Purification of 2-Oxoaldehyde Dehydrogenase and its Dependence on Unusual Amines

By JOHN DUNKERTON and SYBIL P. JAMES

*Department of Biochemistry, University of Birmingham, P.O. Box 363,
 Birmingham B15 2TT, U.K.*

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1. 2-Oxoaldehyde dehydrogenase was purified from sheep liver and gave one band on polyacrylamide-gel electrophoresis. 2. The enzyme was completely dependent for its activity on the presence of Tris or one of a number of related amines, all of general structure:

When more than one R group was hydrogen no enzyme activity was observed. 3. Only one of these amines is known to exist in living tissues and large concentrations of all amines were required for maximum activity. L-2-Aminopropan-1-ol was the most effective amine on the basis of substrate K_m and V_{max} values and the amine K_m values. 4. The enzyme was activated by phosphate which lowered the K_m values for methylglyoxal, amine and NAD^+ . 5. The pH optimum of the enzyme was 9.3 and there was no activity at pH values below 7.8. A search for activators that might produce activity at pH 7.4 proved unsuccessful. 6. The enzyme was inhibited by rather large concentrations of barbiturates (6–46 mM) and nitro-alcohol analogues of the activating amines (66–139 mM).

It has been suggested that 2-oxoaldehydes might regulate cell division by reversible binding to essential -SH groups in the cell (Szent-Györgyi *et al.*, 1962, 1963; Szent-Györgyi, 1965; Együd & Szent-Györgyi, 1966). The 2-oxoaldehyde methylglyoxal was shown to inhibit protein synthesis in cultured mouse lymphoma cells and to inhibit growth, the inhibition being reversed by equimolar cysteine (Gregg, 1968). It was further suggested that the cellular concentration of methylglyoxal was controlled by the action of glyoxalase I (EC 4.4.1.5), which is known to convert methylglyoxal and GSH into S-lactoylglutathione (Szent-Györgyi *et al.*, 1967; Szent-Györgyi, 1968).

Kikuchi *et al.* (1972) showed that 0.01 mM-methylglyoxal gave 50% inhibition of L-glutamine-fructose 6-phosphate aminotransferase (EC 5.3.1.19) and they suggested that this could be a point at which regulation of cell division by methylglyoxal might occur. A double-label technique, capable of detecting a minimum concentration of methylglyoxal of 50 nmol/g of fresh tissue (Walton & McLean, 1971), failed to detect any methylglyoxal in rat liver, suggesting that a very efficient process existed for removal of methylglyoxal which is synthesized both enzymically and

non-enzymically in the cell (Cooper & Anderson, 1970; Bonsignore *et al.*, 1970; Elliot, 1959, 1960; Urata & Granick, 1963; Gibson *et al.*, 1958). However, it is difficult to attribute the lack of measurable methylglyoxal to glyoxalase I alone because the K_m values for methylglyoxal are 0.3 mM for yeast glyoxalase I and 0.19 mM for pig erythrocyte glyoxalase I (Cliffe & Waley, 1961; Mannervik *et al.*, 1972). It seems possible that another enzyme might be responsible for the observed lack of methylglyoxal in liver.

2-Oxoaldehyde dehydrogenase has been shown to catalyse the oxidation of methylglyoxal to pyruvate (Monder, 1967; Jellum & Elgjo, 1970; Willets & Turner, 1970) by using NAD^+ or NADP^+ . The 2-oxoaldehyde dehydrogenase of sheep liver was shown to be specific for 2-oxoaldehydes and its activity in Tris-HCl buffer was shown to increase up to pH 9.3 without reaching a maximum (Monder, 1967). The latter effect, which might represent an activation of the enzyme by Tris buffer, increased with increasing pH owing to deprotonation of the amine group ($\text{p}K_a$ 8.09, 25°C), and this forms the basis of this investigation of the properties of 2-oxoaldehyde dehydrogenase. Such

a study seemed necessary before attempting to find inhibitors that might be expected to possess cytotoxic properties.

