ACKNOWLEDGEMENTS

The authors are indebted to Dr. F. M. DEAN for helpful discussions and to Miss J. D. KERR for technical assistance.

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Biochim. Biophys. Acta, 56 (1962) 440–444

THE PURIFICATION AND PROPERTIES OF ALDEHYDE OXIDASE

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(Received April 29th, 1961)

SUMMARY

1. Highly purified aldehyde oxidase has been prepared from pig liver by reverse ammonium sulphate fractionation, heat treatment and chromatography on DEAE-cellulose and hydroxyl-apatite.

2. Although pure by electrophoresis the enzyme shows several minor components in the ultracentrifuge. The flavin content is 5 mμmoles/mg, and the molybdenum content 1–2 mμmoles/mg. It was not possible to remove the metal from the enzyme by dialysis under several conditions.

3. The presence of large amounts of haem in the preparation can be accounted for by the catalase activity of the preparation.

4. The influence of pH and of chain length of substrate on the activity of the enzyme has been investigated and the protection of the enzyme by ammonium ion noted.

INTRODUCTION

Aldehyde oxidase was first isolated from pig liver in 1940 when it was tentatively identified as a flavoprotein[1]. Since that time the enzyme has also been obtained from

Abbreviations: BSA, bovine serum albumin; DCIP, dichlorophenol-indophenol; TCA, trichloroacetic acid; PMS, phenazine methosulfate.

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the liver of rabbit[2], rat[3] and horse[4]. More recently the pig-liver enzyme has been reinvestigated and its flavoprotein nature confirmed[5]. A metal, molybdenum, and a haem were also implicated in the catalytic mechanism and, on the basis of spectroscopic evidence, it was proposed that the haem was reduced before the flavin. In view of this unusual observation it was decided to investigate further the role of the various constituents of this enzyme. Despite numerous attempts to use methods published earlier[5] no satisfactory preparations were obtained; considerable losses of activity being encountered during the early stages of purification. Furthermore very large blank reactions (no substrate) persisted throughout the initial fractionation and were still in evidence after several ammonium sulphate fractionations. Consequently it was considered worthwhile to devise an alternative method for the purification of this enzyme.

MATERIALS AND METHODS

The most suitable method for the determination of the activity is to follow the reduction, spectrophotometrically, of a hydrogen acceptor. In this work the enzymic reduction of DCIP, ferrycyanide and cytochrome *c* were followed at 600 mμ, 420 mμ and 550 mμ respectively, in the thermostatically controlled cell holder of the Unicam SP500 spectrophotometer at 25°. Optical cells of 1 cm light path were used throughout and the total volume in each assay was 3 ml. Each cell contained 140 μmoles Tris–phosphate buffer pH 9.0, 3 μmoles EDTA pH 7.0, 2 mg of BSA, 400 μmoles of ammonium sulphate, 100 μmoles of acetaldehyde (50 μmoles in the cytochrome *c* assay) and either 1.5 μmoles of potassium ferricyanide, 0.1 μmoles of DCIP or, 0.1 μmole of cytochrome *c*. The acetaldehyde was added immediately before the enzyme. The final pH was 8.5. Spectrophotometric measurements were made 15 sec after the addition of the enzyme and at 15-sec intervals for 2 min. In each assay system one unit of enzyme activity is defined as the amount of enzyme necessary to produce an absorbancy change of 1.00/min.

Flavin estimations

Flavin was estimated in three ways: Bound flavin was determined from the decrease in absorbancy at 450 mμ on addition of acetaldehyde or dithionite to the enzyme, using a coefficient of 10.3 for the decrease in absorbancy at 450 mμ of a millimolar solution of FAD[6].

FAD, in suitable extracts, was also assayed enzymically using the D-amino acid oxidase test system, and by fluorimetry. D-Amino acid oxidase was prepared by the method of MASSEY, PALMER AND BENNET[7] and split into apoenzyme according to NEGELEIN AND BROMEL[8]. The activity of the system was measured manometrically[9]. Fluorescence measurements were made in a fluorimeter designed and constructed by Dr. F. W. J. TEALE. Excitation of fluorescence was achieved with the 366-mμ mercury line isolated with a Chance OXI filter. The fluorescence emitted was analysed with a Chance OGrI filter (Maximum band pass at 540 mμ), 10^{-8} *M* FMN was used as a standard. At pH 7 the fluorescence of FMN is 4.8 times that of the same concentration of FAD[10].

Extracts of the enzyme suitable for analysis were prepared as follows (during these manipulations the solutions were protected from light as much as possible):

0.20 ml of enzyme + 0.05 ml of 20 % (w/v) TCA were incubated together at

room temperature for 20 min, 1.25 ml of water were added and the denatured protein removed by high speed centrifugation. The pH of the supernatant was adjusted to either pH 7.0 (fluorimetric assay) or 8.3 (enzymic assay) by the addition of 1 *N* NaOH. This method of making the extract avoids a high final concentration of trichloroacetate which has been found to inhibit markedly the enzymic assay of FAD. Flavin extracts were also prepared by proteolytic digestion[11].

Molybdenum estimations

These were performed by the toluene–dithiol method of CLARK AND AXLEY[12] as modified by BRAY, MALMSTRÖM AND VANNGARD[13] further modified by reducing the volume of extracting solvent from 5 ml to 1.25 ml so that the same sensitivity could be obtained in 1-cm light path cells (volume, 0.90 ml) in place of the 4-cm light path cells (volume 4.0 ml) recommended. This method has permitted the reliable estimation of 0.2 μg of molybdenum. The enzyme was prepared for analysis by digestion with sulphuric acid and hydrogen peroxide[13].

