Purification and Properties of Two Glyoxylate Reductases from a Species of *Pseudomonas*

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1. Two enzymes that catalyse the reduction of glyoxylate to glycollate have been separated and purified from a species of *Pseudomonas*. Their molecular weights were estimated as 180 000.
2. Reduced nicotinamide nucleotides act as the hydrogen donors for the enzymes. The NADH-linked enzyme is entirely specific for its coenzyme but the NADPH-linked reductase shows some affinity towards NADH.
3. Both enzymes convert hydroxypyruvate into glycerate.
4. The glyoxylate reductases show maximal activity at pH 6.0–6.8, are inhibited by keto acids and are strongly dependent on free thiol groups for activity.
5. The Michaelis constants for glyoxylate and hydroxypyruvate were found to be of a high order.
6. The reversibility of the reaction has been demonstrated for both glyoxylate reductases and the equilibrium constants were determined.
7. The reduction of glyoxylate and hydroxypyruvate is not stimulated by anions.

While studying the malate synthase enzyme [L-malate glyoxylate-lyase (CoA-acetylating), EC 4.1.3.2] in a species of *Pseudomonas*, Hassall & Hullin (1962) discovered that only 45% of the glyoxylic acid was converted into malate. The remainder was shown to have been converted into glycollate by an enzyme, glyoxylate reductase (glycollate-NAD oxidoreductase, EC 1.1.1.26). The present paper reports that the reduction of glyoxylate in the organism is carried out by two separate enzymes. These enzymes have been isolated and their properties compared in an attempt to indicate why two enzymes were necessary to catalyse the same reaction.

METHODS AND MATERIALS

The organism used was first isolated from soil by Professor S. Dagley of this Department. It can grow on butane-2,3-diol as sole carbon source and has been identified as *Pseudomonas fluorescens* by Dr G. M. Williamson, formerly of the Department of Bacteriology, Leeds.

The maintenance of cultures, composition of growth medium, growth procedure, together with the harvesting and disintegration of the cells, have been described by Hullin & Hassall (1962).

Chemicals. Reduced and oxidized nicotinamide nucleotides, DEAE-cellulose and haemoglobin were purchased from the Sigma London Chemical Co., London. Butane-2,3-diol, cytochrome c, DL-glyceraldehyde, glycolaldehyde, α -oxoglutarate, semicarbazide, deoxyribonuclease and

ribonuclease were obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks. Other materials used and their sources are as follows: lithium hydroxypyruvate (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.); sodium glyoxylate (Carl Roth, Karlsruhe, Germany); sodium [1-¹⁴C]glyoxylate (The Radiochemical Centre, Amersham, Bucks.); ferric perchlorate, non-yellow in 4 oz. bottles (G. Frederick Smith Chemical Co., Columbus, Ohio, U.S.A.); lysozyme (Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex). Sephadex G-200, Sephadex G-200 Superfine and Blue dextran were obtained from Pharmacia, Uppsala, Sweden. All other chemical compounds used were of the highest purity available from British Drug Houses Ltd., Poole, Dorset.

Determination of protein. The protein content of the material, obtained in stages 1 and 2 of the purification procedure, was determined by the biuret method of Gornall, Bardawill & David (1949) and in stages 3–6 by the spectrophotometric method of Warburg & Christian (1941).

Determination of chloride. The chloride content of eluents from DEAE-cellulose columns was determined by the method of West & Coll (1957).

Pretreatment of DEAE-cellulose. This was carried out according to the method of Peterson & Sober (1962).

Pretreatment of Sephadex G-200. The Sephadex G-200 was used throughout for gel-filtration according to the methods of Flodin (1962).

Column packing. A chromatographic column (internal diam. 2.25 cm.), fitted at its lower end with a coarse, sintered-glass disk, was mounted vertically and the outlet closed. The column was half-filled with 0.1 M-phosphate buffer, pH 7.0, and the gel suspension then added to fill the column completely. When the gel had sedimented to a depth of 2 cm., the outlet was opened and the gel suspension added to keep the column full of liquid. This process was continued until the desired bed length had been obtained.

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The column was then removed for permanent keeping at 2° and washed with 3–5 bed vol. of buffer to stabilize the bed and also to remove any material which might absorb at 280 m μ . It was not found necessary to stabilize the bed surface mechanically. Since a feature of the method of gel filtration is that substances present in the sample are eluted without change of eluent, the column is regenerated during the experiment and the gel can be used again.

Thin-layer gel filtration. The type of gel used for this method was Sephadex G-200 Superfine (particle size 10–40 μ , water regain 20), 4g. of which was poured on to the surface of 100 ml. of 0.1M-phosphate buffer, pH 7.0, and soaked by the liquid. This concentration was a critical factor in the success of the method. The suspension was allowed to swell for at least 72 hr. at room temp., care being taken to ensure that no aggregates were present. The amount of gel suspension prepared was sufficient for four 20 cm. $\times$ 20 cm. glass plates, which were thoroughly cleaned successively in detergent (Pyroneg; Diversey Ltd., London, W. 1) and distilled water before use. The clean, dry plates were coated with a layer (0.9 mm. thick) of the gel by means of a Unoplan thin-layer spreader (Shandon Scientific Co. Ltd., London, N.W. 10). The plates were stored until required in a horizontal position in a closed chamber containing a dish of the solvent.

Before the plates were used, 0.1M-phosphate buffer, pH 7.0, was allowed to flow through the gel for 12 hr. After equilibration, the plates were mounted horizontally over a white background and samples (5 μ l.), containing approx. 5–10 μ g. of protein, were applied to the gel as a series of spots about 1.5 cm. apart in a line 5 cm. from one edge of the plate. In order not to damage the soft gel layer, a micrometer syringe was used for the application of the samples, the zones of which did not exceed 3 mm. diam.

