The Metabolism of C_2 Compounds in Micro-Organisms

7. PREPARATION AND PROPERTIES OF CRYSTALLINE TARTRONIC SEMIALDEHYDE REDUCTASE*

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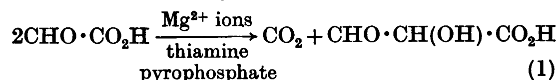
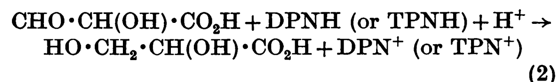
The biosynthesis of cell materials by *Pseudomonas* spp. growing on glycollate (Kornberg & Gotto, 1959, 1961), on glycine (Callely & Dagley, 1959), on oxalate (Quayle & Keech, 1959) or on other substrates catabolized to glyoxylate (Gray, Gerhart & Brooke, 1959) has been shown to be effected by a sequence of reactions in which, initially, glyoxylate is converted into glycerate. This transformation is achieved by the successive action of two adaptively formed enzymes: glyoxylate carboligase (Krakow & Barkulis, 1956; Krakow, Hayashi & Barkulis, 1959; Krakow, Barkulis & Hayashi, 1961), which catalyses the condensative decarboxylation of glyoxylate to tartronic semialdehyde:



$$2CHO \cdot CO_2H \xrightarrow[\text{thiamine pyrophosphate}]{Mg^{2+} \text{ ions}} CO_2 + CHO \cdot CH(OH) \cdot CO_2H \quad (1)$$

and tartronic semialdehyde reductase (Gotto & Kornberg, 1961*a*), which catalyses the reduction of the tartronic semialdehyde thus formed to glycerate:



$$CHO \cdot CH(OH) \cdot CO_2H + DPNH \text{ (or TPNH)} + H^+ \rightarrow HO \cdot CH_2 \cdot CH(OH) \cdot CO_2H + DPN^+ \text{ (or } TPN^+) \quad (2)$$

It is the main purpose of this paper to describe a method for the preparation of this latter enzyme in a crystalline state and to discuss some of the properties of the enzyme thus prepared. Preliminary reports of portions of this work have been published (Gotto & Kornberg, 1961*a*, *b*).

MATERIALS AND METHODS

Enzymic preparation of tartronic semialdehyde. Tartronic semialdehyde was prepared enzymically by the treatment of glyoxylate with glyoxylate carboligase. The main compartment of a Warburg manometer flask contained: 50 μmoles of potassium phosphate, pH 6·5; 0·5 μmole of thiamine pyrophosphate; 5 μmoles of $MgCl_2$; 0·05 ml. of purified glyoxylate carboligase (see below). Side arm 1 contained 80 μmoles of sodium glyoxylate and side arm 2 contained 0·20 ml. of 20% perchloric acid. The flasks were equilibrated at 30° under N_2 for 10–15 min., after which time the glyoxylate was added from the first side arm. When evolution of CO_2 ceased (approx. 10 min.), the perchloric acid was added from the second side arm, the cup was detached from its manometer and the contents of the manometer flask were centrifuged immediately at 2°. (Continued shaking of the flask, after addition of the perchloric acid, was found to result in considerable decarboxylation of the tartronic semialdehyde.) After centrifuging at 20 000*g* for 2 min., the pH was adjusted to 7·0–7·5 by dropwise addition of 2N-KOH, and the potassium perchlorate was removed by centrifuging at 2° for 15 min. The concentration of tartronic semialdehyde in the neutralized solution was calculated from the total change in $E_{340 m\mu}$ when samples were incubated with the purified tartronic semialdehyde reductase (see below) in the presence of an excess of reduced DPN (DPNH). The yields obtained varied between 30 and 60% of the amounts expected from the measured quantities of CO_2 evolved from glyoxylate. Since the product is rather unstable, the tartronic semialdehyde solutions are best prepared freshly each day.

Chemical preparation of tartronic semialdehyde. Authentic tartronic semialdehyde was prepared by Professor D. B. Sprinson from the reductive cleavage of the benzyl *cyclo*acetal derivative (Fischer, Baer & Nidecker, 1937). Since this material became available only after most of the work described in this paper had been completed, the enzymically prepared tartronic semialdehyde was used for most experiments. However, no significant difference was detected between the properties of the enzymic and chemical products.