Electrophoresis

The electrophoresis analysis was performed with a Kern Micro-electrophoresis apparatus Type LK30 (Kern and Co., Aarau, Switzerland) equipped with Jamin interference optics, and the results confirmed by zone electrophoresis on a cellulose column, similar to that described by PORATH[14]. The supporting medium, acetylated cellulose, was prepared by the method of CAMBELL AND STONE[15].

Ultracentrifuge experiments were performed with a Spinco Model E ultracentrifuge. Spectral experiments were performed in the thermostatically controlled cell holder of a Beckman model DU spectrophotometer. The anaerobic experiment was performed as described previously[16].

Chemicals

BSA was the crystalline product of The Armour Laboratories, (Eastbourne) and EDTA, DCIP and toluene 3,4-dithiol were obtained from British Drug Houses, Limited. FAD was purchased from the California Foundation for Biochemical Research and cytochrome *c* was obtained from beef heart[17]. Solka Floc SWB Wood cellulose was purchased from The Brown Company (500 Fifth Avenue, New York) and 2-chloro-triethylamine hydrochloride from L. Light and Co. (Poyle, Bucks). This latter reagent was recrystallized from absolute methanol before use. All other chemicals were of analytical reagent quality and glass distilled water was used throughout. DEAE-cellulose was prepared by the method of PETERSON AND SOBERS[18] and hydroxyl-apatite according to TISELIUS, HJERTEN AND LEVIN[19]. Dialysis tubing was soaked in 1 m*M* EDTA for several hours and thoroughly washed with distilled water before use. All pH measurements were made with a Pye Universal pH meter.

Preparation of columns

15 g of DEAE-cellulose were suspended in 300 ml of water and the pH adjusted to 9.5 by the addition of 1 *M* glycine pH 9.5. The suspension was evacuated at a water pump for 15 min to remove entrained air and the slurry carefully poured into a vertical column of 2.2 cm diameter. This was allowed to pack under gravity. The height of the column was 28 cm and the flow rate 190 ml/h.

The hydroxyl-apatite–cellulose column was packed in a similar fashion and was composed of 700 mg (wet wt. of gel) and 2 g of Whatman's Standard Grade cellulose. Immediately before use both columns were washed with 2 m*M* glutathione and 5 m*M* EDTA. The buffers employed for the column chromatography also contained 2 m*M* glutathione and 5 m*M* EDTA.

Determination of catalase activity

Catalase activity was determined by the spectrophotometric method of BEERS AND SIZER[20] in the Optica recording spectrophotometer at room temperature. A temperature coefficient of 1.1 was employed to correct the rate measurements to 0° (see ref. 21). Freshly diluted enzyme was transferred by means of a micropipette to an adder-mixer[22] and the reaction initiated by addition of the enzyme to the cell situated in the spectrophotometer.

Protein estimations

Protein was determined by the biuret reaction[23].

RESULTS

Preparation of the enzyme

Two pigs livers are cleaned of obvious fat and connective tissue and passed through an electric mincer. Two kilogrammes of the minced liver are extracted with 6 l of cold glass distilled water at 4° for 3 h, and the suspension spun at $1400 \times g$ for 30 min. The supernatant is poured off carefully from the loose precipitate. Solid ammonium sulphate (292 g/l of supernatant) is added and the pH adjusted to 8.0 with concentrated ammonia (this is a convenient stage to leave the preparation overnight). This suspension is centrifuged at $10000 \times g$ for 1 h, and the supernatant discarded. The ppt. is resuspended in cold 48% saturated ammonium sulphate to give fraction ASI. 0.33 volume of cold glass distilled water is carefully added with thorough stirring; after standing for 20 min the suspension is centrifuged at $10000 \times g$ for 1 h, and the supernatant once more discarded. The precipitate is suspended in cold 38% saturated ammonium sulphate and 0.50 volume of cold water added. This suspension is centrifuged for 1 h at $40000 \times g$: the precipitate is rejected. 0.268 volume of alkaline ammonium sulphate (0.94 volume cold saturated ammonium sulphate + 0.6 volume concentrated ammonia) is added to the supernatant and the precipitate gathered by centrifuging at $10000 \times g$ for 20 min. The precipitate is dissolved in 0.05 *M* borate buffer pH 9.5 to give a final protein concentration of 25 mg/ml and solid EDTA added (1 g/100 ml of enzyme solution). The enzyme is rapidly heated to 55° by immersing in a water bath at 70° and held at this temperature for 10 min when it is quickly cooled in a cold bath at −10°. The denatured protein is removed by centrifuging at $10000 \times g$ for 20 min. The supernatant (H1) is concentrated by adding 0.43 volume of the alkaline ammonium sulphate gathering the precipitate by centrifugation ($10000 \times g$ for 20 min) and dissolving it in 0.05 *M* glycine pH 9.5 to give fraction AS III. This is then dialysed with stirring for 4 h against 2 l of the same buffer, the dialysing fluid being renewed after 2 h. About half the dialysed enzyme is applied to a DEAE-cellulose column which is washed with 0.25 *M* glycine buffer pH 9.5 until the E_{280} of the effluent has dropped to 0.05. The enzyme is then eluted with 0.25 *M* glycine pH 9.5 + 0.10 *M* NaCl as a sharp brown

band. The active eluate from two such column treatments is placed directly on a hydroxyl-apatite–cellulose column and impurities removed by washing with 0.10 *M* glycine pH 9.5 + 1 % (w/v) ammonium sulphate. The enzyme is eluted with 0.10 *M* glycine pH 9.5 + 3 % (w/v) ammonium sulphate. Solid ammonium sulphate (30 g/100 ml enzyme solution) is added carefully to the enzyme solution and the pH maintained constant by the cautious addition of concentrated ammonia (2.5 ml/100 ml of enzyme). After standing for thirty minutes the precipitate is gathered by centrifuging at 25000 × *g* for 20 min and dissolved in 0.05 *M* glycine pH 9.5 to yield fraction AS IV.