The plates were developed by inclining them at an angle in a chamber consisting of a large glass chromatography tank inverted over a sheet of plate glass. The upper end of the plate was connected to the solvent reservoir by means of a wick made from Whatman no. 3MM filter paper. Excess of liquid was prevented from accumulating at the bottom of the plate by a filter-paper pad which was moistened to ensure good contact with the gel layer. The solvent was allowed to flow through the gel at a rate which could be measured by observing the rate of movement of a concentrated solution of blue dextran (Pharmacia), which is completely excluded from the gel and therefore travels with the solvent front. A flow rate of 2 cm./hr. was satisfactory and this could be achieved by inclining the plate at an angle approx. 20° to the horizontal. When development was complete, the plate was removed from the chamber and supported horizontally. The filter-paper wick was removed and the smooth side of a piece of Whatman no. 3MM filter paper, 20 cm. $\times$ 20 cm., was applied to the gel surface, the paper being rolled on to the gel to prevent the trapping of air bubbles. The line where the samples were applied to the gel was marked on the filter paper and the covered plate dried in an oven at 80° for 30 min.

The areas of protein on the filter paper covering the plate were then identified by means of a highly sensitive stain, 1% Naphthalene Black 10 B (E. Gurr and Co., London). The stain, dissolved in methanol–water–acetic acid (5:4:1, by vol.), was poured evenly over the plate and, after 1 min., washed with liberal amounts of the mixed solvent until all the excess of stain had been removed and the blue–black

spots were easily visible on a white or pale-blue background. During this procedure, the paper prints became detached from the plate, where the washing was completed, and were then dried at room temp.

Methods involved in the use of [14 C]glyoxylate. Experiments with [14 C]glyoxylate were carried out in tubes made from 5 cm. lengths of 5 mm. internal-bore soft glass sealed at one end. Components of the reaction mixture were added from Pasteur pipettes, 12–15 cm. long, made from the same glass tubing drawn out to a very fine tip of about 0.5 mm. at one end. Calibration of each pipette was effected by transferring the contents of a standard 0.05 ml. pipette filled with water into the capillary and marking the position of the meniscus. The calibration and accuracy of delivery were checked by weighing samples pipetted into small, airtight polythene containers (30 mm. $\times$ 15 mm.) obtained from the Loughborough Glass Co. Ltd.; an accuracy of $\pm 2\%$ by wt. was obtained.

Paper chromatography of 14 C-labelled carboxylic acids. Samples (0.05 ml.) of the clear, supernatant solution from reaction mixtures were applied to a sheet of Whatman no. 4 chromatography paper (10 in. $\times$ 8 in.) and allowed to develop until the solvent had risen 10 in. The carboxylic acids were separated by the following solvent systems: phenol–formic acid–water (Kornberg, 1958); butan-1-ol–water–propionic acid (Calvin & Benson, 1949); butan-1-ol–acetic acid–water (12:3:5, by vol.) as used by Smith (1958); ether–acetic acid–water (13:3:1, by vol.) used by Denison & Phares (1952); benzene–ether–90% (v/v) formic acid–water (15:35:7:5, by vol.) as used by Weimberg (1959); butan-1-ol–pyridine–water (1:1:1, by vol.) used by Morrison (1953).

Of these solvent systems, that of Kornberg (1958) was most frequently used since it gave good separation of the products with low R_F for glyoxylate.

Detection of spots on the chromatogram was by the aniline–xylose reagent of Nordmann & Nordmann (1960). The chromatograms were either sprayed with or dipped into the reagent, dried at ambient temperature in air for a few minutes and then conditioned at 100° for 5–10 min. Brown spots developed on a white background, which gradually darkened. Radioautography of the chromatogram was carried out in the conventional manner.

Elution and assay of radioactive areas on chromatograms. The radioactive areas of chromatograms were cut out in the shape of shields and attached by surface tension to a wick of Whatman no. 3MM filter paper hanging from a trough of water. Elution was initially carried out in the descending manner in an apparatus open to the atmosphere so that the resulting evaporation prevented the formation of droplets and caused concentration of the radioactive material at the lower tip of the paper shield. The apparatus was then enclosed in a large glass vessel and the radioactive material eluted quantitatively into a small, weighed polythene container. The container, holding about 0.2 ml. of eluent, was sealed to prevent evaporation. The volume of eluent was determined by re-weighing.

Samples (0.02 ml.) of the eluent were applied to the smooth side of 25 mm.-diam. disks of filter paper. The disks were dried under an infrared lamp for 1 min. and mounted with smooth side uppermost on aluminium planchets, and the radioactivity was determined with a Nuclear Chicago automatic gas-flow counter.

Direct counting of radioactive areas on chromatograms.

The requisite areas of the chromatogram were cut out, mounted on aluminium planchets and assayed in the automatic gas-flow counter. When it was desirable not to destroy the chromatogram, the radioactivity of the compounds was determined by direct counting, *in situ*, with a mica end-window Geiger-Müller tube (General Electric Co. type EHM 2/5). A variety of masks was used to prevent the counting of more than one spot.

Identification of ^{14}C -labelled reaction products. The identity of an unknown radioactive compound on a chromatogram was first indicated by comparing its position (R_F) with the positions of known compounds run in the same solvent. After elution from the chromatogram, the unknown radioactive spot was mixed with excess of the unlabelled compound which it was suspected to be. The mixture was then applied to the origins of a number of sheets of Whatman no. 4 chromatography paper and developed in the ascending manner with the various solvent systems previously described. The position of the ^{14}C -labelled compound was located by radioautography and the location of the non-radioactive suspected compound determined by spraying the chromatogram with the aniline-xylose reagent.