Experimental

Materials

Fresh sheep liver was obtained from the slaughterhouse and was used immediately. Sephadex G-25 and G-200 were obtained from Pharmacia, Uppsala, Sweden and DEAE-cellulose (DE-52) and CM-cellulose (CM-52) from Whatman Biochemicals Ltd., Maidstone, Kent, U.K. NAD^+ , 2-mercaptoethanol, 2-aminopropane-1,3-diol (DL-serinol), methylglyoxal [aq. 40% (w/v) solution], phosphocellulose, hydroxyapatite, spermidine, hexobarbitone, 5'-AMP, 5'-ADP, 3':5'-cyclic AMP and cellulose powder were obtained from Sigma Chemical Co., Kingston-upon-Thames, Surrey, U.K. Tris base was obtained from Fisons Scientific Apparatus Ltd., Loughborough, Leics., U.K. 2-Amino-2-methylpropan-1-ol, 2-amino-2-methylpropane-1,3-diol, 1-aminopropan-2-ol, 2-aminopropane, 2-nitropropan-1-ol, tris(hydroxymethyl)nitromethane and β -dimethylaminopropionitrile were purchased from Koch-Light Ltd., Colnbrook, Bucks., U.K. L-2-Aminopropan-1-ol, L-2-aminobutan-1-ol, 3-aminopropane-1,2-diol, 2-nitro-2-methylpropane-1,3-diol, 2-nitro-2-methylpropan-1-ol and Ches [3-(cyclohexylamino)ethanesulphonic acid] were purchased from Ralph N. Emmanuel Ltd., Wembley, Middx., U.K. Diethanolamine, triethanolamine, sodium barbitone, Bicine [*N*N-bis-(2-hydroxymethyl)glycine], cyanogum, ammonium persulphate, polyvidone, 8-anilino-naphthalene-1-sulphonate (Na^+ salt) and Tes [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid] were purchased from BDH Chemicals Ltd., Poole, Dorset, U.K. Amylobarbitone, phenobarbitone and pentobarbitone were obtained from the Queen Elizabeth Hospital, Edgbaston, Birmingham, U.K. All buffers and amine solutions were freshly made up before use as described by Gomori (1955) and the pH of the solutions was adjusted with either 4M-HCl or 4M-NaOH at the temperature intended for use.

Methods

Phosphocellulose preparation. Phosphocellulose (20g) was swollen in water overnight and the fines were removed. It was stirred with 1M-NaOH (350ml) for 30min, washed with water (3-4 litres), and then stirred with 1M-HCl (350ml) for 2min, washed as before and the pH adjusted to 7.0. It was then suspended in the starting buffer (5mM-potassium phosphate buffer-5mM-2-mercaptoethanol, pH 7.0, 4°C) and cooled to 4°C, the pH being finally adjusted

to 7.0. Starting buffer (500ml) was run through each column of phosphocellulose before chromatography.

Methylglyoxal preparation. Methylglyoxal (150mM aqueous solution) was purified by chromatography on a column (36cm $\times$ 2cm) of Dowex-1 (HCO_3^- form) as described by Monder (1967) and was standardized by the method of Alexander & Boyer (1971) by using semicarbazide. The methylglyoxal solution was freshly made up before use and stored at pH 7.4 and 4°C.

Enzyme assay. The reaction mixture used for the assay of the enzyme in the purification consisted of Tris (20mM), methylglyoxal (5mM), NAD^+ (0.5mM), sodium pyrophosphate buffer (70mM) and 0.1ml of enzyme solution at pH 9.3 and 25°C in a final volume of 3ml.

The reaction mixture used in kinetic experiments consisted of methylglyoxal (5mM), L-2-aminopropan-1-ol (40mM), K_2HPO_4 (10mM), Ches buffer (50mM), NAD^+ (0.5mM) and 100 μg of enzyme protein at 25°C and pH 9.3 in a final volume of 3ml. Details of any other additions to the assays are given in the text.

The rate of reaction was measured by the increase in E_{340} due to the reduction of NAD^+ , by using a Unicam SP.700 spectrophotometer, and was linear with time for at least 5min. No reaction occurred if any one component of the reaction other than enzyme was omitted, but the reaction mixture in the absence of enzyme always gave a very small increase in E_{340} and this was corrected for. This non-enzymic increase was probably due to polymerization of methylglyoxal at high pH; this slowly produces a yellow colour with an absorption peak at 320nm (Bonsignore *et al.*, 1972). All enzyme assays were carried out in duplicate and duplicates always agreed to within $\pm 5\%$. Protein was measured by the method of Warburg & Christian (1941) as described by Kalckar (1947), and pyruvate by that of Friedemann & Haugen (1943).

Enzyme purification. All steps (a-f below) in the purification were carried out at 4°C and all buffers contained 5mM-2-mercaptoethanol. Fresh sheep liver (480g) was homogenized in 5mM-potassium phosphate buffer, pH 7.5 (1920ml), in a domestic food blender. The homogenate was centrifuged at 1500g in an MSE bench centrifuge for 5min and the sediment discarded.

(a) Solid $(\text{NH}_4)_2\text{SO}_4$ was added to the enzyme extract to give a final concentration of 2M, and the precipitated protein was removed by centrifugation at 80000g for 1½h and discarded. The supernatant was made 2.8M with respect to $(\text{NH}_4)_2\text{SO}_4$ and the precipitate collected by a similar centrifugation for 1½h. This precipitate was resuspended in a minimum volume of 5mM-potassium phosphate buffer, pH 7.4. The solution was desalted by chromatography on a column (3.5cm $\times$ 80cm) of Sephadex G-25 equilibrated with the same buffer.