The details of a typical preparation are shown in Table I. Because of the presence of other enzymes *e.g.* xanthine oxidase, which contribute to the apparent enzyme activity during the initial stages of the preparation, it was not possible to calculate accurately the overall efficiency of this method of preparation. However, it is thought that a purification of 150–200 fold is quite likely with an overall recovery of 10%.

Enzyme activity

The activity of the pure enzyme was 2.4, 0.36 and 0.035 μmoles of acetaldehyde oxidised/mg protein/min with ferricyanide, DCIP and cytochrome *c* as acceptors. Under the conditions of the DCIP assay employed by MAHLER *et al.*[5] the activity was 0.21 μmole acetaldehyde oxidized/mg protein at 25°. IGO AND MACKLER[24] recently reported values ranging from 0.10–0.17 μmoles of acetaldehyde oxidized/mg protein at 38° using enzyme prepared by the method of MAHLER *et al.*[5] and with DCIP as acceptor.

Electrophoresis

Samples of the enzyme which had a specific activity of 1.6 (DCIP assay) appeared homogeneous at pH's 6.7 and 9.5 in the Kern Microelectophoresis apparatus. There was no suggestion of a second component but the fringe band broadens at a rate faster than one would have anticipated from diffusion alone; a phenomenon which has been observed with other rapidly migrating proteins in this instrument[25]. The observed

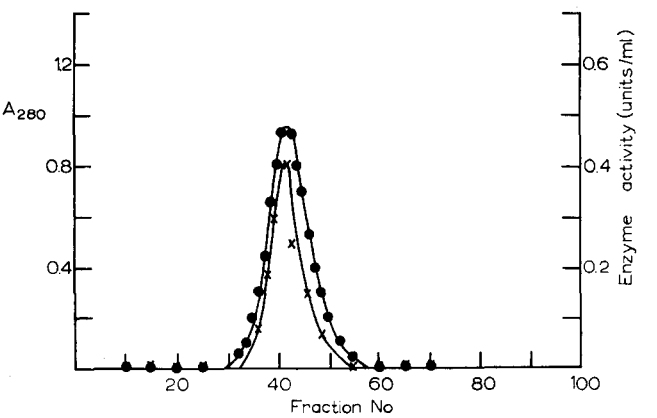

Fig. 1. Elution pattern of enzyme subjected to electrophoresis on a cellulose column for 8 h at a current of 8 mA and potential of 240 V in 0.05 *M* glycine pH 9.5. The eluate collected in 3-ml fractions, was analysed for protein spectrophotometrically at 280 mμ, ●—●, and enzyme activity with DCIP as acceptor, ×—×.

TABLE I

PURIFICATION OF ALDEHYDE OXIDASE

Treatment	*Volume (ml)*	*Protein (mg/ml)*	*Ferricyanide activity*		*DCIP activity*		*Cytochrome c activity*	
			Total units	*Specific activity (units/mg)*	*Total units*	*Specific activity (units/mg)*	*Total units*	*Specific activity (units/mg)*
Aqueous extract	4920	31	6400	0.042	12 800	0.084	4000	0.029
0–0.48 ammonium sulphate suspension	1387	72	3500	0.035	5500	0.055	970	0.01
0–0.38 ammonium sulphate suspension	840	94	3250	0.040	4800	0.070	800	0.008
0–0.25 ammonium sulphate supernatant	620	36	2300	0.105	3520	0.16	480	0.022
Ammonium sulphate II 0.25–0.40	230	57	1840	0.115	2700	0.17	415	0.032
Heat extract	450	8.0	1125	0.313	1673	0.50	450	0.120
Ammonium sulphate III 0–0.40	70	41	1190	0.330	1680	0.50	445	0.125
DEAE-cellulose eluate	45	19	656	0.76	1000	1.15	200	0.225
Hydroxyl-apatite eluate	80	2.8	375	1.6	590	2.42	118	0.480
Ammonium sulphate IV 0–0.40	7	33	380	1.61	582	2.40	115	0.480

electrophoretic mobilities were $-1.1 \cdot 10^{-4}$ cm²/V/sec at pH 9.5 in 0.05 *M* borate and $-8 \cdot 10^{-5}$ cm²/V/sec at pH 6.7 in 0.05 *M* phosphate. Zone electrophoresis at pH 9.5 on a cellulose column confirmed these observations: the elution pattern displayed one symmetrical peak which contained the enzyme activity. As there is over 95 % loss of activity during the electrophoresis, the subsequent enzymic assays were not accurate. Nevertheless the enzyme activity appeared to be regularly distributed within the protein peak (Fig. 1).

On examination of this preparation in the analytical ultracentrifuge, three major components were observed. Earlier workers have obtained preparations of similar purity (flavin content) which yielded similar results[5].

Preparations of the enzyme which had a specific activity of 2.4 (DCIP assay) revealed only one major component in the ultracentrifuge $s_{20,w} = 10.6$. This comprised 45–50 % of the total protein present, there being appreciable amounts of both faster and slower components.