Potassium D-*glycerate.* The quinine salt of D-glycerate, which was a gift from Dr P. W. Kent, was treated with Dowex 50 resin in the H^+ form. The solution thus obtained was titrated with 0·2N-KOH to pH 7: the glycerate content was calculated from the amount of alkali required. High-voltage paper electrophoresis of a sample of the glycerate solution, in 0·5M-pyridine/0·5M-acetic acid adjusted to pH 4·0 (Gross, 1959), showed glyceric acid to be the sole acid constituent detectable.

Sodium [1-^{14}C]*glyoxylate.* Calcium [1-^{14}C]glyoxylate was purchased from The Radiochemical Centre, Amersham, Bucks., and was converted into the sodium salt by treatment with Dowex 50 resin in the Na^+ form. It was purified, in collaboration with Dr I. Zelitch, as follows.

The [1-^{14}C]glyoxylate solution was loaded on to a chromatography column (0·7 cm. diam.), filled to a height of

* Part 6: Kornberg & Gotto (1961).

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6 cm. with Dowex 1 (X8) resin in the acetate form. The material was washed with water and the [1-^{14}C]glyoxylic acid was eluted with 4M-acetic acid. Those fractions of the eluate containing [1-^{14}C]glyoxylic acid, as determined by the method of Friedemann & Haugen (1943) and by radio-assay, were combined and the acetic acid was removed by freeze-drying. The [1-^{14}C]glyoxylic acid was neutralized with 0·2M-KOH: it was found to be 100% pure by chromatography and radioassay of the 2:4-dinitrophenyl-hydrazone (El Hawary & Thompson, 1953).

Lithium hydroxypyruvate. The lithium hydroxypyruvate used was a gift from Professor F. Dickens, F.R.S., and Mr D. H. Williamson.

Malonic semialdehyde. Ethyl ββ-diethoxypropionate, which was a gift from Professor M. J. Coon, was converted into malonic semialdehyde as described by Robinson & Coon (1961).

Mesoxalic semialdehyde. Dihydroxyfumaric acid was converted into mesoxalic semialdehyde by the procedure of Fenton (1905).

Reductone. Reductone was a gift from Dr J. G. Morris.

Other reagents used. Reduced and oxidized pyridine nucleotides, crystalline lactic dehydrogenase, phospho*enol*pyruvate and 3-phosphoglycerate were purchased from C. F. Boehringer und Soehne (Mannheim, Germany); β-hydroxypropionic acid was from the California Corp. for Biochemical Research (Los Angeles, U.S.A.); thiamine pyrophosphate was from the Nutritional Biochemicals Corp. (Cleveland, Ohio, U.S.A.); 2:6-dichlorophenol-indophenol, DL-glyceraldehyde, dihydroxyfumaric acid and glyoxylic acid were from British Drug Houses Ltd. (Poole, Dorset); protamine sulphate ('ex herring') and L-malic acid were from L. Light and Co. Ltd. (Colnbrook, Bucks.). Oxaloacetic acid, sodium pyruvate and L-lactate were gifts from Professor Sir Hans Krebs, F.R.S. All other reagents used were of the highest purity commercially available.

Preparation of tartronic semialdehyde reductase

Mass cultures of glycollate-grown *Pseudomonas ovalis* Chester (Dixon, Kornberg & Lund, 1960) were generously supplied by Mr R. Elsworth (Ministry of Aviation, Microbiological Research Establishment, Porton, Wilts.). Tartronic semialdehyde reductase and other enzymes required were prepared from the frozen packed cells as follows.

Step 1: *preparation of ultrasonic extracts.* Frozen cells (80 g.) were thawed and diluted to 200 ml. with 5 mM-sodium potassium phosphate, pH 7·0 (prepared by mixing 61·1 vol. of 5 mM-Na_2HPO_4 with 38·9 vol. of 5 mM-KH_2PO_4). The suspension was subjected (in 20 ml. batches) for 5 min. to the output of a 600 w Mullard magnetostrictor oscillator operating at 3·5 A. After this treatment, the batches were combined and centrifuged for 1 hr. at 35 000g in a MSE Speed 17 centrifuge at 2°. The precipitate was discarded. All further operations were carried out at 0–2°, the enzymes being assayed at each step as described below.

