Biochem. J. (1967) 104, 953

The Microbial Oxidation of Methanol

PURIFICATION AND PROPERTIES OF THE ALCOHOL DEHYDROGENASE OF *PSEUDOMONAS* SP. M27

BY C. ANTHONY* AND L. J. ZATMAN
Department of Microbiology, University of Reading

(*Received* 30 *December* 1966)

1. A method for the purification of the nicotinamide nucleotide-independent alcohol dehydrogenase of *Pseudomonas* sp. M27 is described. 2. In the analytical ultracentrifuge, the purified enzyme shows a single major component of molecular weight 146000. 3. On electrophoresis in polyacrylamide gels between pH 5·0 and 9·55, it shows only one protein band and the isoelectric point appears to be between pH 7·0 and 8·0. 4. Spectrographic analysis indicates no significant metal content. 5. Amino acid analysis shows an unusually small number of cysteine/cystine residues per molecule as well as about 4·1% of glucosamine. 6. The role of ammonia as enzyme activator has been investigated.

In previous papers Anthony & Zatman (1964*b*, 1965) have described some of the properties of the alcohol dehydrogenase of *Pseudomonas* sp. M27, a pink organism that grows on methanol as sole source of carbon and energy (Anthony & Zatman, 1964*a*). The enzyme is unusual in many respects; a wide range of primary alcohols including methanol is oxidized, activity is independent of nicotinamide nucleotides but dependent on ammonia or methylamine as activator and on *N*-methylphenazonium methosulphate (phenazine methosulphate) as primary hydrogen acceptor. The present paper describes further work on the purification, properties and mode of action of this enzyme.

MATERIALS AND METHODS

Unless otherwise stated materials were obtained from the sources given by Anthony & Zatman (1965).

Buffer solutions. Buffer solutions were prepared according to the data of Dawson, Elliott, Elliott & Jones (1959).

Spectrophotometry. All spectrophotometric measurements were made with a Unicam SP. 800 recording spectrophotometer.

Protein estimation. Two methods were used: the method of Lowry, Rosebrough, Farr & Randall (1951) and the spectrophotometric method of Warburg & Christian (1941); the procedures were those of DeMoss & Bard (1957). Standard curves were prepared from crystallized bovine plasma albumin obtained from Armour Pharmaceutical Co. Ltd. (Eastbourne, Sussex).

Standard spectrophotometric enzyme assay. The cuvettes (10mm. light-path) contained the following, in a total volume of 3ml.: 0·3m-mole of tris–HCl, pH 9·0; 20μmoles of methanol; 3·3μmoles of *N*-methylphenazonium methosulphate (phenazine methosulphate); 0·13μmole of 2,6-dichlorophenol-indophenol; 45μmoles of NH_4Cl. The reference cuvette contained deionized water. Enzyme solution was blown in from a pipette and the initial rate of dye reduction taken as twice the change in E_{600} occurring between 15 and 45sec. after addition of enzyme. The amount of enzyme was always adjusted to give a rate of change of E_{600} of less than 0·6/min.

Enzyme units. One unit of enzyme activity as measured by the spectrophotometric assay is defined as the amount of enzyme that produces a change in E_{600} of 0·01/min. between 15 and 45sec. after addition of the enzyme. Assuming that the molar extinction coefficient of 2,6-dichlorophenol-indophenol at 600mμ is $1{\cdot}91 \times 10^7$ cm.^{2}mole^{-1} (Basford & Huennekens, 1955), 570 of these units are equivalent to 1 standard unit as defined in *Enzyme Nomenclature* (1965).

Ion-exchange cellulose and gel-filtration media. Ion-exchange celluloses were obtained from Bio-Rad Laboratories, Richmond, Calif., U.S.A. Sephadex for gel filtration was obtained from Pharmacia (G.B.) Ltd., London, W. 13. The materials were prepared and columns set up according to instructions in the Companies' manuals. Fractions were collected with a Central fraction collector made by Aimer Products Ltd., London, N.W. 1.