Spectrum

The spectrum of the enzyme is presented in Fig. 2. There is considerable absorption throughout the ultraviolet and visible regions of the spectrum with maxima at 278 (not shown) and 405 mμ, with a pronounced shoulder from 450–480 mμ. The enzyme reduced with either acetaldehyde or dithionite is characterized by a general decrease in absorption throughout the spectrum. The difference spectrum (oxidised — reduced) has two peaks, maxima at 360 and 460 mμ, and is similar to that obtained with xanthine oxidase[26], succinic dehydrogenase[27] and earlier preparations of this enzyme[5] in that the resemblance to a pure flavin spectrum is obscured by a decrease in absorption extending as far as 600 mμ. The critical ratios $A_{780}/A_{450} = 7.5$ and $A_{410}/A_{450} = 2.1$ are somewhat different from those reported earlier[5].

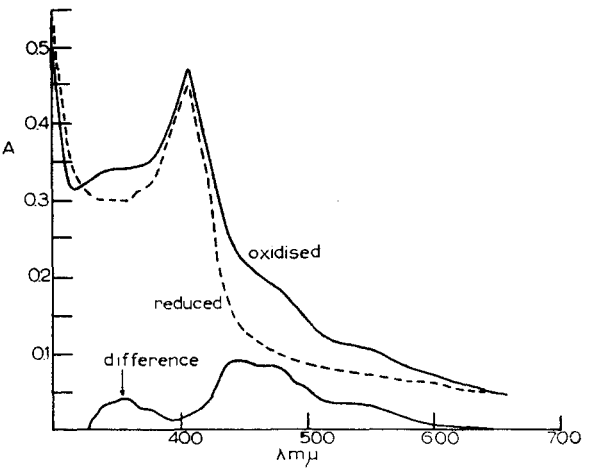

Fig. 2. Spectrum of aldehyde oxidase (0.19 %) in 0.05 *M* Tris–phosphate pH 9.0. Reduction was achieved by the addition of 10 μmoles of acetaldehyde and the difference spectrum (oxidised — reduced) obtained by calculation.

The results of a comprehensive flavin analysis are presented in Table II. As the flavin reactivates the D-amino acid oxidase test system then it is, in all likelihood, FAD. Furthermore, the flavin content of the best preparations would appear to be

5 mμmoles/mg of protein. Acid denaturation only liberates half of the total flavin present in the preparation; a similar incomplete release of flavin has also been observed with liver xanthine dehydrogenase[28] whilst the flavin of succinic dehydrogenase is only liberated after extensive proteolytic digestion[11].

TABLE II

FLAVIN ANALYSIS OF ENZYME

Method of extraction	*Assay*	*Flavin content (mμmoles flavin/mg protein)*
Nil	Spectrophotometric	4.6
TCA extract	Fluorimetric	2.4
TCA extract	Enzymic	2.8
Proteolytic digestion	Fluorimetric	5.0
Proteolytic digestion	Enzymic	5.4

Fluorescence

The enzyme demonstrates only slight green fluorescence when excited at 366 mμ with a mercury lamp; this fluorescence is highly polarized, suggesting that it is produced by enzyme bound flavin. The intensity of this fluorescence increases markedly on proteolytic digestion of the enzyme preparation.

Molybdenum content

Analysis of several preparations of the enzyme for molybdenum has confirmed the presence of this metal, although the actual molybdenum content has varied between 1 and 2 mμ moles/mg of protein, the smaller value being much more frequent.

The metal could not be removed by dialysis against 0.05 *M* glycine (pH 9.5) containing 1% (w/v) ammonium sulphate and EDTA for 48 h. At the end of this time the cytochrome reductase activity had decreased by about a half whereas both the DCIP and ferricyanide activities were unaffected (Table III). Dialysis against this buffer mixture containing the extra additions of either *o*-phenanthroline or potassium cyanide for 24 h followed by further dialysis against the standard buffer mixture

TABLE III

EFFECT OF DIALYSIS ON THE ENZYME ACTIVITIES AND THE MOLYBDENUM CONTENT OF ALDEHYDE OXIDASE

5-mg aliquot of enzyme dialysed as shown for 24 h at 4°, and for a further 24 h *vs.* buffer A. The samples so obtained, together with untreated enzyme, were analysed for metal content and enzymic activities.

	*Ferricyanide activity**	*DCIP activity**	*Cytochrome c activity**	*Mo content***
Before dialysis	1.6	2.4	0.55	0.11
Dialysis *vs.* (A) 0.05 *M* glycine pH 9.5 + 0.5 m*M* EDTA + 1% (w/v) ammonium sulphate	1.7	2.3	0.25	0.08
Dialysis *vs.* A + 0.2 m*M* *o*-phenanthroline	0.78	1.15	0.05	0.096
Dialysis *vs.* A + m*M* KCN	0	0	0	0.011

* Enzyme units/mg protein.
** μg Mo/mg protein.

for 24 h likewise produced no loss in protein bound molybdenum (Table III). These alternative treatments had rather drastic effects on the various enzyme activities; dialysis against the cyanide produced virtually complete inhibition of all activities, whilst *o*-phenanthroline produced a very large loss in the cytochrome *c* but rather smaller losses in both DCIP and ferricyanide activities. It is worth noting that incubation of the enzyme with 10^{-3} *M* cyanide at 0° produces complete and virtually instantaneous loss of enzyme activities when assayed in all three systems. On the other hand incubation of the enzyme with 10^{-3} *M* *o*-phenanthroline at 0° produces a large (approx. 80 %) inhibition of the cytochrome reductase activity instantaneously; the extent of inhibition does not increase with time at least over a two hour period. Under these conditions no inhibition of either DCIP or ferricyanide activities by *o*-phenanthroline could be detected.