Steps 2–3: *treatment with protamine sulphate and alumina* C_γ*-gel.* After centrifuging, 2% (w/v) protamine sulphate solution (1·5 mg. of protamine sulphate for each 10 mg. of soluble protein) was added slowly to the supernatant solution. The precipitate was removed by centrifuging for 30 min. at 35 000g. To each 100 ml. of supernatant solution was added 20 ml. of alumina C_γ-gel [30 mg. dry wt./ml.; prepared as described by Colowick (1955)]. The precipitate was removed by centrifuging and discarded.

Step 4: *fractionation with ammonium sulphate.* To each 100 ml. of supernatant solution was added 24·3 g. of solid $(NH_4)_2SO_4$ (40% saturation). The precipitate was collected by centrifuging and was discarded. To each 100 ml. of the supernatant solution was added 24·5 g. of solid $(NH_4)_2SO_4$ (75% saturation); the precipitate was collected by centrifuging and was dissolved in 15–20 ml. of 5 mM-phosphate buffer, pH 7·0, containing 1 mM-mercapto-ethanol. This material was dialysed at 2° against 2 l. of 5 mM-sodium potassium phosphate, pH 7·0, and 1 mM-mercaptoethanol. This buffer mixture was replaced by 2 l. of fresh buffer mixture after 9 hr. and dialysis was continued for a further 9 hr. The small quantity of material precipitated during dialysis was removed by centrifuging.

Step 5: *column chromatography.* A batch of 7 g. of diethylaminoethylcellulose (DEAE-cellulose; Whatman DE 50) was suspended in 500 ml. of 5 mM-sodium potassium phosphate buffer, pH 7·0, and the pH of the suspension was re-adjusted to 7·0 by the addition of 2N-HCl. The cellulose was washed several times with 5 mM-phosphate and particles which did not sediment were removed by decantation. The slurry was then poured into a chromatographic column (2 cm. × 30 cm.), the lower end of which was closed with glass wool. The column was equilibrated at 2° by allowing 1 l. of 5 mM-sodium potassium phosphate, pH 7·0, to run through it. The dialysed solution was applied to the DEAE-cellulose column at a rate just sufficient to keep the top of the column moist: by this means it was possible to adsorb the material in a narrow band at the top of the column. A linear gradient of KCl was now applied to the column by allowing 400 ml. of a solution containing 5 mM-sodium potassium phosphate, pH 7·0, 1 mM-mercapto-ethanol and 50 mM-KCl to mix, with constant stirring, with 400 ml. of a solution containing 5 mM-sodium potassium phosphate, pH 7·0, 1 mM-mercaptoethanol and 450 mM-KCl: this mixture was allowed to flow through the column at 40–50 ml./hr. Fractions, each containing approx. 8 ml. (95 drops), were collected with a Locarte automatic fraction collector.

The hold-up volume, i.e. the material which was not adsorbed on the column, was rich in DPNH-dehydrogenase activity, but was devoid of tartronic semialdehyde reductase. It was precipitated by the addition of solid $(NH_4)_2SO_4$ to 75% of saturation (516 mg./ml.). The precipitated material was dissolved in 5 mM-phosphate, pH 7·0 and stored at 2°. Samples, suitably diluted in buffer, were used as the source of DPNH-dehydrogenase in the procedures described below.

Tartronic semialdehyde reductase was eluted from the column between 110 and 180 mM-KCl, the peak of activity appearing at 130 mM-KCl (Fig. 1). Those fractions containing the enzyme at specific activities (see below) greater than 30 were combined and the enzyme was precipitated by the addition of $(NH_4)_2SO_4$ to a final concentration of 75% of saturation.