Preparation of crude extracts of Pseudomonas *sp. M*27. Preparation of some extracts and growth of organisms were essentially as described by Anthony & Zatman (1964*a*,*b*). Organisms were grown in a mineral medium with either methanol (0·5%, v/v) or methanol (0·5%, v/v)+sodium lactate (0·2%, w/v) as carbon and energy source either in 2l. conical flasks containing 1l. of medium on a gyratory shaker at 30°, or in two 12l. batches in a fermentor under similar conditions of aeration and agitation. The fermentor was built by L. H. Engineering Co. Ltd., Stoke Poges, Bucks. The organisms were harvested at room temperature in a Griffin–Christ centrifuge with the continuous-flow rotor at

* Present address: Department of Physiology and Biochemistry, University of Southampton.

12000*g* and a flow rate of about 150ml./min. When large amounts of extract were required they were prepared by disrupting the organisms in a Braun MSK homogenizer (Shandon Scientific Co. Ltd.). About 50g. of dry ballotini beads (0·11mm. diam.) were placed in the 75ml. glass vessel, which was then three-quarters filled with a suspension of organisms (1ml. of 20mM-phosphate buffer, pH7·0, per g. wet wt. of organisms). The machine was operated at about 5° for 3min. at 4000rev./min. The beads were separated from the homogenate by using a coarse-grade sintered-glass filter. The homogenate was centrifuged at 38000*g* for 1hr.; the resulting supernatant liquid is referred to as crude extract.

Purification of the enzyme. The pH of crude extract was lowered very carefully with N-HCl to 4·0 at room temperature and the heavy precipitate removed by centrifugation at 38000*g* for 20min. The pH of the supernatant liquid was raised to 6·0 with N-NaOH and solid $(NH_4)_2SO_4$ added to 65% saturation; after removal of the precipitate by centrifugation, $(NH_4)_2SO_4$ was added to give 85% saturation. The precipitated enzyme was dissolved in 20mM-tris–HCl buffer, pH8·0, and passed through a column of DEAE-cellulose, equilibrated with the same buffer. The enzyme was not adsorbed; it was eluted in the solvent front with 20mM-tris–HCl buffer, pH8·0. The pH of the pooled active fractions was lowered to 6·0 and solid $(NH_4)_2SO_4$ slowly added. Any precipitate that formed at less than 65% $(NH_4)_2SO_4$ saturation was removed and the 65–80%-saturated fraction was dissolved in the minimum volume of 0·10M-tris–HCl buffer, pH8·0. This solution was then passed through a large column of Sephadex G-150 equilibrated with the same buffer; the total volume of the column was about 20 times that of the sample. The main protein peak (as indicated by E_{280}) was collected and pooled. If there was any absorption of light at 550mμ due to cytochrome *c* the pooled fractions were concentrated with $(NH_4)_2SO_4$ and passed through a similar column. The pooled active fractions were dialysed against 1000vol. of deionized water at 2° before freeze-drying. This is referred to as purified enzyme.

For experiments on the role of ammonia as enzyme activator, the enzyme was purified without the use of ammonium salts. Nucleic acid and much inactive protein were removed from crude extract by lowering the pH to 4·0 with N-HCl and the neutralized supernatant liquid was then passed through a large DEAE-cellulose column (equilibrated with 20mM-tris–HCl buffer, pH8·0) on which the enzyme was not adsorbed (Table 1, omitting steps 3 and 5). This yielded a preparation of specific activity about 300units/mg. of protein [about the same as the $(NH_4)_2SO_4$ fraction used by Anthony & Zatman (1964*b*, 1965)]. Dialysis of this enzyme (when required) was done against 1000vol. of 20mM-tris–HCl buffer, pH8·0, for 46hr. at 2°.

Gel electrophoresis. This was done with the help of Dr J. D. Oram of the National Institute for Research in Dairying, Shinfield, Reading. Polyacrylamide gels were prepared by dissolving 5g. of acrylamide and 0·1g. of *NN'*-methylenebisacrylamide in 100ml. of 10mM-tris–citrate buffer (at the required pH for the run) followed by addition of 1·0ml. of a 30% (w/v) solution in ethanol of *NNN'N'*-tetramethylenediamine and 1·0ml. of a 30% (w/v) solution of ammonium persulphate. The solution was poured into shallow Perspex troughs and left to gel in the absence of air. About 50μl. (containing 1mg. of protein) of enzyme solution was introduced into narrow slots in the gel near the end to be connected to the negative terminal of the Shandon Universal electrophoresis tank (Shandon Scientific Ltd., London). The tank buffer was 0·1M-tris–HCl (at the required pH for the run) containing 0·1M-NaCl. A potential difference of 5–10v/cm. was applied for 5–16hr. in the cold room (4°) and gels were stained by immersion for 5min. in a solution containing Amido Black 10B (1g.), methanol (250ml.), acetic acid (10ml.) and distilled water (700ml.). The stained strips were left in 10% acetic acid overnight and then washed in acetic acid–methanol–water (1:5:5, by vol.) until the gel was washed free of excess of stain.