Stability of the enzyme

The enzyme is only stable in the pH range 9–9.5, and activity is lost rapidly at neutral pH. One preparation of the enzyme has been kept for ten months at —15° with only 15 % loss in activity. However, dialysed preparations lose activity at an appreciable rate: One such preparation lost 25 % of its activity at 4° and 12 % at —15° in four days. There is a certain amount of evidence to indicate that the enzyme is stabilised by ammonium ion. GORDON *et al.*[1] found that the enzyme rapidly loses activity on dialysis, an observation confirmed by CARPENTER[4] who showed that this loss of activity could be restored by addition of ammonium ion to the assay. On the basis of these observations CARPENTER proposed that the reaction mechanism of the enzyme involved the formation of acetaldehyde ammonia. This restoration of activity by NH_4^+ has been partially confirmed in this work. When the activity of dialysed enzyme is estimated both in the absence and presence of ammonium sulphate, no differences could be observed in the initial rate of the reaction (Fig. 3). However, as the reaction proceeded, the velocity of the reaction in the absence of ammonium sulphate began to decrease more rapidly than did the velocity of the reaction with ammonium

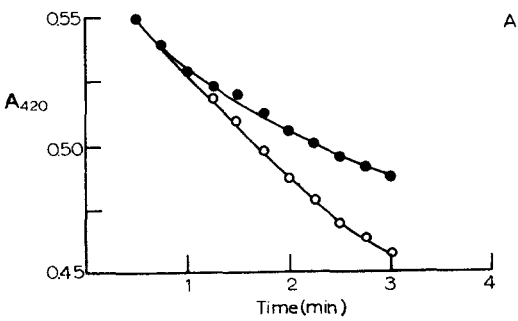

Fig. 3. The reduction of potassium ferricyanide by aldehyde oxidase in the presence, O—O, and absence, ●—●, of ammonium sulphate (400 μmoles) under otherwise standard assay conditions.

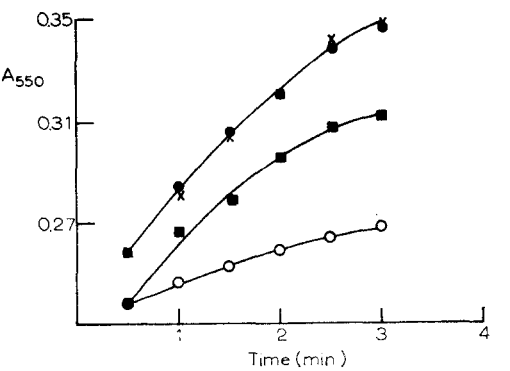

Fig. 4. The reduction of cytochrome *c* by aldehyde oxidase in 0.05 *M* Tris–chloride pH 9.0, O—O, and 0.05 *M* Tris–phosphate pH 9.0, ■—■, in the absence of both BSA and EDTA, and in 0.05 *M* Tris–chloride pH 9.0, ●—●, and 0.05 *M* Tris–phosphate pH 9.0 in the presence of 1 μmole of EDTA, ×—×.

sulphate present, suggesting that ammonium ions protect the enzyme from inactivation. (The possibility that the protection is some function of ionic strength was ruled out by the demonstration that only ammonium salts are active in this role.) As CARPENTER[4] employed the static Thunberg tube assay such an inactivation would manifest itself as an apparent decrease in enzyme activity. The second piece of evidence relating to this phenomenon was obtained from dialysis experiments. Highly purified enzyme is irreversibly inactivated by dialysis, 40 % of the total activity being lost after 4 h dialysis against 0.05 *M* glycine pH 9.5. After addition of 1 % (w/v) ammonium sulphate to the dialysis fluid, no loss in activity could be detected, even after 48 h dialysis.

As the enzyme is sensitive to heavy metals, addition of BSA or EDTA to the assay system routinely produces a marked increase in activity: the complete inhibition produced by 1 μmole of Cu^{2+} is abolished by the quantities of metal binding agents employed in the assays. It has been reported earlier that the reduction of cytochrome *c* by this enzyme is stimulated by phosphate[5]. This has been confirmed, the enzyme exhibiting rather more activity in Tris–phosphate than in Tris–chloride buffer (Fig. 4). In the presence of EDTA, however, the same activity is observed in both buffers suggesting that the apparent stimulation observed with phosphate is due to the weak sequestering powers of this ion reversing the inhibition produced by trace amounts of heavy metals. The stimulation of succinic dehydrogenase by phosphate has also been attributed to this property of the phosphate cation[29].

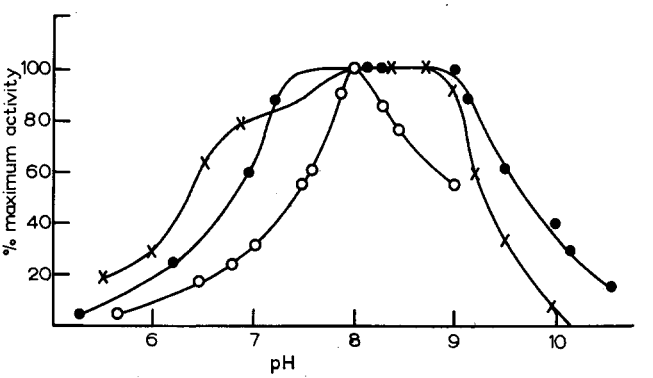

Fig. 5. Variation of enzyme activity with pH using acetaldehyde as substrate and DCIP, ×—×, ferricyanide, ●—●, and cytochrome *c*, ○—○, as acceptors. 0.05 *M* Tris–maleate buffers were employed in the pH range 5–8.6, and 0.05 *M* glycine buffers over the pH range 8.6 to 10.5. The pH of each assay was checked at the end of each reaction. The reduction of DCIP was followed at 530 mμ where the absorption is isosbestic with pH.