Glyoxylate carboligase (see below), which was eluted between 200 and 250 mM-KCl, was similarly precipitated by the addition of solid $(NH_4)_2SO_4$ to 75% of saturation. This material retained full enzymic activity for several months when stored at 2° in the presence of crystalline bovine serum albumin and 5 mM-sodium potassium phos-

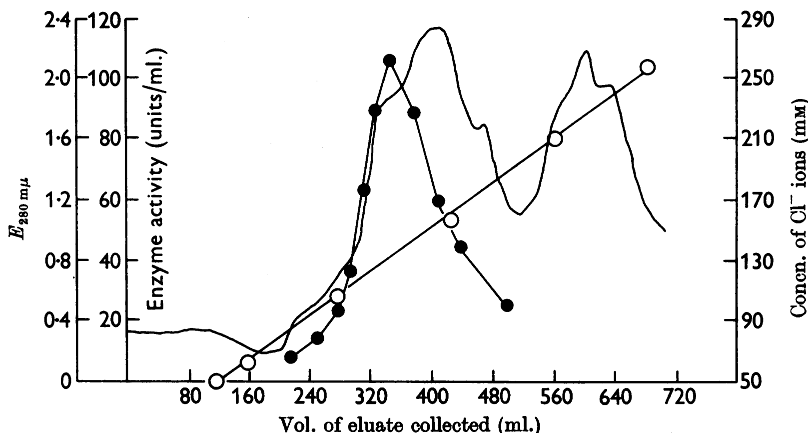


Fig. 1. Elution of tartronic semialdehyde from DEAE-cellulose. For conditions see text. The continuous line represents the protein concentration, measured as $E_{280\,m\mu}$. ●, Tartronic semialdehyde-reductase activity; ○, gradient of chloride concentration.

phate, pH 7·5, containing 1 mM-$MgCl_2$ and 0·1 mM-thiamine pyrophosphate.

Steps 6–7: *crystallization.* The $(NH_4)_2SO_4$ precipitate of tartronic semialdehyde reductase was collected by centrifuging and was dissolved in 3–4 ml. of 5 mM-phosphate, pH 7·0. Any material which did not dissolve at this point was removed by centrifuging. The supernatant solution was brought to first turbidity (approx. 40% of saturation) by the slow addition of solid $(NH_4)_2SO_4$. The material was allowed to warm slowly to room temperature (21°), during which time crystallization began, and was maintained at this temperature for 1 hr. and at 0° for a further period of 4–5 hr. The crystalline precipitate was collected by centrifuging, dissolved in the minimal volume of 5 mM-sodium potassium phosphate buffer, pH 7·0, and recrystallized three times with $(NH_4)_2SO_4$ as described above. These successive recrystallizations were not accompanied by any significant change in the specific activity of the enzyme. The crystals (Fig. 2) appeared to have the shape of two pyramids fused at their bases. The material sedimented in the ultracentrifuge as a single symmetrical peak (Fig. 3).

The simple and reproducible purification procedure is summarized in Table 1.

D-*Glyceric dehydrogenase.* D-Glyceric dehydrogenase (Stafford, Magaldi & Vennesland, 1954; Holzer & Holldorf, 1957) was prepared in collaboration with Dr I. Zelitch. Tobacco leaves (four), Havana variety, weighing 35 g. after removal of the midribs, were ground with a little sand and 35 ml. of water, 310 mg. of cysteine–HCl and 250 mg. of $KHCO_3$. The extract was filtered through a double layer of cheese cloth and the filtrate was centrifuged twice at 20 000g for 20 and 10 min. periods respectively. To the supernatant solution, solid $(NH_4)_2SO_4$ was added to 40% saturation. The precipitate was dissolved in 4·5 ml. of 10 mM-potassium phosphate, pH 7·0, containing 5 mM-cysteine–HCl, and was stored frozen at −12°.

Assay of enzymes

All spectrophotometric assays were performed in silica cells, 1·5 ml. vol., 1 cm. light-path, in a Cary model 14 recording spectrophotometer at 23 ± 1°.