Fig. 1. DEAE-cellulose chromatography of 2-oxoaldehyde dehydrogenase

Enzyme from the $(\text{NH}_4)_2\text{SO}_4$ stage was adjusted to pH 8.9 and chromatographed on a column ($1.4\text{ cm} \times 12\text{ cm}$) with a linear gradient (---) of NaCl (0–0.2M) in 2-amino-2-methylpropane-1,3-diol (5mM, pH 8.9) containing 2-mercaptoethanol (5mM). The flow rate was 20 ml/h and the volume of each fraction was 2 ml. The assay used for lactate dehydrogenase (○) consisted of sodium pyruvate (1mM), NADH (0.2mM), potassium phosphate buffer (50mM) and 0.02 ml of enzyme in a final volume of 3 ml at 25°C and pH 7.5. The assay used for 2-oxoaldehyde dehydrogenase (●) consisted of methylglyoxal (5mM), NAD^+ (0.5mM), Tris (20mM), sodium pyrophosphate buffer (70mM) and 0.1 ml of enzyme solution in a final volume of 3 ml at 25°C and pH 9.3.

(b) The desalted enzyme solution was passed through a column ($1.5\text{ cm} \times 12\text{ cm}$) of DEAE-cellulose equilibrated with 5mM-2-amino-2-methylpropane-1,3-diol buffer, pH 8.9. Adsorbed enzyme was eluted with a linear gradient (100 ml) of NaCl (0–0.2M), and the appropriate fractions were pooled and adjusted to pH 7.4 with 50mM- KH_2PO_4 . The enzyme solution was then desalted on Sephadex G-25 as before. The DEAE-cellulose step is important since it completely removes lactate dehydrogenase from the enzyme, which is essential since the products measured in the reaction under study are NADH and pyruvate, substrates for the lactate dehydrogenase. Fig. 1 shows the chromatography of 2-oxoaldehyde dehydrogenase on DEAE-cellulose; it is evident that only a small concentration of salt is required to elute the enzyme.

(c) The enzyme solution was adjusted to pH 7.0 and passed through a column ($1.5\text{ cm} \times 12\text{ cm}$) of phosphocellulose equilibrated with 5mM-potassium phosphate buffer, pH 7.0. 2-Oxoaldehyde dehydrogenase

did not bind to phosphocellulose under these conditions. The enzyme was then concentrated by adsorption on DEAE-cellulose as before and eluted with 0.1M-NaCl in 5mM-2-amino-2-methylpropane-1,3-diol, pH 8.9. The enzyme was then adjusted to pH 7.4 and desalted on Sephadex G-25.

(d) The enzyme sample was next applied to a column ($3.5\text{ cm} \times 56\text{ cm}$) of Sephadex G-200 and eluted with 5mM-potassium phosphate buffer, pH 7.4. Fractions (2 ml) having the highest 2-oxoaldehyde dehydrogenase specific activity were pooled. The peak of enzyme activity occurred in fraction 110 and its elution continued until fraction 130, after which no other protein was eluted. Protein was first eluted in fraction 50, the main protein peaks occurring in fractions 75 and 95. The results suggest that 2-oxoaldehyde dehydrogenase has a relatively low molecular weight.

(e) The enzyme solution was passed through a column ($16\text{ cm} \times 2\text{ cm}$) of hydroxyapatite-cellulose (1:5, w/w), equilibrated in 5mM-potassium phosphate buffer, pH 7.0. The enzyme was eluted with a linear gradient (100 ml) of potassium phosphate buffer, pH 7.0 (0–0.2M), and the active fractions were pooled and desalted as before on Sephadex G-25.

(f) The enzyme was adsorbed to DEAE-cellulose as before and eluted with a linear gradient (100 ml) of NaCl (0–0.2M) in 5mM-2-amino-2-methylpropane-1,3-diol, pH 8.9. The enzyme fractions coincident with the main protein peak were pooled and adjusted to pH 7.4 with 50mM-HCl. The enzyme was stored in solution at 4°C. The purification is summarized in Table 1.

Affinity chromatography. Attempts to bind 2-oxoaldehyde dehydrogenase to a column of Sepharose-6-*N*-(6-aminoethyl)-AMP were unsuccessful at pH 7.4, or pH 8.9 in the presence or absence of 1mM-methylglyoxal, 10mM-2-amino-2-methylpropane-1,3-diol, or a mixture of both amine and methylglyoxal.