Effect of pH and chain length of substrate on enzyme activity

The variation of activity with pH for each of the acceptors commonly employed is shown in Fig. 5. Whilst the curves with both DCIP and ferricyanide exhibit a broad maximum between pH's 8 and 9, that with cytochrome *c* has a very sharp optimum at pH 8. The pH curve with DCIP has a pronounced shoulder at pH 7.5. The following salts produced no significant change in this curve; sodium chloride, 100 μmoles; sodium sulphate, 120 μmoles; sodium acetate, 100 μmoles; sodium phosphate, 60 μmoles and ammonium chloride, 120 μmoles.

The activity of the enzyme with a series of related aldehydes has been determined. These experiments were performed under the standard conditions of the ferricyanide assay and the rate measured at infinite concentration of aldehyde by the double reciprocal method[29]. The amount of ferricyanide employed in this assay, was known

TABLE IV

ACTIVITY OF ENZYME WITH A SERIES OF RELATED SUBSTRATES

Activity determined at infinite concentration of each aldehyde under the standard conditions of the ferricyanide assay.

	V_{max} (μmoles aldehyde oxidised/min/mg protein)	Michaelis constant (mM)
Formaldehyde	1.7	380
Acetaldehyde	7.9	100
Propionaldehyde	8.7	30
Butyraldehyde	20.6	25
Valeraldehyde	4.1	1.25
Heptaldehyde	0.54	1.3
2-Methyl propionaldehyde	1.47	40
2-Hydroxy butyraldehyde	4.1	28
2-Methyl butyraldehyde	20.6	25
2-Ethyl butyraldehyde	10.0	1.25
Crotonaldehyde	13.0	6.7

to be in great excess at least when acetaldehyde was the substrate. There is a marked variation in enzyme activity with the chain length of the aliphatic aldehyde, maximum activity being observed with the four carbon straight chain butyraldehyde (Fig. 6, Table IV), the rates with formaldehyde and heptaldehyde being only a small fraction of that observed with butyaldehydes whilst octaldehyde was oxidised extremely slowly. The K_m's decreased sharply from 380 mM for formaldehyde to 1 mM with heptaldehyde, with increase in chain length of the substrate (Table IV). In a recent report IGO AND MACKLER[24] have demonstrated that preparations of aldehyde oxidase

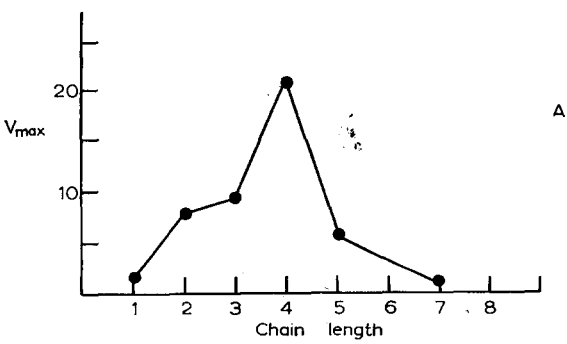

Fig. 6. Variation of enzyme activity with increase in chain length of aldehyde. The measurements were performed at 25° under the conditions of the ferricyanide assay and V_{max}. obtained at infinite aldehyde concentration.

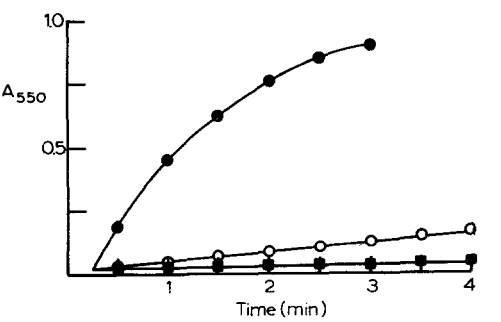

Fig. 7. The reduction of cytochrome *c* by aldehyde oxidase, ■—■, and the effect of adding 1 μmole of silicomolybdate, ○—○, or 3.2 μmoles of PMS, ●—●, under standard assay conditions. The reaction with PMS was performed with 0.1 the amount of enzyme of the other reactions and the rate obtained multiplied by ten for presentation in this figure.

prepared by the method of MAHLER *et al.*[5] had slight activity with both hypoxanthine and DPNH. These activities have also been demonstrated with this preparation of the enzyme; hypoxanthine activity could be detected with DCIP and oxygen as acceptors, whilst DPNH activity was apparent with DCIP and ferricyanide as acceptors. Whilst the activity of DPNH with DCIP had maximum activity in the neutral pH range, ferricyanide reduction was maximal in the range pH 5–6, a phenomenon which has been observed with several flavoproteins[30]. The DPNH oxidising activity of xanthine oxidase has also been found to be significant under these conditions[31] in contrast to earlier observations. Activity was also detected with cinchonine, pyridoxal, and N-methyl nicotinamide but not with quinine or pyridoxal phosphate as substrates.

Effect of phenazine methosulphate

The reduction of cytochrome *c* by aldehyde oxidase is stimulated by the addition of molybdenum to the assay system, a phenomenon which is due to the silicomolybdate anion present in solution. This anion can act as an electron acceptor for the enzyme and the reduced acceptor is able to reduce cytochrome *c* non-enzymically[32]. It has been found that phenazine methosulphate is extremely active in this capacity (Fig. 7) producing a thirty-fold stimulation of activity indicating that the absolute requirement is for an acceptor of suitable potential and reactivity; and that the observed stimulation by solutions of molybdate is not a unique property of certain complexes of molybdenum as has been claimed previously[33].