If the size, shape and position of the two spots were identical in all solvent mixtures tested, it was assumed that the radioactive product corresponded with the unlabelled compound.

Assay of the two glyoxylate reductases. The enzymes were assayed by measuring the rate of oxidation of either NADH or NADPH in the presence of enzyme and glyoxylate. Determinations were carried out spectrophotometrically in 3 ml. silica cells of 1 cm. light-path containing 0.3 ml. of m -phosphate buffer, pH 6.8, 0.1 ml. of m -sodium glyoxylate and an appropriate amount of enzyme. The reaction was started by the addition of 0.1 ml. of 4 mM-NADH or 4 mM-NADPH and the extinction at 340 μm observed with a Hilger-Gilford recording spectrophotometer at 25°. At some stages, it was necessary to measure the rates of oxidation of NADH and NADPH in the absence of glyoxylate and use the values to determine the true enzyme activity.

One unit of enzyme is defined as that quantity which catalyses a decrease in extinction at 340 μm of 0.001/min. under the conditions of assay. Specific activity is defined as units of enzyme/mg. of soluble protein.

Separation of two glyoxylate reductases

Stage 1: preparation of a crude extract. Frozen, crushed cells (35 g.) were thawed, diluted to 110 ml. with 5 mM-phosphate buffer, pH 7.0, and deoxyribonuclease (1 mg.) and ribonuclease (1 mg.) added to the suspension. Precautions were taken to prevent excessive bubbling during this treatment. The suspension was centrifuged at 70 000 g for 30 min. in a Spinco model L ultracentrifuge, with the type 30 rotor at 2°. The sediment was discarded. All further operations were carried out at 2°.

Stage 2: ultracentrifugation. The supernatant solution was centrifuged at 140 000 g for 3 hr. in a Spinco model L ultracentrifuge with the type 50 rotor. The ribosomal pellet was discarded.

Stage 3: fractionation with ammonium sulphate. The requisite amount of solid $(\text{NH}_4)_2\text{SO}_4$ (specially prepared free from heavy metals by British Drug Houses Ltd.) was slowly added to the supernatant solution to produce 30% saturation (17.6 g./100 ml. of extract). The solution was

agitated continuously for 20 min. and then centrifuged. The precipitate was discarded and a further amount of $(\text{NH}_4)_2\text{SO}_4$ added to the supernatant solution to produce 60% saturation (19.8 g./100 ml. of extract). The previous procedure was repeated, except that the precipitate was retained, dissolved in 30 ml. of 5 mM-phosphate buffer, pH 7.0, and dialysed against 10 l. of 5 mM-phosphate buffer, pH 7.0, which was replaced three times with 10 l. of fresh buffer at intervals of 1 hr. Continuous agitation was essential to prevent excessive precipitation of the protein and this was achieved by using a magnetic stirrer. The small quantity of material which was precipitated during dialysis was removed by centrifuging.

Stage 4: column chromatography. A batch of treated DEAE-cellulose suspended in 5 mM-phosphate buffer, pH 7.0, was poured into a chromatographic column (internal diam. 1.8 cm.) fitted at its lower end with a coarse sintered-glass disk. Equilibration was carried out at 2° by allowing 1 l. of 5 mM-phosphate, pH 7.0, to pass through the column. The protein was applied to the DEAE-cellulose column (20 cm. $\times$ 1.8 cm.) at a rate just sufficient to keep the top of the column moist: by this means the material was adsorbed as a narrow band at the top of the column. The unadsorbed material was removed by passing 60 ml. of 5 mM-phosphate buffer, pH 7.0, through the column. A linear gradient of NaCl was then applied to the column by allowing 200 ml. of 0.5 M-NaCl in 5 mM-phosphate buffer, pH 7.0, to mix, with constant stirring, with 200 ml. of 5 mM-phosphate buffer, pH 7.0. The mixture was allowed to flow through the column at about 30 ml./hr. Fractions, each containing 7.75 ml. (180 drops), were collected with a Beaumaris automatic fraction collector (Beaumaris Instrument Co. Ltd., Beaumaris, Anglesey). The chloride content of the fractions was determined.

Two glyoxylate reductases were eluted from the column (Fig. 1). Those fractions which showed an increase in specific activity over the previous stage were combined and the protein was precipitated by adding $(\text{NH}_4)_2\text{SO}_4$ to final concn. 60% saturation (39 g./100 ml. of soln.). (i) NADPH-linked enzyme was eluted between 40 and

Fig. 1. Elution of NADH-linked and NADPH-linked glyoxylate reductases from DEAE-cellulose. For conditions, see text. —, Protein concn. measured as $E_{280 \text{ m}\mu}$. ○, Activity of NADH-linked glyoxylate reductase; ●, activity of NADPH-linked glyoxylate reductase; ▲, gradient of chloride concn.

100mM-NaCl, with the peak of activity appearing at 65mM-NaCl. The fractions used for further purification were 12-15 inclusive. (ii) NADH-linked enzyme was eluted between 150 and 230mM-NaCl, with the peak of activity appearing at 175mM-NaCl. The fractions used for further purification were 23-26 inclusive.

Stage 5: gel-filtration, Sephadex G-200. The bed surface of a Sephadex G-200 column (55 cm. $\times$ 2.25 cm.) was allowed to become just exposed. The precipitate containing the NADPH-linked or NADH-linked enzyme was dissolved in 1 ml. of 0.1M-phosphate buffer, pH 7.0, and carefully placed on top of the column with a pipette. At the moment the solution disappeared from the surface, a small amount of 0.1M-phosphate buffer, pH 7.0, was added to wash the surface. When this in turn had disappeared, a larger amount was added. The column was eluted with 300 ml. of 0.1M-phosphate buffer, pH 7.0, at a rate of 5.5 ml./hr. Fractions each containing 2.75 ml. (70 drops) were collected with a Beaumaris fraction collector. The NADPH-linked enzyme was eluted between 115 and 154 ml. of buffer collected (Fig. 2). Those fractions with activity greater than 300 units/ml. were combined, namely fractions 44-51

inclusive. The NADH-linked enzyme was eluted between 100 and 132 ml. of buffer collected (Fig. 3) and those fractions with activity greater than 3000 units/ml., namely fractions 40-44 inclusive, were combined.