Criteria of purity. Polyacrylamide gels were made by dissolving cyanogum (1g), ammonium persulphate (40mg) and β -dimethylaminopropionitrile (0.04 ml) in buffer (20 ml). Tris-HCl buffer (40mM) was used for pH 8.5 and 40mM-phthalate buffer for pH 5.5. The gel was left to set in electrophoresis tubes ($8\text{ cm} \times 0.5\text{ cm}$). The electrophoresis tank buffer contained 5mM-2-mercaptoethanol, and before electrophoresis of the sample, electrophoresis was carried out for 2 h to remove excess of persulphate from the gels.

A sample of enzyme solution was concentrated by dialysis in aq. 40% (v/v) polyvidone for 15 min and a small drop applied to each electrophoresis tube together with 1 drop of aq. 50% (v/v) glycerol to weigh the sample down. Electrophoresis was carried out for 0.5, 1, 2, 3 and 4 h at 20°C and with a current of 3 mA/tube. Protein was detected by treating the gels with 3M-HCl for 1 min and 0.001% 8-anilino-naphthalene-

Table 1. *Purification of 2-oxoaldehyde dehydrogenase from 480g of sheep liver*

Full details are given in the text.

Procedure	Total volume (ml)	Total activity ($\mu\text{mol/min}$)	Total protein (mg)	Specific activity ($\mu\text{mol/min per mg}$)	Yield (%)
Crude enzyme	1920	66.4	44300	0.0015	100
(NH_4) ₂ SO ₄ (2.0–2.8M)	380	46.4	4752	0.0097	69
DEAE-cellulose (1)	83	36.9	1592	0.0232	55
Phosphocellulose	91	26.8	701	0.0382	40
Sephadex G-200	196	14.7	157	0.0938	22
Hydroxyapatite	136	9.8	84	0.1166	14
DEAE-cellulose (2)	32	5.6	42	0.1334	8.4

Table 2. *Effect of amine structure on kinetic parameters of 2-oxoaldehyde dehydrogenase*

The assay mixture for the measurement of the amine K_m values consisted of methylglyoxal (5mM), amine, potassium phosphate (10mM), Ches buffer (50mM), NAD⁺ (0.5mM) and 100 μg of enzyme protein in a final volume of 3ml at 25°C and pH 9.3. For measurement of methylglyoxal K_m values and V_{max} , the same assay mixture was used and included 40mM-amine. Double-reciprocal plots were made of the results.

Amine	K_m for amine (mM)	K_m for methylglyoxal (mM)	V_{max} ($\mu\text{mol/min per mg}$)
Tris	2.4	4.0	0.22
L-2-Aminopropan-1-ol	5.0	2.08	0.27
2-Amino-2-methylpropane-1,3-diol	4.3	5.0	0.33
2-Amino-2-methylpropan-1-ol	15.0	2.6	0.20
2-Aminopropane-1,3-diol	7.7	5.5	0.24
2-Aminopropane	16.6	—	—
2-Aminobutan-1-ol	20.0	3.8	0.25

1-sulphonate for 3 min. The gels were then observed under u.v. light.

The enzyme migrated as single bands at pH 8.5 and 5.5 with mobilities of 1.2 and 0.2cm/h respectively.

Results

Purification of 2-oxoaldehyde dehydrogenase

Table 1 summarizes the purification of 2-oxoaldehyde dehydrogenase from sheep liver. It demonstrates that the enzyme has only been purified times 89 with respect to the crude enzyme. The specific activity of the crude enzyme may be a low value owing to contamination by lactate dehydrogenase. However, the rates of reaction were linear for at least 4min, and at pH 9.3 it is unlikely that interference from lactate dehydrogenase will be very great. The 2-oxoaldehyde dehydrogenase now described resembles that first studied by Monder (1967) in that it was found, in the present investigation, to oxidize methylglyoxal with the formation of stoichiometric amounts of pyruvate and NADH; the enzyme has the same pH behaviour as that of Monder (1967) and it also uses either NAD⁺ or NADP⁺ in the oxidation of the methylglyoxal. The purified enzyme was not assayed for the presence of other contaminating dehydrogenases but it was

shown that there was a stoichiometric reduction of NAD⁺ and oxidation of methylglyoxal at the first DEAE-cellulose stage of the purification and there was no reduction of methylglyoxal when NADH was used.

Effect of enzyme concentration on the rate of reaction

A linear relationship was found to exist between the concentration of enzyme and the rate of reaction up to an enzyme concentration of 50 $\mu\text{g/ml}$, much greater than that used in all subsequent kinetic work (33.3 $\mu\text{g/ml}$).