Ultracentrifugal studies. We thank Dr P. A. Charlwood for doing this work at the National Institute for Medical Research, Mill Hill, London, N.W. 7. Purified enzyme solution was dialysed in 0·15M-NaCl in 25mM-tris–HCl buffer, pH7·2 before ultracentrifugation, which was done in a Spinco model E ultracentrifuge at 20°. Sedimentation-velocity measurements were made at 59780rev./min. with a solution of purified enzyme (about 0·4% protein, refractive-index increment 0·00078), and a slightly less pure preparation at 0·7% concentration. The apparent molecular weight of the enzyme was measured by the Archibald (1947) method. For this purpose the solution of 0·4% concentration was examined at 6570rev./min. In all calculations the partial specific volume, $\bar{v}$, was assumed to be 0·73 and other details were as given by Charlwood (1961).

Amino acid analysis of purified enzyme. We are grateful to Dr A. W. Phillips of Wellcome Research Laboratories for doing both the amino acid and spectrographic analyses. Purified enzyme (10ml.) was dialysed against three 2l. volumes of deionized water over 48hr. at 2°. The contents of the dialysis sac were then freeze-dried and the amino acid analysis was done on the hydrolysate prepared from about 1mg. of the resultant powder. The powder was hydrolysed in an evacuated sealed tube with constant-boiling HCl at 110°; two periods of hydrolysis were used: 20hr. and 48hr. The analysis was done on a Beckman Spinco model 120 amino acid analyser as described by Spackman, Stein & Moore (1958). β-2-Thienylalanine was used as internal standard for the 150cm. column run and 2-amino-3-guanidinopropionic acid for the 50cm. column run. The content of threonine and serine was determined from the amounts present in the 20hr. and 48hr. hydrolysates by extrapolation to zero time. The spectrophotometric method used for tryptophan was that of Beaven & Holiday (1952).

Spectrographic analysis of purified enzyme. Purified enzyme was dialysed and freeze-dried as described for the amino acid analysis. The sample was packed into an acid-washed carbon electrode and then burned for 30sec. in a d.c. arc at 66v and 11A by using the cathode-layer technique. The spectrum was photographed between 3500Å and 2450Å. The resulting plates were compared with standard spectra obtained with known amounts of the various elements and with electrode blanks.

RESULTS

Purification of the enzyme. For this work it was necessary to extract and purify large amounts of the enzyme and many alternative methods were tried. *Pseudomonas* sp. M27 was grown in a mineral medium containing methanol or methanol + sodium

Table 1. *Purification of the enzyme*

Activities were measured in the standard spectrophotometric assay and protein was measured by the method of Lowry *et al.* (1951).

Fraction	Sp. activity (units/mg. of protein)	Volume (ml.)	Total units	Yield (%)	Purification
(1) Crude extract	43	415	1050000	100	—
(2) pH 4·0 supernatant	220	385	893000	85	4·9
(3) $(NH_4)_2SO_4$ fraction (65–80% saturation)	326	34	650000	62	7·6
(4) Pooled fractions from DEAE-cellulose	384	34	495000	47	8·9
(5) Pooled fractions from Sephadex G-150	420	60	347000	33	9·8

lactate as carbon and energy source; a higher yield of organisms was obtained with the latter mixture. The specific activity of crude extracts prepared from cells grown on methanol was the same as when grown on methanol + lactate and was independent of the method of cell disruption. The specific activity of crude extracts was the same whether prepared from cells stored at −22° for 1 year or from freshly harvested cells.