Stage 6: precipitation with ammonium sulphate. The enzymes were precipitated from their respective pooled fractions by adding $(\text{NH}_4)_2\text{SO}_4$ to give final concn. 60% saturation (39 g./100 ml. of soln.). Each precipitate was removed by centrifuging and dissolved in 2 ml. of water. When required, these enzyme solutions were further diluted with buffer (1:10 dilution for the NADPH-linked enzyme; 1:20 dilution for the NADH-linked enzyme). The purified enzymes retained their activities for about 6 weeks when stored as a suspension in $(\text{NH}_4)_2\text{SO}_4$ soln. at 2°. Activity was lost very quickly when frozen or when kept as an $(\text{NH}_4)_2\text{SO}_4$ precipitate. The results of each stage of the purification procedure are summarized for the NADPH-linked glyoxylate reductase in Table 1 and for the NADH-linked enzyme in Table 2.

RESULTS

Coenzyme specificity. The relative rates of oxidation of both NADH and NADPH were determined for each purified enzyme. Endogenous

Fig. 2. Elution of NADPH-linked glyoxylate reductase from a Sephadex G-200 column (55 cm. $\times$ 2.25 cm.). For conditions, see text. —, Protein concn. measured as $E_{280 \text{ m}\mu}$. $\circ$, Activity of NADPH-linked glyoxylate reductase.

Fig. 3. Elution of NADH-linked glyoxylate reductase from a Sephadex G-200 column (57 cm. $\times$ 2.25 cm.). For conditions, see text. —, Protein concn. measured as $E_{280 \text{ m}\mu}$. $\circ$, Activity of NADH-linked glyoxylate reductase.

Table 1. Summary of the purification of the NADPH-linked glyoxylate reductase

Stage	Vol. of soln. (ml.)	Protein (mg.)	Activity (units)	Recovery (%)	Sp. activity (units/mg. of protein)	Purification
1. Crude extract	94	1860	46800	100	25.2	1.0
2. Ultracentrifugation (140000g)	84	1250	43700	93	35.0	1.4
3. $(\text{NH}_4)_2\text{SO}_4$ precipitate (30-60% saturation)	38	538	39600	85	73.6	2.9
4. DEAE-cellulose (fractions 12-15)	27	32	22400	48	692	27.4
5. Gel-filtration (Sephadex G-200 fractions 44-51)	21	2.7	13400	29	4920	195
6. $(\text{NH}_4)_2\text{SO}_4$ precipitate (60% saturation)	2	2.2	12200	26	6660	220

Table 2. Summary of the purification of the NADH-linked glyoxylate reductase

Stage	Vol. of soln. (ml.)	Protein (mg.)	Activity (units)	Recovery (%)	Sp. activity (units/mg. of protein)	Purification
1. Crude extract	94	1860	231 800	100	124.7	1.0
2. Ultracentrifugation (140000g)	84	1250	219 300	95	175.5	1.4
3. (NH ₄) ₂ SO ₄ precipitate (30–60% saturation)	38	538	176 800	76	328	2.6
4. DEAE-cellulose (fractions 23–26)	28	90	103 500	45	1150	9.2
5. Gel-filtration (Sephadex G-200 fractions 40–44)	13	7.8	61 800	27	7930	63.7
6. (NH ₄) ₂ SO ₄ precipitate (60% saturation)	2	5.9	58 200	25	9870	79

activity towards both cofactors had been removed. The NADH-linked glyoxylate reductase showed no activity towards NADPH whereas the NADPH-linked enzyme showed some activity towards NADH. The relative activities of this latter enzyme towards NADPH and NADH respectively were in the ratio 4.4:1.

pH optimum. The variation of enzyme activity with pH was investigated with the following ranges of buffer solutions: 0.1M-NaH₂PO₄-Na₂HPO₄, pH 5.8–8.0; 0.1M-KH₂PO₄-citrate, pH 4.0–5.8; 0.1M-glycine adjusted to pH 8.6 with 2N-sodium hydroxide. The standard assay procedure was used except that 2.7ml. of the required buffer was added to the cuvette. The pH was determined at the end of the reaction with a Pye single glass electrode. Maximal activity for both enzymes occurred between pH 6.0 and 6.8; the NADPH-linked glyoxylate reductase retained its activity over a wider range of pH values (60% activity at pH 5.0 and pH 8.6) than the NADH-linked glyoxylate reductase (60% activity at pH 5.2 and pH 7.6).

Substrate specificity. The activities of the enzymes towards various substrates are shown in Table 3. The substrates, neutralized with either sodium hydroxide or hydrochloric acid, were added to give a final concentration 100 μ moles/3ml. cuvette. Activity is expressed as a percentage of the rate of oxidation observed with glyoxylate as substrate.