Dependence of 2-oxoaldehyde dehydrogenase on amines

2-Oxoaldehyde dehydrogenase was completely dependent on Tris, L-2-aminopropan-1-ol, 2-amino-2-methylpropane-1,3-diol, 2-amino-2-methylpropan-1-ol or 2-aminopropane. Table 2 gives the amine K_m values together with values of K_m and V_{max} for methylglyoxal in the presence of each amine. L-2-Aminopropan-1-ol gives the lowest K_m for methylglyoxal, a high V_{max} value, a low K_m for amine and was used in all subsequent work. L-2-Aminopropan-

1-ol also possesses a carbon skeleton that resembles known physiological compounds more closely than do Tris and its close derivatives.

Spermidine, 1-aminopropan-2-ol, 3-aminopropane-1-2-diol, ethanolamine, diethanolamine, triethanolamine, noradrenaline, lysine and arginine did not activate the enzyme. Diethanolamine (50mM) caused 40% inhibition of the enzyme activated by L-2-aminopropan-1-ol (40mM) and was considered unsuitable for use as a buffer. The results suggest that

only amines of the basic structure:

will activate the enzyme, where not more than one of the R groups is hydrogen.

Fig. 2 shows that L-2-aminopropan-1-ol activates 2-oxoaldehyde dehydrogenase by increasing $V_{\max}$, and has no effect on the K_m value for methylglyoxal.

Fig. 2. Effect of L-2-aminopropan-1-ol concentration on K_m and $V_{\max}$ of 2-oxoaldehyde dehydrogenase for methylglyoxal

The assay mixture contained methylglyoxal, NAD^+ (0.5mM), potassium phosphate (10mM), Ches buffer (50mM), L-2-aminopropan-1-ol (40mM, ● or 5mM, ○) and 100 μg of enzyme protein in a final volume of 3ml at 25°C and pH9.3.

Activation by P_i and PP_i

The enzyme was activated by P_i and PP_i . P_i produced more activity than PP_i and as the effective concentrations of P_i were in the physiological range for liver (Veech *et al.*, 1970; Schulz *et al.*, 1967) 10mM-potassium phosphate was used in all experiments.

Table 3 shows that the presence of 10mM-phosphate had no effect on $V_{\max}$, but lowered the K_m values for methylglyoxal, amine and NAD^+ . The significance of the phosphate activation is shown in the observations made on the stability of the enzyme on storage.

Effect of phosphate on 2-oxoaldehyde dehydrogenase prepared from sheep liver acetone-dried powder

The properties of 2-oxoaldehyde dehydrogenase from sheep liver homogenized in potassium phosphate buffer and from an acetone-dried sheep liver extract as prepared by Monder (1967) were compared (Dunkerton, 1974). The enzyme was partially purified in each case and it was found that high concentrations of P_i (10–20mM) inhibited the enzyme prepared from the acetone-dried sheep liver extract. Other slight differences were observed but the complete dependence on amines for activity, the response to pH

Table 3. Effect of phosphate on the K_m and $V_{\max}$ values of 2-oxoaldehyde dehydrogenase for methylglyoxal, NAD^+ and L-2-aminopropan-1-ol

To determine the K_m of methylglyoxal, the concentrations of L-2-aminopropan-1-ol and NAD^+ were 40mM and 0.5mM respectively, with 0.3–5mM-methylglyoxal. To determine the K_m of L-2-aminopropan-1-ol, the concentrations of methylglyoxal and NAD^+ were 5mM and 0.5mM respectively, with 2–60mM-L-2-aminopropan-1-ol, and to determine the K_m of NAD^+ , the concentrations of methylglyoxal and L-2-aminopropan-1-ol were 5mM and 40mM respectively, with 0.05–0.5mM- NAD^+ . All the assay mixtures contained Ches buffer (50mM), enzyme protein (100 μg), potassium phosphate (0.0 or 10mM), and assays were carried out at 25°C and pH9.3 in a final volume of 3ml. K_m and $V_{\max}$ values were calculated from linear double-reciprocal plots.

P_i (mM)	$V_{\max}$ ($\mu\text{mol/min per mg}$)	K_m (mM)		
		Methylglyoxal	NAD^+	L-2-Aminopropan-1-ol
0.0	0.28	7.14	0.32	36.0
10.0	0.27	2.08	0.11	5.0

change and the methylglyoxal K_m values were unaffected by the initial treatment of the sheep liver with acetone.

Stability of enzyme on storage

It was observed that after 20 days storage at 4°C and pH 7.4 in the presence of 50mM-NaCl and 5mM-2-amino-2-methylpropane-1,3-diol, the enzyme still possessed 97% of its original catalytic activity but had completely lost its response to phosphate. This suggests that phosphate binds at a site on the enzyme other than the active site and that it may be an allosteric effector of 2-oxoaldehyde dehydrogenase. It

was also observed that in the absence of amine and salt, the enzyme lost its response to phosphate after only 4 days but further work on the effect of amines or salt on the stability of the enzyme has not yet been carried out.