The purification procedures are described in detail in the Materials and Methods section. During this purification the enzyme separated on the Sephadex G-150 column as a golden-yellow band travelling in front of the red cytochrome *c*. Examination of the pooled enzyme fractions from the Sephadex G-150 column in the analytical ultracentrifuge showed the presence of a small percentage of material of much lower molecular weight and also of protein of very high molecular weight. For all analytical work on the enzyme protein and its prosthetic group, this heavy material and also most of the smaller material were removed by a second run through Sephadex G-150. Removal of cytochrome *c* was the most difficult step in the purification procedure and no alternatives to gel filtration were successful. A major attempt at crystallization of the enzyme was not made. Results obtained in a typical purification procedure are shown in Table 1. Assuming the purified enzyme is 100% pure, the data in Table 1 indicate that this alcohol dehydrogenase accounts for some 10% of the total soluble protein of the organism.

Stability of the purified enzyme. Freeze-dried purified enzyme (prepared with or without a second run through Sephadex G-150) stored for 2 months at −22° showed no loss of activity. Solutions of the purified enzyme in 20mM-tris–hydrochloric acid buffer, pH 8·0, stored in the frozen state showed about 15% loss of activity after 2 months at −22°; these same solutions were stable at 2° for up to about a week.

The enzyme lost little or no activity during 18hr. at room temperature in buffers covering a wide range of pH values (Table 2).

Table 2. pH *tolerance of the purified enzyme*

Purified enzyme (2mg.) was dissolved in 1ml. of the appropriate buffer solution. After standing for 18hr. at room temperature, the pH value of the solution was adjusted to 9·0 and the activity assayed spectrophotometrically. All the activities are expressed as percentages of the activity of the control system with a solution of the enzyme in 0·10M-phosphate buffer, pH 7·0, that had not been kept at room temperature.

pH value	Buffer (0·10M)	Activity (% of control)
2·2	Glycine–HCl	0
3·0	Glycine–HCl	0
4·0	Sodium acetate–acetic acid	90
4·8	Sodium acetate–acetic acid	95
5·6	Sodium acetate–acetic acid	95
6·0	Sodium phosphate	98
7·0	Sodium phosphate	100
8·0	Tris–HCl	100
9·0	Tris–HCl	95
10·0	Glycine—NaOH	83
10·4	Glycine–NaCl–NaOH	69
11·0	Glycine–NaCl–NaOH	71
12·0	Glycine–NaCl–NaOH	19

Criteria of purity of the purified enzyme. (1) The final step in the preparation of enzyme involved gel filtration on Sephadex G-150; the enzyme molecule entered the gel matrix. All fractions in the main peak had identical specific activities and identical relative extinction values measured at 260, 280, 350 and 410mμ. Fig. 1 shows that a further passage of purified enzyme through a column of Sephadex G-100 gave only one protein peak, and that all fractions had the same specific activity.

(2) The following techniques were used in an attempt to achieve further purification of the enzyme: chromatography on columns of guanidino-cellulose, CM-cellulose or sulphoethyl-cellulose, adsorption on calcium phosphate or alumina C_γ gels and fractional precipitation with cold acetone. In all these procedures the specific activities and relative extinction values of each fraction at 260,

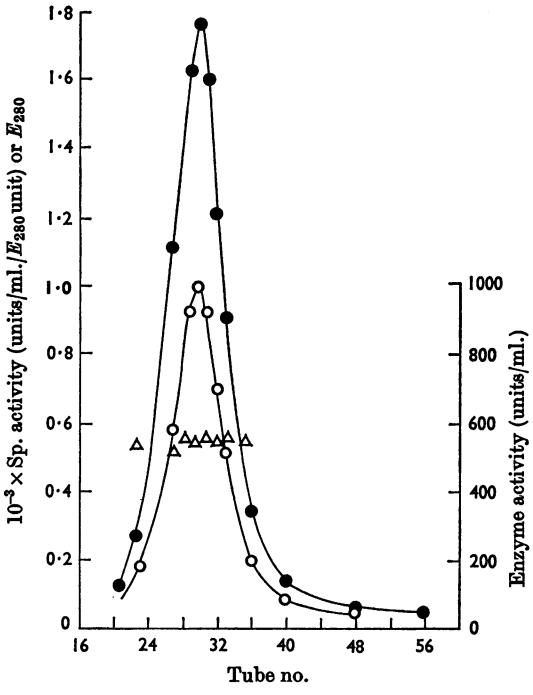

Fig. 1. Passage of purified enzyme through a column of Sephadex G-100. A solution (2ml.) of purified enzyme (35mg. of protein/ml.) was passed through a column (50cm. × 2cm.) of Sephadex G-100 previously equilibrated with 20mM-tris–HCl buffer, pH 8·0, and 2ml. fractions were collected. ●, E_{280}; ○, enzyme activity (units/ml.); △, 10^{-3} × specific activity (units/ml./E_{280} unit).