Both enzymes catalysed the reduction of hydroxypyruvate but at a lower rate than glyoxylate. The fact that glyoxal served as a substrate for these enzymes was not surprising in view of the similarity of its structure to glyoxylate. The NADPH-linked glyoxylate reductase showed no significant activity towards any other substrate tested, but the preparation containing the NADH-linked enzyme reduced both oxaloacetate and pyruvate at a significant rate because of contamination of the protein with malate and lactate dehydro-

Table 3. Substrate specificity of the two glyoxylate reductases

Substrate	Rate of reaction	
	NADH-linked enzyme	NADPH-linked enzyme
Glyoxylate	100*	100†
Hydroxypyruvate	56	71
Glyoxal	50	18
Oxaloacetate	27	5
Pyruvate	23	0
DL-Glyceraldehyde	10	8
Glycolaldehyde	5	0
Oxalate	2	8
α -Oxoglutarate	0	5
Formate	0	0
Oxamate	0	0
Formaldehyde	0	0
Acetaldehyde	0	0

* Corresponds to a change in $E_{340\text{ m}\mu}$ of 0.12/min.

† Corresponds to a change in $E_{340\text{ m}\mu}$ of 0.038/min.

genases. At each stage of the purification procedure, the activities of both malate dehydrogenase and lactate dehydrogenase were compared with the activity of the NADH-linked glyoxylate reductase; a gradual but not total removal of the contaminating enzymes was observed. Similar investigations also demonstrated that, in addition to the two glyoxylate reductases, the bacterium contained another enzyme that catalysed the reduction of hydroxypyruvate.

Inhibition of enzyme action. The effect of *p*-chloromercuribenzoate, which forms mercaptides with the thiol groups of proteins (Hellerman, Chinard & Deitz, 1943), on the activities of the two glyoxylate reductases in the presence of their respective cofactors was determined (Table 4).

The inhibitor exerted a much greater effect on

Table 4. *Inhibition by p-chloromercuribenzoate*

The two enzymes were assayed, as described in the Methods and Materials section, by measuring the rate of oxidation of either NADH or NADPH in the presence of glyoxylate after the prior incubation of the enzyme at 25° for 5 min. with the compounds listed.

Added compound	Rate of reaction	
	NADH-linked enzyme	NADPH-linked enzyme
None	100*	100†
<i>p</i> -Chloromercuribenzoate (0.01 mM)	12	100
<i>p</i> -Chloromercuribenzoate (0.1 mM)	6	50
L-Cysteine (1 mM) + <i>p</i> -chloromercuribenzoate (0.01 mM)	92	98
L-Cysteine (10 mM) + <i>p</i> -chloromercuribenzoate (0.1 mM)	90	78
L-Cysteine (1 mM)	100	100

* Corresponds to a decrease in $E_{340\text{ m}\mu}$ of 0.12/min.

† Corresponds to a decrease in $E_{340\text{ m}\mu}$ of 0.034/min.

the NADH-linked than on the NADPH-linked enzyme. The addition of L-cysteine before *p*-chloromercuribenzoate afforded almost complete protection against the inhibition.

The effect of other inhibitors on the enzymes is shown in Table 5.

The rate of reduction of glyoxylate catalysed by the glyoxylate reductases was not significantly affected by glycine, sodium fluoride, EDTA, *N*-ethylmaleimide or 1 mM-oxaloacetate, 1 mM-arsenite and 1 mM-*o*-phenanthroline.

Potassium cyanide and oxalate affected principally the NADPH-linked enzyme, whereas 1 mM-oxamate, 10 mM-pyruvate and 10 mM-oxaloacetate significantly decreased the rate of reduction by the NADH-linked reductase only. Both enzymes were strongly inhibited by semicarbazide and 10 mM-oxamate, 10 mM-arsenite and 10 mM-*o*-phenanthroline, but only partially inhibited by $\alpha\alpha'$ -bipyridyl. 1 mM-Iodoacetamide and 1 mM-pyruvate exerted a slight effect on the reaction catalysed by the NADH-linked enzyme.

Kinetic properties. Effect of substrate concentration on the initial reaction rate was determined at pH 6.8 in the presence of a constant amount of enzyme and NADH or NADPH respectively. Maximal rate of glyoxylate reduction by both enzymes occurred with 33.3 mM-sodium glyoxylate. Oxidation rates of NADH and NADPH decreased at glyoxylate concentrations less than or exceeding this value demonstrating that substrate concen-

Table 5. *Inhibition of the two glyoxylate reductases*

The two enzymes were assayed, as described in the Methods and Materials section, by measuring the rate of oxidation of either NADH or NADPH in the presence of glyoxylate after the prior incubation of the enzyme at 25° for 5 min. with the compounds listed below.

Added compound	Rate of reaction	
	NADH-linked enzyme	NADPH-linked enzyme
None	100*	100†
Semicarbazide (100 μ moles)	56	26
Potassium cyanide (100 μ moles)	76	37
Glycine (100 μ moles)	98	91
Sodium fluoride (10 mM)	98	100
Iodoacetamide (1 mM)	79	100
<i>N</i> -Ethylmaleimide (1 mM)	88	100
$\alpha\alpha'$ -Bipyridyl (10 mM)	67	79
EDTA (10 mM)	96	95
Sodium arsenite (10 mM)	5	18
Sodium arsenite (1 mM)	95	85
Oxamate (10 mM)	12	62
Oxamate (1 mM)	60	97
<i>o</i> -Phenanthroline (10 mM)	21	50
<i>o</i> -Phenanthroline (1 mM)	86	91
Oxalate (10 mM)	82	12
Oxalate (1 mM)	88	41
Pyruvate (10 mM)	30	94
Pyruvate (1 mM)	78	94
Oxaloacetate (10 mM)	32	78
Oxaloacetate (1 mM)	95	100

* Corresponds to a decrease in $E_{340\text{ m}\mu}$ of 0.12/min.

† Corresponds to a decrease in $E_{340\text{ m}\mu}$ of 0.034/min.

tration was a critical factor in the assay procedure adopted for both glyoxylate reductases. The Michaelis constant (K_m) for glyoxylate, determined from the plot of the reciprocal rate of NADH or NADPH oxidation against the reciprocal substrate concentration (Lineweaver & Burk, 1934), was 7 mM for the NADH-linked enzyme and 14 mM for the NADPH-linked enzyme.