Effects of pH on the enzyme

Fig. 3 shows that the pH optimum of the enzyme is 9.3 and that it possesses no activity below pH 7.8. The pH optimum was unchanged when pyrophosphate was substituted for phosphate and when other activating amines were substituted for L-2-aminopropan-1-ol. The results in Table 4 show the effect of pH on the K_m and V_{max} values for methylglyoxal and L-2-aminopropan-1-ol. Increase in pH value appears to lower the K_m for methylglyoxal but has little effect on V_{max} . This is difficult to interpret since increase in pH would increase the free amine concentration and, according to Fig. 2, this would be expected to increase V_{max} ; however, an increase in pH would probably have other related effects such as conformational changes of both substrate- and phosphate-binding sites. The results in Table 4 also suggest that the decrease in the amine K_m values and the increase in V_{max} with the increase in pH is due to deprotonation of the amine and the consequent increase in the free amine concentration.

Fig. 3. Effect of pH on 2-oxoaldehyde dehydrogenase

The assay mixture consisted of methylglyoxal (5mM), NAD⁺ (0.5mM), potassium phosphate (10mM), Ches buffer (50mM), Bicine buffer (50mM), L-2-aminopropan-1-ol (40mM) and 100 μg of enzyme protein in a final volume of 3ml at 25°C.

Preincubation of substrates

Preincubation of the reaction mixture in the absence of enzyme for 20, 40 and 60min did not result in any increase in enzyme activity when the enzyme was finally added.

Other effectors of the enzyme

At pH 9.3, under the standard assay conditions used, ATP (1–4mM) had no effect on the enzyme

Table 4. Effect of pH on kinetic parameters of 2-oxoaldehyde dehydrogenase

To determine the K_m of methylglyoxal, the concentrations of L-2-aminopropan-1-ol and NAD⁺ were 40mM and 0.5mM respectively, with 0.3–5mM-methylglyoxal. To determine the K_m of L-2-aminopropan-1-ol the concentrations of methylglyoxal and NAD⁺ were 5mM and 0.5mM respectively, with 2–60mM-L-2-aminopropan-1-ol. All the assay mixtures contained Ches (or Bicine) buffer (50mM), potassium phosphate (10mM), enzyme protein (100 μg), and assays were carried out at 25°C in a final volume of 3ml. K_m and V_{max} values were calculated from linear double-reciprocal plots.

pH	Methylglyoxal		L-2-Aminopropan-1-ol	
	K_m (mM)	V_{max} (μmol/min per mg)	K_m (mM)	V_{max} (μmol/min per mg)
8.5*	7.7	0.26	18.0	0.09
8.9	4.7	0.28	—	—
9.2	2.4	0.31	—	—
9.3	2.08	0.28	5.0	0.29

* 50mM-Bicine used as buffer.

alone or in the presence of 4mM-Mg²⁺. AMP, ADP and 3':5'-cyclic AMP (0.1–0.5mM) gave weak inhibition of the enzyme (10–30%) and carbonate, sulphate and pyrophosphate (1mM) had very little effect.

At pH7.5, in the presence of 50mM-potassium phosphate buffer, 5mM-methylglyoxal, 5mM-L-2-aminopropan-1-ol and 0.5mM-NAD⁺, ATP, ADP, AMP, 3':5'-cyclic AMP, sulphate, carbonate and pyrophosphate had no effect. The L-amino acids (0.1 or 1.0mM) glycine, alanine, valine, leucine, isoleucine, serine, threonine, cysteine, glutamate, aspartate, lysine, arginine, histidine, phenylalanine, tyrosine, tryptophan and proline had no effect either, under the above conditions or in the absence of activating amine.

When 50mM-Tes buffer, pH7.5, was used instead of phosphate buffer, 0.01–5mM-Mg²⁺, -Ca²⁺, -Mn²⁺, -Fe³⁺, -Fe²⁺, -Ni²⁺ or -Zn²⁺ did not affect enzyme activity.

Inhibition of 2-oxoaldehyde dehydrogenase

Table 5 shows the inhibition of the enzyme by barbiturates and by nitro-alcohol analogues of the activating amines. It is clear that very high concentrations of nitro-alcohols are required for inhibition. This is probably the result of competition with the high concentrations of amine required for activity. However, preliminary experiments indicate that 2-nitro-2-methylpropane-1,3-diol shows non-competitive inhibition with respect to methylglyoxal and mixed inhibition with respect to amine. Barbitone is a considerably weaker inhibitor than the other barbiturates.