Reduced diphosphopyridine nucleotide dehydrogenase. This was estimated by measurement of the rate of change of $E_{600\,m\mu}$, consequent upon the enzymic reduction of 2:6-dichlorophenol-indophenol in the presence of the enzyme and DPNH. The complete system contained, in 1 ml.: 0·4 ml. of 0·01% (w/v) 2:6-dichlorophenol-indophenol; 0·05 ml. of 0·1 M-KCN adjusted to pH 8·5; 0·1 ml. of 1 M-disodium hydrogen phosphate, pH 8·5; appropriate amounts of enzyme. $E_{600\,m\mu}$ of this solution was measured for 1–2 min., after which time the reaction was started by addition of 0·1 ml. of 10 mM-DPNH. One unit of enzyme was defined as $\Delta E_{600\,m\mu} = -1{\cdot}0$ unit/min.

Glyoxylate carboligase. This was assayed manometrically as described by Kornberg & Gotto (1961) or spectrophotometrically by measurement of the rate of oxidation of DPNH when this enzyme was incubated with glyoxylate, in the presence of its required cofactors Mg^{2+} ions and thiamine pyrophosphate, and an excess of tartronic semialdehyde reductase. The complete system contained, in 1 ml.: 0·1 ml. of 1 M-potassium phosphate, pH 7·5; 0·05 ml. of 0·1 M-$MgCl_2$; 0·05 ml. of 10 mM-thiamine pyrophosphate; 0·02 ml. of 10 mM-DPNH; an excess (determined as described later) of crystalline tartronic semialdehyde reductase. $E_{340\,m\mu}$ was recorded for 1–2 min., after which time the reaction was started by addition of 0·03 ml. of 0·2 M-sodium glyoxylate. One unit of enzyme is defined as that quantity catalysing the oxidation of 1 μmole of DPNH/min., thus giving $\Delta E_{340\,m\mu} = -6{\cdot}28$ units/min.

Tartronic semialdehyde reductase. This was assayed by measurement (*a*) of the rate of oxidation of DPNH in the presence of the enzyme and tartronic semialdehyde, or (*b*) of the rate of reduction of 2:6-dichlorophenol-indophenol in the presence of the enzyme, D-glycerate, DPN^+ and excess of DPNH-dehydrogenase.

(*a*) In the former assay, the complete system contained, in 1 ml.: 0·1 ml. of 1 M-disodium hydrogen phosphate, pH 8·5; 0·02 ml. of 10 mM-DPNH; an appropriate amount of enzyme. $E_{340\,m\mu}$ was recorded for 1–2 min., after which time the reaction was started by the addition of 0·05 ml. of 10 mM-tartronic semialdehyde. One unit of enzyme is defined as that quantity which catalyses the oxidation of 1 μmole of DPNH/min. and thus gives $\Delta E_{340\,m\mu} = -6{\cdot}28$ units/min.

(*b*) In the latter assay, the complete system contained, in 1 ml.: 0·4 ml. of 0·01% (w/v) 2:6-dichlorophenol-indophenol; 0·1 ml. of 1 M-disodium hydrogen phosphate, pH 8·5; 0·05 ml. of 0·1 M-KCN; 0·02 ml. of DPNH-dehydrogenase; 0·1 ml. of 10 mM-DPN^+; an appropriate amount of the enzyme. $E_{600\,m\mu}$ was recorded for 1–2 min., after which time the reaction was started by the addition of 0·05 ml. of 70 mM-potassium D-glycerate. One unit of enzyme in this assay is defined as that quantity which catalyses $\Delta E_{600\,m\mu} = -1{\cdot}0$ unit/min. This quantity is equivalent to 0·39 unit of enzyme determined under the conditions of the former assay (*a*).

Specific activity. A specific activity of 1 is defined as 1 unit of enzyme/mg. of soluble protein.