280, 350 and 410mμ were identical with those of the starting material (purified enzyme).

(3) Velocity ultracentrifugation of the purified enzyme revealed only one main peak (Fig. 2), together with a small peak that indicated the presence of less than 5% of a contaminant of much lower molecular weight.

(4) Only one protein band was obtained on polyacrylamide-gel electrophoresis at pH 5·0, 6·5, 8·6 or 9·55 and the mobilities obtained at these pH values suggested that the isoelectric point of the enzyme is between pH 7·0 and pH 8·0. This value would explain the failure of the enzyme to adsorb on anion-exchange cellulose at pH 8·0, whereas nucleic acids and much inactive protein in the crude extract are adsorbed under these conditions. This property provides a very rapid and efficient method of purification; thus passage of a crude extract through a column of DEAE-cellulose at pH 8·0 yields enzyme of nearly the same specific activity as the most purified material.

Metal content of the purified enzyme. Spectrographic analysis of the purified enzyme showed the absence of significant amounts of any metal. Copper and silicon were present in amounts between 10 and 100p.p.m. There were also traces (less than 0·1 p.p.m.) of iron, nickel, silver, zinc, tin, aluminium, lead, sodium and magnesium. This result is compatible with the relative insensitivity of the enzyme to metal-chelating agents shown by Anthony & Zatman (1965).

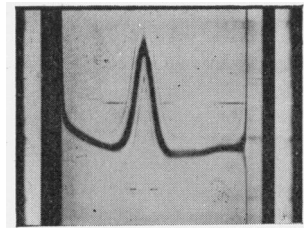

Fig. 2. Ultracentrifuge pattern obtained with purified enzyme. Sedimentation (right to left) was at 59780rev./min. for 60min. at 20° in 0·15M-NaCl in 25mM-tris–HCl buffer, pH 7·2. Protein concentration was approx. 0·4% (refractive index increment 0·00078).

Amino acid analysis of the purified enzyme. Results of an analysis are given in Table 3. A correction has been made for the partial destruction of serine, threonine and glucosamine during hydrolysis. The tryptophan content was calculated from measurements of E_{280} and E_{294} made on the intact protein. The unusually low content of cysteine + cystine could be accounted for by 6–8 free cysteine residues or 3–4 cystine bridges per molecule, or some free cysteine and some bridges. If all the cysteine is present in cystine bridges there is probably one cystine bridge per protein sub-unit as a protein of molecular weight 146000 is likely to have at least 3–4 sub-units (Reithel, 1963). The presence of glucosamine in an enzyme protein is unusual and the significance of this finding is at present obscure.

Molecular weight of the purified enzyme. Velocity ultracentrifugation of a solution of the purified enzyme revealed only one main peak (Fig. 2), together with a small peak that indicated the presence of less than 5% of a contaminant of much lower molecular weight. For the main component the sedimentation coefficients ($S_{20,w}$) were $7{\cdot}7_8$s and $7{\cdot}5_5$s at 0·4% and 0·7% concentration respectively. The molecular weight derived from the results of the low-speed experiment was 146000 as measured by the Archibald (1947) method. This value is consistent with the results of velocity ultracentrifugation, since a protein having $S^0_{20,w}$ 8s must have a minimum molecular weight of approx. 112000. However, the molecular weight obtained in the ultracentrifuge conflicts with the value of 120000 obtained by Anthony & Zatman (1964*b*)

Table 3. *Amino acid analysis of the purified enzyme*

The analysis was done in a Beckman Spinco model 120 amino acid analyser with about 1 mg. of purified enzyme. A correction was applied for partial destruction of serine, threonine and glucosamine during hydrolysis. The tryptophan value was obtained from measurements of E_{280} and E_{294} made on the intact protein. Full details are given in the Materials and Methods section. The total yield of products was only 72·77% (w/w) and the values in the first column of the Table were obtained by multiplying the actual experimental values (expressed per 100 g. of purified enzyme) by 100/72·77.

