Extracts of these bacteria grown on non-inducing media did not cross-react with antiserum. Whereas there was complete fusion of precipitin lines in the cases of *E. coli* K12 and *K. aerogenes*, 'spurring' of the lines occurred with *S. typhimurium*. Extracts of the strain of *Clostridium* used as the source of the ethanolamine ammonia-lyase originally studied, gave a faintly visible cross-reaction with the antiserum, but only one precipitin line was detectable and this crossed the corresponding (inner) precipitin line formed with the *E. coli* enzyme. No cross-reaction was observed between the antiserum and extracts of *Corynebacterium aquaticum* (N.C.I.B. 9460) known to possess weak ammonia-lyase activity (Blackwell *et al.*, 1976) or *Erwinia carotovora* (N.C.P.P.B. 1280), grown on the appropriate ethanolamine media. The results of some of these experiments are shown in Fig. 3.

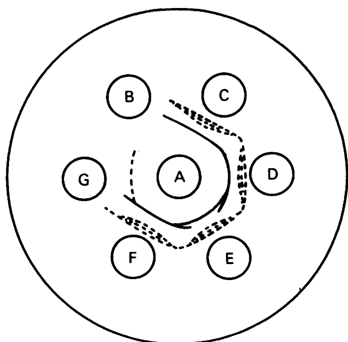

Fig. 3. *Cross-reactions between antiserum to ethanolamine ammonia-lyase of E. coli and cell-free extracts of other bacteria*

Reactions between the antibody in rabbit serum and enzyme preparations were detected by the double-diffusion technique of Ouchterlony (1949) as described in the Materials and Methods section. Bacteria were grown under conditions known to induce enzyme formation, as appropriate, and cell-free extracts were prepared (Scarlett & Turner, 1976). Undiluted antiserum was placed in the centre well A; extracts of *E. coli* type 1 grown on non-inducing and inducing media in wells B and C respectively; extract of *E. coli* K12 in well D; extract of *S. typhimurium* in well E; extract of *K. aerogenes* in well F and extract of *Clostridium* sp. in well G. Additional information is given in the text. The results were recorded photographically and are given in the form of a composite diagram.

Discussion

A comparison of the ethanolamine ammonia-lyase of *E. coli* and the *Clostridium* species (Bradbeer, 1965; Kaplan & Stadtman, 1968*a*,*b*) reveals a number of basic similarities. Both enzymes are narrowly specific for ethanolamine, which also protects against inactivation, and have an absolute requirement for coenzyme B_{12} which is tightly bound. Each enzyme is optimally active at about pH 7.5, is inactivated by incubation with coenzyme in the absence of substrate and is competitively inhibited by substrate analogues L-2-aminopropan-1-ol and DL-1-aminopropan-2-ol. Both are non-competitively inhibited by hydroxo-, cyano- and methyl-cobalamins, which act irreversibly and lack cofactor activity themselves. The reaction catalysed by both enzymes has a detectable lag period at low concentrations of the coenzyme. Both enzymes are large, with molecular weights well in excess of half a million, made up of eight to ten apparently identical subunits. Both contain essential thiol groups. These similarities are the more remarkable when the dissimilarity of *E. coli* and *Clostridium* species themselves (Breed *et al.* 1957) is considered, although some enzyme characteristics are common to other adenosylcobalamin-dependent enzymes of bacterial origin (Babior, 1975).