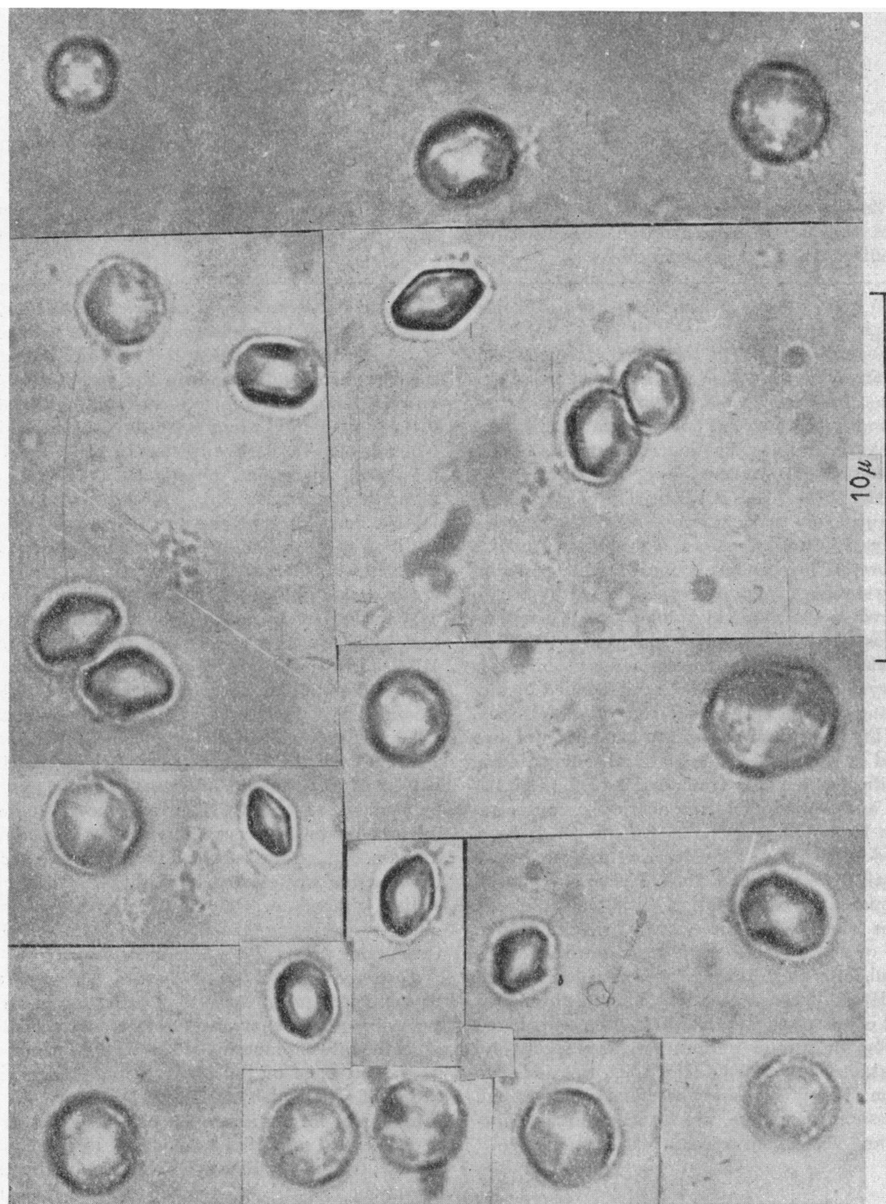


Fig. 2. Crystals of tartronic semialdehyde reductase; composite photograph to show crystalline structure (magnification ×3380). Photograph by Dr R. Barer (Department of Human Anatomy, University of Oxford).