The effect of substrate concentration on the initial reaction rate was investigated in a similar manner with lithium hydroxypyruvate as substrate. Maximal rate of hydroxypyruvate reduction by the NADH-dependent enzyme was observed with 8.3 mM-substrate. The NADPH-dependent enzyme was saturated with substrate at 2.7–8.3 mM. Once again, both enzymes were inhibited by excess of substrate, the effect being more marked on the NADPH-linked reductase. K_m for hydroxypyruvate was 5 mM for the NADH-linked enzyme and 7 mM for the NADPH-linked enzyme; that is, in each case, less than for glyoxylate.

Equilibrium of the reaction. The apparent and

thermodynamic equilibrium constants, K and K_H respectively, for the reaction:

were calculated from the expressions

$$K = \frac{[\text{glyoxylate}^-] [\text{reduced cofactor}]}{[\text{glycollate}^-] [\text{oxidized cofactor}]}$$

and

$$K_H = K [\text{H}^+]$$

for both glyoxylate reductases.

Since the equilibrium of the systems greatly favoured glycollate formation, it proved extremely difficult to demonstrate directly the enzymic formation of glyoxylate and NAD or NADP from glycollate. With high concentrations of the reactants and a H^+ ion concentration approx. 8.0, it was just possible to detect an increase in extinction at 340 m μ . Because of this small rise in $E_{340 \text{ m}\mu}$, it became impossible to use a blank cell in its normal role since all the components in the reaction mixture showed varying levels of absorption at the wavelength used. Zelitch (1955), in demonstrating the reversibility of the glyoxylate reductase from tobacco leaves, stated that his readings were taken against a blank cell containing all the constituents of the reaction mixture except NAD^+ . In his experiment, he showed an increase in $E_{340 \text{ m}\mu}$ of 0.06 over 30 min. as measured with a Beckman spectrophotometer. At the concentration Zelitch used, the oxidized nicotinamide-adenine dinucleotide will also give a reading of 0.06 at 340 m μ and it seems doubtful if an increase in extinction of that

order over 30 min. could be detected accurately by the instrument used.

To overcome these difficulties a cuvette was prepared containing all the reactants except 10 μg . of the enzyme; this was used as a partial blank cell in calibrating the instrument, a Hilger-Gilford automatic recording spectrophotometer. The automatic recorder was started at the same time as the enzyme was added to the cuvette to begin the reaction and $E_{340 \text{ m}\mu}$ of both the partial blank cell and experimental cell were determined until there was no further change. The readings at 340 m μ of the experimental cell were extrapolated back to zero time and this extinction value used as the true blank value. The difference between this reading and the final reading was used to determine the amount of reduced dinucleotide formed at equilibrium; any variation in the reading of the partial blank cell was taken into account.

The apparent and thermodynamic equilibrium constants calculated for five experiments are given in Tables 6 and 7 for the NADH- and NADPH-linked enzymes respectively.

Conversion of $[1\text{-}^{14}\text{C}]$ glyoxylate into $[^{14}\text{C}]$ glycollate. $[1\text{-}^{14}\text{C}]$ Glyoxylate of relatively high activity was used to examine the products of the reaction catalysed by the two glyoxylate reductases. Samples of the deproteinized solutions were examined by paper chromatography and radioautography. The conversion of glyoxylate into glycollate was quantitatively determined by assaying the radioactivity of the appropriate spots from the chromatogram.

The complete system, in a small glass tube, contained in 0.15 ml.: 10 μmoles of phosphate buffer, pH 6.8; 0.05 μmole (0.25 μc) of sodium $[1\text{-}^{14}\text{C}]$ glyoxylate; 0.2 μmole of either NADH or NADPH;

Table 6. *Equilibrium constants for the oxidation of glycollate to glyoxylate by the NADH-linked glyoxylate reductase*

The reaction mixture contained, in a total volume of 3.0 ml.: 300 μmoles of tris-HCl buffer at the pH specified; 13.1 μmoles of NAD^+ ; various amounts of sodium glycollate; an appropriate amount of enzyme. When equilibrium had been attained, as indicated by no further change in extinction at 340 m μ , the pH was determined with a Pye single glass electrode. The concn. of NAD^+ was calculated from $E_{280 \text{ m}\mu}$ and the equilibrium concn. of NADH from $E_{340 \text{ m}\mu}$ as described in the text. The concn. of glycollate, NAD^+ and H^+ were assumed not to vary throughout the reaction since the amounts utilized were less than 1% of those added. The concn. of glyoxylate formed was taken to be equal to that of the NADH.

pH	Equilibrium concn.				Equilibrium constants	
	$[\text{H}^+]$ (μmM)	$[\text{NAD}^+]$ (mM)	$[\text{NADH}]$ (μM)	$[\text{Glycollate}]$ (M)	$10^{10} \times K$	$10^{18} \times K_H$
8.00	10.00	13.1	4.1	1.7	7.6	7.6
8.05	8.92	13.1	3.3	1.3	6.4	5.7
8.07	8.52	13.1	2.6	1.0	5.2	4.4
8.09	8.13	13.1	3.2	1.5	5.2	4.2
8.12	7.58	13.1	5.2	1.9	10.8	8.2
Average					7.0	6.0

Table 7. *Equilibrium constants for the oxidation of glycollate to glyoxylate by the NADPH-linked glyoxylate reductase*

The conditions and method used were identical with those described in Table 6 for the NADH-linked enzyme, except that the reaction mixture contained 12.2 μ moles of NADP⁺ in place of NAD⁺.