Table 5. *Inhibition of 2-oxoaldehyde dehydrogenase by barbiturates and by nitro-alcohol analogues of the activating amines*

The enzyme assay mixture consisted of methylglyoxal (5mM), L-2-aminopropan-1-ol (40mM), NAD⁺ (0.5mM), Ches buffer (50mM), potassium phosphate (10mM) and 100μg of enzyme protein at 25°C and pH9.3 in a final volume of 3ml. Reciprocal velocity plotted against inhibitor concentration was linear.

Inhibitor	Concn. of inhibitor for 50% inhibition (mM)
Tris(hydroxymethyl)nitromethane	139.0
2-Nitro-2-methylpropane-1,3-diol	66.0
2-Nitro-2-methylpropan-1-ol	70.0
2-Nitropropan-1-ol	83.0
Barbitone	46.0
Phenobarbitone	9.0
Hexobarbitone	5.8
Amylobarbitone	12.0
Pentobarbitone	7.2

It is possible that the hydrophobic substituent group replacing one of the ethyl groups of barbitone interacts with a hydrophobic-binding site on the enzyme.

Discussion

In some investigations of 2-oxoaldehyde dehydrogenase activity, enzyme assays have been carried out in Tris-HCl buffer (Monder, 1967; Jellum 1968; Jellum & Elgjo, 1970). The results of the present investigation suggest that in these cases the enzyme was dependent on Tris for its activity. Assays of *Bacillus subtilis* extracts, some assays of sheep liver extracts and an adenocarcinoma extract were carried out in diethanolamine-HCl buffer or phosphate buffer in the absence of Tris (Jellum, 1968; Jellum & Elgjo, 1970; Willetts & Turner, 1970). These results, especially those from assays of sheep liver extracts, are difficult to interpret in view of the properties of sheep liver 2-oxoaldehyde dehydrogenase now described. In the present study diethanolamine did not activate 2-oxoaldehyde dehydrogenase, but inhibited the enzyme activated by L-2-aminopropan-1-ol. Slow oxidation of diethanolamine by NAD⁺ and crude sheep liver extracts in the absence of methylglyoxal was also observed.

It is difficult to compare the properties of purified 2-oxoaldehyde dehydrogenase with those of Monder's enzyme since the latter had been partially purified from a sheep liver which had been dried with acetone at -20°C and this treatment had been shown to have an effect on the enzyme's properties (Dunkerton, 1974). Further, the kinetic work carried out by Monder (1967) was done at 30°C and pH8.5 and so it is of little value comparing the kinetic properties of the two enzyme preparations although the *K_m* values for methylglyoxal and NAD⁺ were of the same order and the enzyme in each case could use either NAD⁺ or NADP⁺ in the oxidation of methylglyoxal. The response to pH change was very similar for both Monder's enzyme and the enzyme in this report. Both enzymes showed little activity at physiological pH but a marked increase as the pH was raised towards 9.3, although Monder failed to demonstrate an optimum pH and did not show that the enzyme was completely dependent on the Tris buffer for activity.

The properties of 2-oxoaldehyde dehydrogenase now described suggest that phosphate may be an allosteric effector of the enzyme. Phosphate activates by lowering the *K_m* values for methylglyoxal, amine and NAD⁺ and the observations made on the stability of the enzyme suggest that phosphate binds at a site other than the active site. Phosphate activation by removal of an inhibitory metal ion is unlikely, since the enzyme has been in contact with 5mM-phosphate throughout most of the purification.

There seems little doubt that the amines form part of the reaction mechanism since different amines

produce different K_m and V_{max} values for methylglyoxal. In addition, phosphate affects the amine-dependence of the enzyme and the nitro-alcohol analogues of the amines inhibit the enzyme. It was shown that activation of the enzyme by L-2-amino-propan-1-ol was effected by an increase in V_{max} , the K_m for methylglyoxal being unaffected. This is difficult to explain and the results obtained do not show whether the substrate for the enzyme is free methylglyoxal or an adduct formed from methylglyoxal and amine. The latter mechanism, if it exists, would be interesting since it is analogous to that of glyoxalase I, where methylglyoxal reacts with GSH to form a thiohemiacetal which is the substrate for that enzyme. If an adduct were the substrate, an increase in amine concentration might be expected to lower the K_m of methylglyoxal owing to the larger fraction present as adduct. However, if the equilibrium for formation of adduct lies well towards the adduct, as might be expected at the high pH used, it is possible that such an effect on the K_m of methylglyoxal would be very small and difficult to demonstrate. This would not, however, explain the large effect of amine concentration on V_{max} . The observed effect of pH does not demonstrate the nature of the substrate. At lower pH values, a smaller concentration of free amine would be available to react with methylglyoxal and consequently the K_m values for amine would be expected to increase as the pH was lowered. This was observed but could also be explained if free methylglyoxal was the substrate and the enzyme was activated by free amine. Since the initial rate is linear and preincubation of substrates before enzyme addition produced no increase in enzyme activity, adduct formation, if it occurs, must be extremely rapid; this is likely at high pH. The kinetics of the reaction are very difficult to explain and further work will be complicated by the fact that with low amine concentrations (<5 mM), methylglyoxal is inhibitory at concentrations above 5 mM such that each enzyme velocity measurement at any given methylglyoxal concentration may be inhibited to some extent. The significance of the complete dependence on amines of a particular structure is difficult to interpret since only one of these, 2-amino-propane, has been detected in living tissue (Patterson & Mottram, 1974). This amine, the least effective of those studied in activating 2-oxoaldehyde dehydrogenase, occurs in pig muscle at a concentration of about 55 µg/kg of fresh tissue which is about 1×10^5 times smaller than that required for enzyme activation *in vitro*.