	Amino acid composition				
		[g. of amino acid/mole of enzyme protein (146000 g.)]†		[No. of residues of each amino acid/mol. of enzyme (to the nearest whole number)]†	
Amino acid	(g. of amino acid/100 g. of sample, based on residue wts., i.e. mol.wt.−18)	(*a*)	(*b*)	(*a*)	(*b*)
Glycine	6·60	7000	9620	92	127
Alanine	5·40	5740	7880	61	84
Valine	5·50	5850	8040	50	68
Leucine	7·20	7620	10480	58	80
Isoleucine	3·85	4100	5640	31	43
Serine	3·00	3185	4380	30	41
Threonine	5·60	6000	8260	50	69
Aspartic acid	14·00	15100	20800	113	155
Glutamic acid	7·15	7600	10450	51	76
Lysine	8·80	9350	12850	63	87
Arginine	3·42	3650	5020	21	29
Histidine	1·73	1840	2530	12	17
Phenylalanine	4·20	4440	6110	27	37
Tyrosine	6·10	6460	8890	35	48
Tryptophan	2·60	2780	3820	13	18
Proline	4·60	4880	6720	42	58
Methionine	8·16	2310	3180	15	21
Cysteine+cystine	<0·70	730	1020	3 or 4‡	3 or 4‡
Ammonia	2·10	2220	3050	19	26
Glucosamine	about 4·10*	4380	6030	22	30

* The actual value obtained for glucosamine was 1·9% (w/w); this has been corrected to about 3% (to allow for destruction during hydrolysis) and then multiplied by 100/72·77 to give the value of 4·1.

† Two values were calculated: (*a*) based on the actual amounts of amino acid obtained in the analysis; (*b*) based on the corrected values (i.e. the first column of the Table).

‡ This represents 3 or 4 cystine bridges or 6 or 8 cysteine residues/mol.

with the gel-filtration method of Andrews (1964). Andrews (1966) has suggested that anomalous behaviour of this sort during gel filtration may be a common feature of enzymes that have prosthetic groups. The results obtained with the ultracentrifuge, if they could be corrected to take account of the finite concentration at which measurements were done, would give a molecular weight slightly greater than 146000, thus increasing the discrepancy.

Absorption spectrum of the purified enzyme. The absorption spectrum of the purified enzyme (Anthony & Zatman, 1967) shows peaks at 280 mμ and about 350 mμ with little or no absorption at or above 450 mμ.

Role of ammonia as enzyme activator. Anthony & Zatman (1964*b*) showed that ammonia base rather than ammonium ion appears to be the activating species for this enzyme. They showed that crude sonic extracts were slightly active in the absence of added ammonium salts; it was thought that this activity might be due to the presence of an alternative enzyme activator. Because of the difficulty of measuring very small amounts of ammonia in very concentrated and coloured protein solutions, the enzyme was purified considerably without the use of ammonium salts for the following work, as described in the Materials and Methods section. Brief dialysis of this enzyme preparation against 100 vol. of 20 mM-tris–hydrochloric acid buffer, pH 8·0, for 1 hr. had no effect on enzyme activity when this was measured in the absence of added ammonium salts. The specific activity of this preparation was about the same as that of the ammonium sulphate fraction used by Anthony & Zatman (1964*b*, 1965). The initial rate of reaction of this enzyme preparation (undialysed) without added ammonium salts was 10–15% of that in a