pH	Equilibrium concn.				Equilibrium constants	
	[H ⁺] (μ M)	[NADP ⁺] (mM)	[NADPH] (μ M)	[Glycollate] (M)	$10^{10} \times K$	$10^{18} \times K_H$
7.77	17.0	12.2	1.8	1.9	1.4	2.4
7.83	14.8	12.2	1.3	1.0	1.4	2.1
7.88	13.2	12.2	2.1	1.0	3.6	4.8
7.90	12.6	12.2	2.7	1.7	3.5	4.4
7.92	12.0	12.2	1.4	1.8	0.9	1.1
			Average		2.2	3.0

Table 8. *Comparison of physical properties of glyoxylate reductases with those of other proteins*

Samples of both glyoxylate reductases, together with haemoglobin, cytochrome *C*, ribonuclease, lysozyme and bovine serum albumin were applied as a series of spots to Sephadex G-200 Superfine gel. After development of the plate and identification of the protein zones, the distances (migration velocities) which the various proteins had travelled were compared with haemoglobin. From these measurements, R_{Hb} , the partition coefficients (Kd) and mol. wts. (M) of the proteins were calculated as described in the text.

Protein	$10^{-3} \times$ Known mol. wt.	Reference	R_{Hb}	Kd	$10^{-3} \times$ Calc. mol. wt.
Haemoglobin	64.5	Gutter, Sober & Peterson (1956)	1.00	0.62	29.5
Cytochrome c	12.4	Margoliash (1962)	0.68	0.85	10.0
Ribonuclease	13.7	Hirs, Moore & Stein (1956)	0.72	0.82	11.5
Lysozyme	14.7	Fromageot & de Garilhe (1950)	0.65	0.88	8.9
Bovine serum albumin	67.0	Phelps & Putnam (1960)	1.37	0.35	105.0
NADH-linked glyoxylate reductase			1.52	0.24	175.0
NADPH-linked glyoxylate reductase			1.55	0.22	190.0

1 μ g. of the NADH-linked enzyme or 15 μ g. of the NADPH-linked enzyme.

After incubation at 30° for various times up to 160 min., the reaction was stopped by placing the tube in boiling water for 1 min., cooling in ice and adding 0.05 ml. of 2N-hydrochloric acid to complete the precipitation of the protein.

The radioautographs showed the progressive conversion of glyoxylate into glycollate by both enzymes in the presence of their respective cofactors. Negligible amounts of glycollate were formed in the absence of reduced pyridine nucleotide or with boiled enzyme in the presence of the required cofactor. With the NADPH-linked glyoxylate reductase, a small amount of glycollate was formed when NADH was the cofactor. The NADH-linked enzyme, however, was entirely specific for its coenzyme and no glycollate was produced when NADPH was the cofactor. This

finding agrees with the results obtained when comparing the rates of oxidation of NADH and NADPH in the presence of the enzymes.

Estimation of the molecular weights of the two glyoxylate reductases. The purified enzymes, together with proteins of known mol. wt., were allowed to traverse a thin layer of Sephadex G-200 Superfine gel. The mol. wt. (M) of the protein was calculated from the expression $\log M = 1.47R_{Hb}^{G200} + 3.0$ (Morris, 1964). R_{Hb} was defined as $R_{Hb} = dp/dHb$, where dp and dHb were the distances travelled respectively by the test protein and haemoglobin during the development of the chromatograms. The relationship between the partition coefficient (Kd) and R_{Hb} values of the protein for Sephadex G-200 is given by $Kd^{G200} = 1.35 - 0.73R_{Hb}^{G200}$ (Morris, 1964). The results obtained are shown in Table 8.

The molecular weights of the NADH-linked and

NADPH-linked glyoxylate reductases were estimated as 175 000 and 190 000 respectively. These values are at the upper limit for determining molecular weights by this method and too much emphasis should not therefore be attached to the actual values, beyond the fact that they appear to be somewhat large molecules.

The resolving power of the method is remarkably high, owing to the small particle size of the gel and a linear flow rate of approx. 1.5 cm./hr. The minimum amount of protein that can be used depends on the sensitivity of the method for locating the protein zones.

The relatively slow migration of lysozyme shown in Table 8 has been observed by Whitaker (1963), Porath (1960), Morris (1964) and by Miranda, Rochat & Lissitzky (1962); it is probably due to an ion-exchange effect. The similar retardation of haemoglobin is unlikely to be due to an ion-exchange or adsorption effect since it has also been observed on agar and polyacrylamide gels (Andrews, 1962); it is probably due to the dissociation of the haemoglobin into α - and β -chains with mol.wt. about 34 000.

Effect of anions. Zelitch (1955) has demonstrated that anions stimulate the enzymic activity of some glyoxylate reductases. The effect of anions on the NADH-linked and NADPH-linked glyoxylate reductases purified in this work was determined with glyoxylate and hydroxypyruvate as substrates. Each cuvette contained, in final vol. 3.0 ml.: 300 μ moles of phosphate buffer, pH 6.9; 0.4 μ mole of either NADH or NADPH; 10 μ moles of glyoxylate or hydroxypyruvate; 100 μ moles of the salt and an appropriate amount of enzyme. The initial rate of oxidation of the cofactor was determined at 25°. The salts used were: sodium sulphate, potassium sulphate, ammonium sulphate, sodium chloride, potassium chloride, ammonium chloride, potassium bromide, potassium nitrate and potassium iodide.

The purified enzymes, when assayed in the presence of the various anions and either glyoxylate or hydroxypyruvate, showed no increase in activity compared with that in the absence of the anions. Since the enzymes were stored in a solution of ammonium sulphate and dialysis was not possible owing to dilution difficulties, it was initially assumed that the presence of ammonium sulphate was affecting the stimulatory action. Accordingly, a crude extract of the organism was prepared and dialysed against 10 l. of 5 mM-phosphate buffer, pH 7.0, which was replaced three times with 10 l. of fresh buffer at 1 hr. intervals. The solution of non-diffusible material was then used as a source of both enzymes and tested for activity in the presence of the various anions. Once again, there was no stimulation of enzymic activity when the

salts listed above were added to the reaction mixture.