The role of the haem component

The spectrum of the enzyme shows clearly the presence of a haem protein in the enzyme preparations; furthermore, a complex series of spectral changes have been observed during the reduction of the enzyme by acetaldehyde, thus confirming the earlier observations[5]. On addition of acetaldehyde to the enzyme there is a large and immediate decrease at 410 mμ to a lower value which is quite stable (Fig. 8). During this time, the absorbancy at 450 mμ increases slowly. After several minutes there is

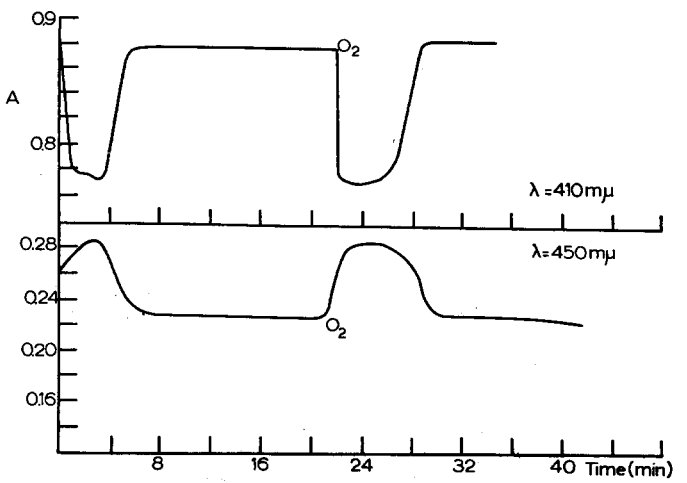

Fig. 8. Effect of acetaldehyde (10 μmoles) on E_{410} and E_{450} of aldehyde oxidase in 0.05 *M* Tris–phosphate pH 9.0 at 25° (total volume 30 ml). Oxygen was introduced at the time shown by gently inverting the spectrophotometer cell three times.

a fairly rapid increase in the absorption at 410 mμ to its original value, with a concomitant decrease at 450 mμ to a new low and stable absorbancy. If the cuvette is shaken gently with air this cycle of spectral events is repeated. When the experiment is repeated under anaerobic conditions (Fig. 9A) the extent of the spectral changes

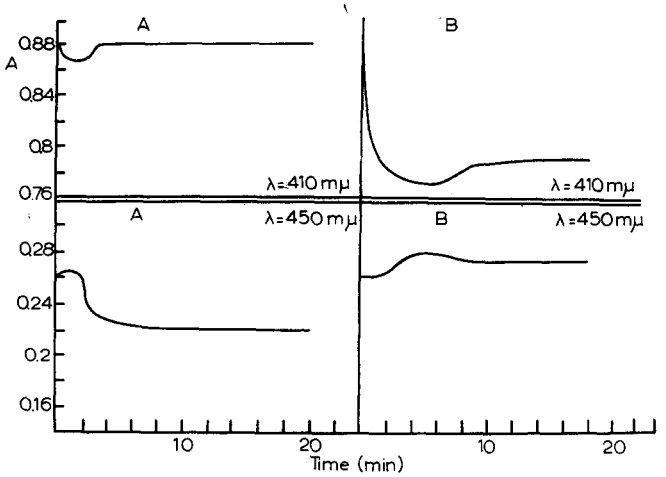

Fig. 9. Effect of acetaldehyde (10 μmoles) on E_{410} and E_{450} of aldehyde oxidase in 0.05 *M* Tris-phosphate buffer pH 9.0 at 25°. A, Under anaerobic conditions; B, aerobically in the presence of 0.10 μmole of arsenite.

at 410 mμ is much smaller, though the final absorbancies attained are just the same. It is probable, that under perfectly anaerobic conditions, not possible to achieve because of the sensitivity of the enzyme to surface forces, there would be no spectral changes at 410 mμ. These results indicate that the pattern of these spectral changes is dependent on the presence of oxygen. As these occur rather quickly it was, at first

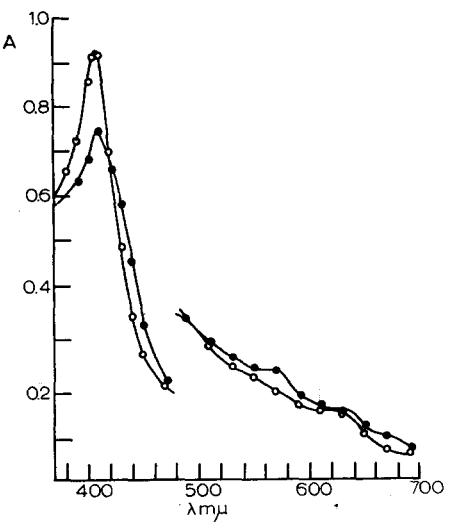

Fig. 10. Spectrum of the intermediate formed on addition of 10 μmoles of acetaldehyde to aldehyde oxidase in the presence of 0.10 μmole of arsenite. The right hand portion of the spectrum is at twice the scale shown on the ordinate. O—O, oxidised enzyme; ●—●, after acetaldehyde.

not possible to obtain a spectrum of the intermediate state. Fortunately it was found that arsenite produced a "stabilisation" of the intermediate (Fig. 9B) and it was thus possible to obtain the spectrum of this arsenite-stabilised transient (Fig. 10). The most striking feature of this spectrum is the large decrease in absorbancy in the Soret region, and the concomitant showing of this peak towards longer wavelengths. There is a general increase in absorption from 420 mμ to 700 mμ and several minor peaks can be detected at 530, 575, and 675 mμ. Addition of cyanide to the enzyme shifted the Soret peak from 407 to 425 mμ with a decrease in absorption in this region. Azide had no obvious effect on the spectrum, but on addition of acetaldehyde the Soret peak is shifted slightly to longer wavelengths and two peaks appear at 560 and 585 mμ. These peaks disappear rapidly and the spectrum soon reverts to its original form.