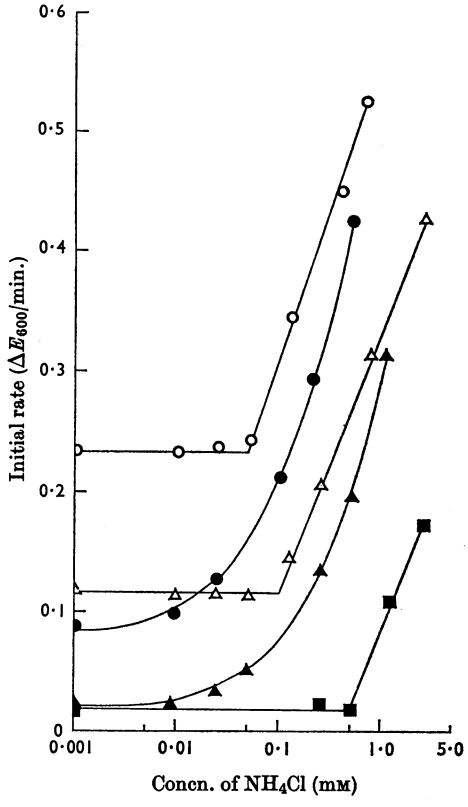

Fig. 3. Rate of enzyme reaction as a function of NH_4Cl concentration. The cuvettes (10mm. light-path) contained the following in a total volume of 0·5ml.: 50μmoles of tris–HCl, pH 9·0; 3μmoles of methanol; 0·55μmole of phenazine methosulphate; 0·02μmole of 2,6-dichlorophenol-indophenol; 0·5μmole of KCN; water to 0·5ml. The following amounts of enzyme were present: ○, 0·3mg. of protein (undialysed); ●, 0·3mg. of protein (dialysed); △, 0·15mg. of protein (undialysed); ▲, 0·15mg. of protein (dialysed); ■, 0·075mg. of protein (undialysed). (KCN was included in the reaction mixture because of the possibility of contamination with cytochromes.)

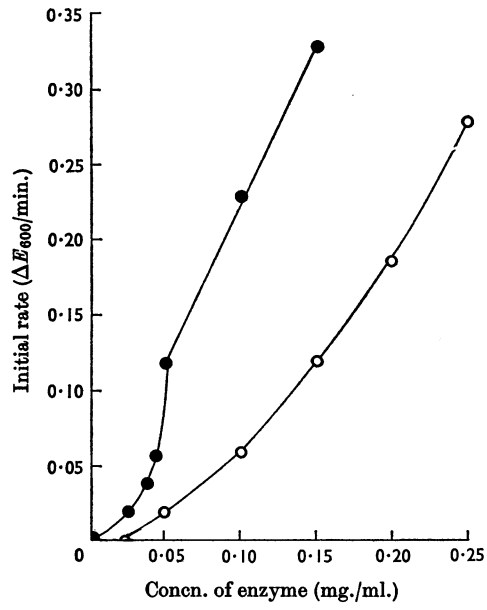

Fig. 4. Rate of enzyme reaction as a function of enzyme concentration in the absence of added ammonium salts. The cuvettes (10mm. light-path) contained the following in a total volume of 0·5ml.: 50μmoles of tris–HCl, pH 9·0; 3μmoles of methanol; 0·55μmole of phenazine methosulphate; 0·02μmole of 2,6-dichlorophenol-indophenol; 0·5μmole of KCN; water to 0·5ml. The following enzyme preparations were used: ●, undialysed enzyme; ○, dialysed enzyme.

complete assay system. Fig. 3 gives the curves obtained by plotting the initial rate of enzyme reaction against the concentration of ammonium chloride (on a logarithmic scale) with extensively dialysed, and undialysed, enzyme preparations; dialysis was against 1000vol. of buffer (20mM-tris–hydrochloric acid buffer, pH 8·0) for 46hr. at 2°. With dialysed preparations there is no break in the curve whereas with undialysed preparations there is a definite break; the latter occurred at higher ammonium chloride concentrations as the concentration of enzyme was decreased. In Fig. 4 the initial rate of enzyme reaction in the absence of added ammonium salts is plotted against the concentration of dialysed or undialysed enzyme. In the presence of ammonium salts and with much smaller amounts of enzyme, linear curves were obtained whether dialysed or undialysed enzyme was used. The results shown in Figs. 3 and 4 are difficult to interpret; they could indicate the presence of a very small amount of enzyme activator in both dialysed and undialysed enzyme preparations. If this activator is ammonia it must be present in the cells before disruption or it must have been produced during disruption. That there is still some activity in the absence of added ammonia when large amounts of extensively dialysed enzyme are used suggests that some ammonia or other activator may remain tightly bound to the enzyme. It is not known whether the relatively high ammonia content revealed on amino acid analysis (Table 3) of the enzyme is due to amide nitrogen or to other 'bound ammonia'. The results shown in Figs. 3 and 4 are typical of the type of complex kinetics obtained in many experiments with relatively large amounts of enzyme and low concentrations of ammonia. Although these results are of considerable interest in connexion with the mechanism of action of the

enzyme and the role of ammonia, the authors consider that further discussion of this aspect of the work must await further experimental data.