Factors which may have been preventing anionic stimulation of the enzymes were investigated. The effects of varying the ratio of salt to substrate concentration, decreasing the buffer concentration, and incubating the salt and enzyme before adding the substrate were studied. None of these modifications affected the activity of the enzymes.

DISCUSSION

The high Michaelis constants determined for the glyoxylate reductases described in this paper were peculiar to the organism used since the NADPH-linked enzyme in tobacco and spinach leaves (Zelitch & Gotto, 1962) showed an affinity for glyoxylate 100 times that of its NADH-linked counterpart (Zelitch, 1955).

D-Glycerate-dehydrogenase activity (Stafford, Magaldi & Vennesland, 1954) has been associated with all the glyoxylate reductases so far purified, although the NADPH-linked enzyme from tobacco and spinach leaves (Zelitch & Gotto, 1962) had very much less of this activity than the NADH-linked enzyme from the same source. The glyoxylate reductases obtained from *Pseudomonas* sp., however, reduced glyoxylate and hydroxypyruvate at approximately equal rates under optimum conditions.

During the purification of the enzyme from tobacco leaves (Zelitch, 1955), and again in this work, another enzyme specific for hydroxypyruvate was either destroyed or removed. It seems highly probable that this enzyme was the true D-glycerate dehydrogenase; its properties were similar to those of the glyoxylate reductases, indicating that they were closely related proteins.

The fact that the NADH-linked glyoxylate reductase is five times as active in the crude extract as the NADPH-linked enzyme might result from a balancing effect to prevent the over-utilization of the nicotinamide nucleotide which is present in the lesser amount in the cell.

The observations that the optimum pH range for the reduction of glyoxylate by both reductases from *Pseudomonas* sp. was 6.0–6.8 and that the NADPH-linked enzyme retained a large percentage of its activity over a wider range of H^+ ion concentration are in accordance with similar findings of Zelitch (1955) and Laudahn (1963) for the glyoxylate reductases from tobacco and spinach leaves.

The inhibitory action of keto acids on the enzymic reduction of glyoxylate indicates that the glyoxylate reductases combine with these acids without reducing them, thus blocking the active sites on the enzyme to glyoxylate molecules.

The effect of *p*-chloromercuribenzoate on glyoxylate reduction shows that the activity of glyoxylate reductases is strongly dependent on free thiol groups. This is in agreement with Zelitch (1955), who presented evidence for the existence of enzyme-inhibitor, enzyme-NADH and enzyme-substrate complexes involving the SH-groups of the glyoxylate reductase from tobacco leaves.

Anions stimulated the rate of oxidation of NADH when hydroxypyruvate but not glyoxylate was the substrate with glyoxylate reductase (Zelitch, 1955) and D-glycerate dehydrogenase (Holzer & Holldorf, 1957). This behaviour can be explained on the assumption that the anions produce changes in the enzyme which enable additional sites of attachment to become available for reaction with the substrate.

Anions did not stimulate the reduction of glyoxylate or hydroxypyruvate by the glyoxylate reductases from *Pseudomonas* sp. either in crude extracts or after purification. It seems probable therefore that additional sites of catalytic attachment were not present on these enzymes, a fact which may be connected with their high Michaelis constants.

The reversibility of the reactions catalysed by the two glyoxylate reductases was demonstrated and the equilibrium constants were determined. Equilibrium conditions favour reduction of glyoxylate. The oxidation of glycollate to glyoxylate by the glyoxylate reductases from *Pseudomonas* sp. is highly endergonic with $\Delta G' + 24$ kcal./mole at pH 8.0. As indicated by Zelitch (1955), if this oxidation could be achieved by an enzyme requiring molecular oxygen and not linked to a nicotinamide nucleotide, the reaction would be exergonic with $\Delta G'$ about -41 kcal./mole at pH 8.0. Such an enzyme, the flavoprotein glycollic acid oxidase, has been found in plants (Zelitch, 1953; Clagett, Tolbert & Burris, 1949) and in bacteria (Katagiri & Tochikura, 1960a).

The glycollate formed in *Pseudomonas* sp. did not appear to be further metabolized nor was it excreted into the growth medium. Glyoxylate is not converted into glycollate on any known metabolic pathway and its position in metabolism seems to be anomalous.

It appears that shuttling of nicotinamide nucleotides to and from the mitochondria is not a normal physiological process but small substrates can partially overcome this permeability barrier. A cyclic process is set up which depends on the interplay of a dehydrogenase outside the mitochondria that reduces a substrate with a nicotinamide nucleotide and an enzyme inside that oxidizes the reduced substrate. Racker (1961) suggested that the glycollate-glyoxylate cycle in leaves (Zelitch & Ochoa, 1953; Zelitch, 1953) could play a

similar function in energy metabolism. Schäfer & Lamprecht (1961) have also postulated a role for this same cycle in transporting hydrogen from NADH in the cytoplasm through the mitochondrial membrane of liver tissue. If the glycollate-glyoxylate system operates, it may interact with NADPH-linked as well as NADH-linked reductases. In view of the similarity in properties of these two enzymes, they might work together, at the same location within the cell, and be connected with a glycollate-glyoxylate cycle. On the other hand, the rate of conversion of glycollate into glyoxylate reported by Katagiri & Tochikura (1960a,b) in coliform organisms was of a very low order, which argues against the participation of the glycollate-glyoxylate system in terminal respiration.

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