Differences in the characteristics of the ethanolamine ammonia-lyases of *E. coli* and the *Clostridium* sp. include the reactivity of essential thiol groups. Although Kaplan & Stadtman (1968*a*) demonstrated enzyme inhibition by *p*-chloromercuriphenyl sulphate, the clostridial enzyme was insensitive to iodoacetamide and *N*-ethylmaleimide, dithiothreitol having no activating effect. Titration with 5,5′-dithiobis-(2-nitrobenzoic acid) showed that the clostridial enzyme possessed several categories of SH- groups and provided evidence for their importance in adenosylcobalamin-dependent catalysis (Kaplan & Stadtman, 1968*b*; Mauck & Babior, 1977). In contrast, the *E. coli* enzyme was sensitive to inhibition by iodoacetamide, and dithiothreitol was an essential protector of enzyme activity, particularly in the absence of substrate. Whereas inactivation of the clostridial enzyme by SH- reagents was prevented by the cofactor, but not by ethanolamine (Mauck & Babior, 1977), inactivation of the enzyme from *E. coli* by iodoacetamide was markedly retarded by ethanolamine. The effect of pH on the K_m for ethanolamine indicated that a group with the same pK_a

as an SH- group (Dixon & Webb, 1964) was important in substrate binding. A thiol group essential for enzyme activity appears to be a feature shared by all adenosylcobalamin-dependent enzymes (Babior, 1975; Mauck & Babior, 1977; Krouwer & Babior, 1977). The pH-titration results with the *E. coli* enzyme also suggested that the imidazole group of a histidine residue may also be involved in substrate binding.

A more absolute difference between the enzymes from *E. coli* and the *Clostridium* sp. is that no univalent cation was required for the activity of the *E. coli* enzyme. Full activity was seen when K^+ ions were rigorously excluded from reaction mixtures, K^+ had no activity effect and Na^+ and Li^+ did not inhibit. The clostridial enzyme, in contrast, required K^+ for catalytic activity (K_m 0.47mM), NH_4^+ and Rb^+ were less effective whereas Li^+ and Na^+ were competitive inhibitors (Kaplan & Stadtman, 1968*a*). Although a number of other adenosylcobalamin-dependent enzymes require univalent metal ions for activity, many others do not (Babior, 1975).

The quaternary structure of ethanolamine ammonia-lyase of *E. coli* is of considerable interest. All coenzyme B_{12}-dependent enzymes about which structural information is available are oligomeric (Krouwer & Babior, 1977). Determinations of molecular weight of the *E. coli* enzyme indicated nine subunits per molecule, although caution and analogy with the clostridial enzyme lead us to suggest that eight to ten subunits are present. No evidence was obtained for the allosteric regulation of enzyme activity, e.g. by any of the potential effectors tested. An anomalous finding was that although SDS/polyacrylamide-gel electrophoresis disclosed only a single band of protein, twin precipitin lines were observed in the immunological studies. Although multiple precipitin lines could be accounted for by the production of antiserum to an impure antigen, or perhaps to apoenzyme contaminated with trace amounts of some cobalamin derivative, this does not appear to be the explanation. Experiments with mutants in which ammonia-lyase formation is constitutive (C. A. Blackwell & J. M. Turner, unpublished work) suggest that two protein components are associated with enzyme activity. The possibility that ethanolamine ammonia-lyase in *E. coli* is made up of two similar oligomers, not separated by SDS/polyacrylamide-gel electrophoresis under the conditions used, cannot be excluded at this stage.

The immunological evidence showing that a variety of Enterobacteriacea formed cross-reacting proteins when grown on enzyme-inducing media confirmed the results of enzyme assays (Blackwell *et al.*, 1976; Scarlett & Turner, 1976). The degree of relatedness between the *E. coli* enzyme and those of the other enterobacteria indicated by the immunodiffusion results, was similar to that deduced by an immunological study of alkaline phosphatase (Cocks & Wilson, 1972). It was notable that a single non-identical precipitin line was formed between antiserum to *E. coli* ethanolamine ammonia-lyase and clostridial extracts. This suggested that if the *E. coli* enzyme is made up of two protein components, only one is related to, but not identical with, the clostridial protein. The non-reaction with extracts of *Erwinia carotovora* grown on ethanolamine medium was consistent with the degradation of ethanolamine by the sequential action of kinase and phospho-lyase (deaminating) enzymes (Jones *et al.*, 1973; Jones & Turner, 1971) in this bacterium.

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