Determination of catalase content

In view of the previous experimental results it was considered that the haem protein present in the enzyme was catalase (see DISCUSSION). Consequently it was deemed necessary to estimate the catalase content of the enzyme. From the observed catalase activity and using published values[21] for Katalasefähigkeit (60000) molecular weight (225000) and molar extinction coefficient at 405 mμ ($340 \cdot 10^3$) it was found that catalase comprised about 11 % of enzyme protein and accounted for 84 % of the light absorption at 405 mμ in preparations of aldehyde oxidase.

DISCUSSION

Although it has not been possible to prepare pure aldehyde oxidase by the method described here, several interesting features of the enzyme appear to be resolved. Preparations of the same purity as those described earlier (based on flavin content and enzyme activity) also appeared homogeneous on electrophoresis although the electrophoretic mobilities were rather different. As reported previously[5] these preparations exhibited several components in the ultracentrifuge. However, the fact that it has been possible to further purify the enzyme to a state where it exhibits one major component when examined in the ultracentrifuge, suggests that the previous proposal for polymeric forms of the enzyme is unlikely and indicates that the presence of several components is the result of major contamination by other proteins.

The studies on the role of the haem component demonstrate clearly that one of the contaminants is catalase. The spectrum of the intermediate obtained in the presence of arsenite (Fig. 11) is similar to that obtained by CHANCE[34] with catalase exposed to low concentrations of peroxide continuously generated by glucose oxidase and glucose. The decrease in the intensity of the Soret band with a small shift to the red end of the spectrum and the general increase in absorbtion throughout the visible spectrum can be equated with the formation of the Catalase I and Catalse II complexes. The latter is produced slowly by the further reaction of Catalase I with a second molecule of peroxide. The initial reduction observed at 410 mμ then, is due to the formation of the Catalase I complex and the slow increase at 450 from the conversion of the primary complex into the secondary complex. When all the oxygen

in solution is exhausted, the peroxide is rapidly utilised and the catalase complexes revert back to free catalase. This results in the increase at 410 mμ and part of the decrease at 450 mμ. Furthermore, the enzyme flavin now becomes reduced and the absorbancy at 450 mμ decreases by the appropriate amount. Introduction of more oxygen by gentle shaking results in the production of more peroxide and a repetition of the spectral events. Under anaerobic conditions, peroxide formation is greatly decreased and the usual changes due to the formation of the catalase complexes are not observed. The apparent stabilisation of the intermediate by arsenite is in all probability due to the partial inhibition of aldehyde oxidase by this agent. The production of peroxide would then be much slower and consequently continue for a longer time.

The spectral changes produced by cyanide and by azide and acetaldehyde are very similar to those reported earlier with catalase with cyanide, and azide and hydrogen peroxide[32]. These observations strongly suggested that the haem component present in the enzyme preparation was catalase, although this possibility has been refuted[5].

Determination of catalase activity revealed that there was indeed a considerable amount of this enzyme present, sufficient, as calculation showed, to account for over 80% of the light absorbtion at 405 mμ. These experiments eliminate the postulated involvement of haem in the reaction mechanism of aldehyde oxidase, a possibility which had been considered earlier[35].

It is interesting, however, that the presence of catalase at this stage is a little unexpected as there are several stages in the preparation where the sepearation of catalase and aldehyde oxidase might have been anticipated. Furthermore the apparent electrophoretic mobility of the preparation is somewhat higher than that reported earlier for catalase[36] and it is an intriguing possibility that catalase and aldehyde oxidase exist in some form of complex as has been suggested previously[1]. In an attempt to resolve the two enzymes, the preparation has been rechromatographed on DEAE-cellulose in the presence of urea. Complete separation was only obtained under conditions which produced denaturation of aldehyde oxidase (unpublished results). It must be pointed out, however, that catalase has been shown to interact strongly with several proteins[36] and the observed phenomena might be an artefact of isolation without any physiological significance.

Although the enzyme is sensitive to metal-chelating agents, it is not at all certain that the molybdenum found in the preparation is the sensitive site, especially as several contaminating proteins appear to be present. The effect of EDTA is unusual in that, contrary to earlier reports[5] it produces a stimulation of enzyme activity, presumably by removal of toxic heavy metals from the enzyme. Cyanide behaves as anticipated, producing complete and instantaneous inhibition of all the activities examined although it has been claimed that a prolonged incubation is necessary to elicit the full response to the inhibitor[3]. The pattern of inhibition obtained with orthophenanthroline is not entirely consistent with the metalloflavoprotein concept[37] the lack of inhibition of the ferricyanide reaction by this agent being incompatible with the concept as originally proposed (ferricyanide like cytochrome *c* is a one electron acceptor).

While in no way disproving the concept, the great stimulation of the aldehyde-cytochrome reductase activity by PMS suggests that the previously described

stimulation by silicomolybdate[30, 32, 33] is due to the same cause, —*viz.* merely functioning as an artificial electron carrier of suitable redox potential and activity.

ACKNOWLEDGEMENTS

The author wishes to express his appreciation of the advice and guidance of Dr. V. MASSEY. This work was supported by a maintenance grant from the Department of Scientific and Industrial Research.

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