That the activity which is lost on prolonged dialysis is due to removal of ammonia from the enzyme, rather than to the removal of some other low-molecular-weight substance, is indicated by the following results. Undialysed enzyme was subjected to pressure dialysis in a collodion sac; the material passing through (the diffusate) contained no protein, as measured by extinction at 280mμ. The rate of reaction of extensively dialysed enzyme was measured with various amounts of this diffusate and the results were plotted graphically; the curve obtained was the same shape as that obtained by plotting the rate of dialysed enzyme reaction against ammonium chloride concentration, e.g. as in Fig. 3, there being no break in the curve. The diffusate gave a positive reaction with Nessler's reagent and became inactive on freeze-drying. The volume of material required for a given rate with extensively dialysed enzyme contained a similar amount of ammonia (as measured with Nessler's reagent) as the amount of ammonium chloride required to give the same rate.

In an attempt to detect whether or not ammonia is involved in association or dissociation of enzyme protein sub-units, extensively dialysed enzyme was passed through columns of Sephadex G-100 equilibrated with either 20mM-tris–hydrochloric acid buffer, pH 9·0, alone or tris buffer in 10mM-ammonium chloride. The molecular weight of the enzyme is about 120000 as estimated by gel filtration and is able to enter the gel matrix of Sephadex G-100. Any smaller sub-units would have a larger elution volume than the parent protein. However, there was only one protein peak from each column and these had identical elution volumes. As there was no apparent dissociation under these conditions it was not possible to conclude whether or not ammonia has any effect on association or dissociation of the enzyme into sub-units.

DISCUSSION

The results given in the present paper emphasize the differences between the alcohol dehydrogenase of *Pseudomonas* sp. M27 and other alcohol dehydrogenases (Sund & Theorell, 1963). The unusual absorption spectrum (Anthony & Zatman, 1967) indicates, further, that this enzyme is not a flavoprotein. Further evidence that the alcohol dehydrogenase is not a flavoprotein is contained in the following paper (Anthony & Zatman, 1967), together with evidence for a new oxidoreductase prosthetic group.

This work was supported by grants from the Agricultural Research Council, the Science Research Council and 'Shell' Research Ltd.

REFERENCES

Andrews, P. (1964). *Biochem. J.* **91**, 222.
Andrews, P. (1966). In *Protides of the Biological Fluids*, vol. 14, p. 573. Ed. by Peeters, H. Amsterdam: Elsevier Publishing Co.
Anthony, C. & Zatman, L. J. (1964*a*). *Biochem. J.* **92**, 609.
Anthony, C. & Zatman, L. J. (1964*b*). *Biochem. J.* **92**, 614.
Anthony, C. & Zatman, L. J. (1965). *Biochem. J.* **96**, 808
Anthony, C. & Zatman, L. J. (1967). *Biochem. J.* **104**, 960.
Archibald, W. J. (1947). *J. phys. Chem.* **51**, 1204.
Basford, R. E. & Huennekens, F. M. (1955). *J. Amer. chem. Soc.* **77**, 3873.
Beaven, G. H. & Holiday, E. R. (1952). *Advanc. Protein Chem.* **7**, 319.
Charlwood, P. A. (1961). *Biochem. J.* **78**, 163.
Dawson, R. M. C., Elliott, D. C., Elliott, W. H. & Jones, K. M. (1959). *Data for Biochemical Research*, pp. 192–209. Oxford: The Clarendon Press.
DeMoss, R. D. & Bard, R. C. (1957). In *Manuals of Microbiological Methods*, p. 179. Society of American Bacteriologists, Committee on Bacteriological Technic. London: McGraw–Hill Book Co. (Inc.) Ltd.
Enzyme Nomenclature (1965). *Recommendations* (1964) *of the International Union of Biochemistry on the Nomenclature and Classification of Enzymes*. London: Elsevier Publishing Co.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
Reithel, F. (1963). *Advanc. Protein Chem.* **14**, 1.
Spackman, D. H., Stein, W. H. & Moore, S. (1958). *Analyt. Chem.* **30**, 1190.
Sund, H. & Theorell, H. (1963). In *The Enzymes*, 2nd ed., vol. 7, p. 25. Ed. by Boyer, P. D., Lardy, H. & Myrbäck, K. London: Academic Press (Inc.) Ltd.
Warburg, O. & Christian, W. (1941). *Biochem. Z.* **310**, 384.