The physiological role of the enzyme is difficult to assess because of its unusual properties. However, there would appear to be four possible roles that might be suggested for the enzyme. It may play a part in regulation of cell growth by controlling intracellular concentrations of 2-oxoaldehydes, it may have a simple detoxifying role as has been suggested for

glyoxalase I (Vander-Jagt *et al.*, 1972), it may be part of a shunt from triose phosphate to methylglyoxal, L-lactate and glycogen similar to that suggested in *Escherichia coli* (Cooper & Anderson, 1970) or it may simply be an artifact and have no connexion with 2-oxoaldehydes *in vivo*. However, 2-oxoaldehyde dehydrogenase is unlikely to be an artifact because it has been demonstrated to exhibit specificity for 2-oxoaldehydes (Monder, 1967) and for particular types of amine. Also, Dakin & Dudley (1913) showed that perfusion of liver with methylglyoxal produced both D- and L-lactate and since no conversion of D-lactate into L-lactate was demonstrated, it was inferred that there was an enzyme other than glyoxalase I acting on methylglyoxal. This suggestion for an enzyme system producing pyruvate from methylglyoxal was the basis of an investigation of 2-oxoaldehyde dehydrogenase by Monder (1967).

Since 2-oxoaldehydes have growth-inhibitory properties, inhibition of enzymes concerned with the metabolism of the 2-oxoaldehyde methylglyoxal could provide a means of controlling tumour growth. Enzymes such as glyoxalase I and 2-oxoaldehyde dehydrogenase, which have been shown to act on methylglyoxal, might also act on other intracellular 2-oxoaldehydes such as 4-hydroxy-2-oxobutyr-aldehyde, which has been discovered in HeLa cells (Sparkes & Kenny, 1969). 4-Hydroxy-2-oxobutyr-aldehyde has more potent cytotoxic properties than methylglyoxal and it is possible that inhibition of methylglyoxal degradation may cause an accumulation *in vivo* of 2-oxoaldehydes other than methylglyoxal.

As an explanation of the unusual pH behaviour of the enzyme, it is possible that a pH value of about 9.3 actually exists locally in the cell, but there is no evidence to suggest that 2-oxoaldehyde dehydrogenase exists in cell organelles where a high pH could occur and the unusual pH behaviour of the enzyme is unlikely to be a sufficient explanation of other unusual properties of the enzyme. Since the weak binding of the enzyme to DEAE-cellulose, lack of binding to phosphocellulose and low electrophoretic mobility suggest that the enzyme is not highly charged, it is possible that it is weakly associated with membranes *in vivo* and that removal from this environment may account for many of its unusual properties. It may be that components of intracellular membranes actually form an integral part of the enzyme and such components are easily removed when the cell is disrupted. Evidence for this possibility is demonstrated by the work of Kempner & Miller (1968), who showed that intracellular centrifugation of *Euglena gracilis* cell contents resulted in sedimentation of cytoplasmic protein at a faster rate than expected, suggesting that protein regarded as 'soluble' cytoplasmic protein might be bound to membranes or cell organelles *in vivo*.

It is also possible that a cofactor is missing from the reaction mixtures used for study of the enzyme *in vitro*. Activation of salivary amylase by Cl^- involves a shift of the pH optimum from about 6.0 to 7.0, suggesting that the pH optimum of 2-oxoaldehyde dehydrogenase can similarly be moved to more physiological pH values by addition of cations or anions (Dixon & Webb, 1964). However, many possible effectors were without effect at either pH 9.3 or pH 7.5, and attempted further development of inhibitors of 2-oxoaldehyde dehydrogenase as possible cytotoxic agents both *in vitro* and *in vivo* would seem to be of little value until the nature of the amine-dependence and the significance of the high pH optimum of the enzyme are more clearly understood.

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