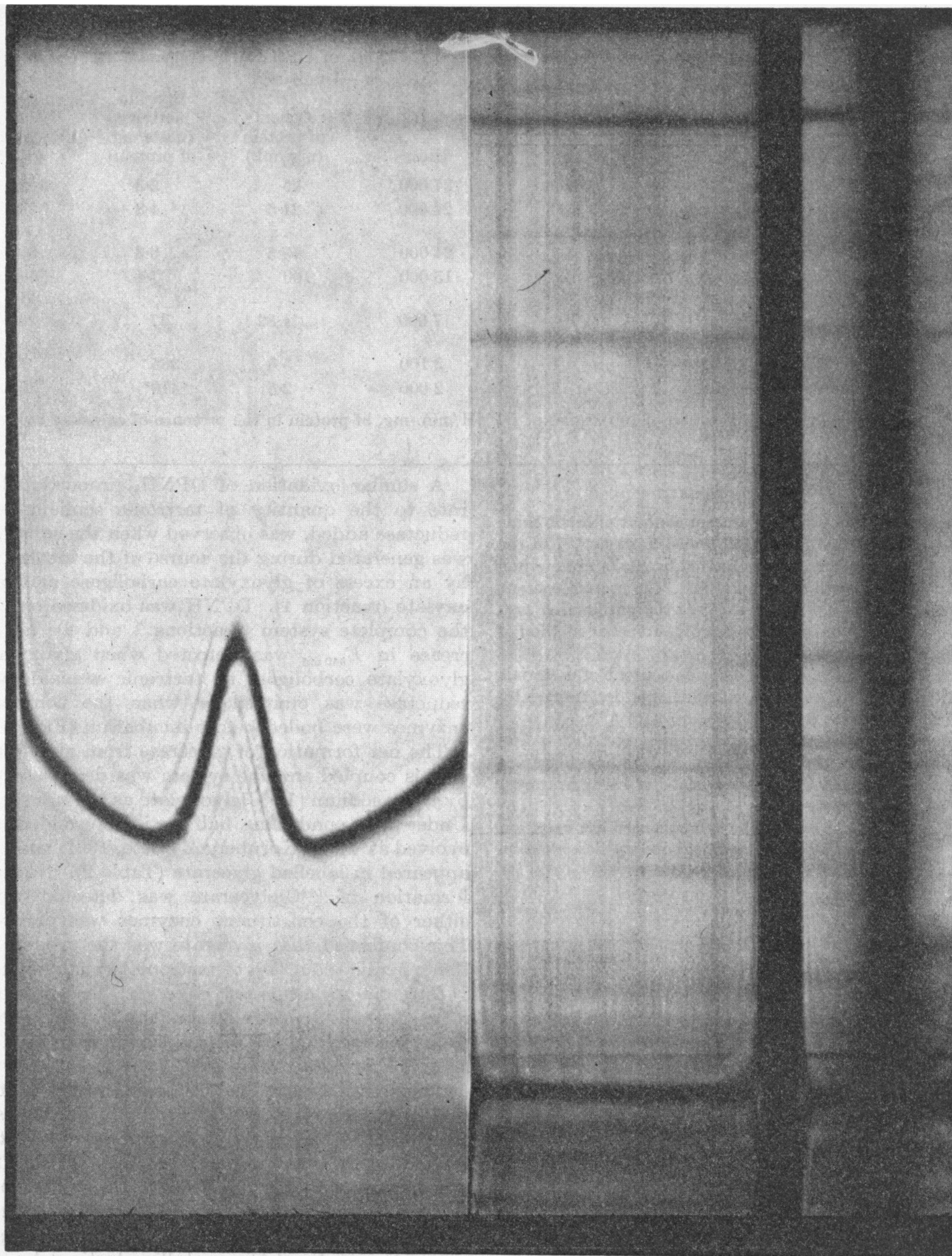


Fig. 3. Sedimentation pattern of crystalline tartronic semialdehyde reductase. The enzyme was dissolved in 0·1 M-sodium potassium phosphate buffer, pH 7·4. Sedimentation was from right to left at phase-plate angle 35° and rotor temperature 3·2°. The photograph was taken after 64 min. at 59 780 rev./min.

Table 1. *Summary of purification of tartronic semialdehyde reductase*

The enzyme was assayed by measurement of the rate of decrease of $E_{600\,m\mu}$. The complete system contained, in 1 ml.: 100 μmoles of disodium hydrogen phosphate, pH 8·5; 5 μmoles of KCN; 3·5 μmoles of D-glycerate; 0·4 ml. of 0·01% 2:6-dichlorophenol-indophenol; 0·5 μmole of DPN^+; 0·02 ml. of DPNH-dehydrogenase and enzyme. One unit of enzyme is defined as that quantity catalysing $\Delta E_{600\,m\mu} = -1$ unit/min.

Step	Vol. of soln. (ml.)	Units	Concn. of protein (mg./ml.)	Specific activity (units/mg. of protein)	Recovery (%)
1. Sonic extract	180	27 000	65	2·3	100
2. Supernatant from protamine sulphate treatment	230	24 000	21·5	4·8	89
3. Supernatant from C_γ-gel adsorption	290	24 000	13·5	6·3	89
4. Ammonium sulphate precipitate, 40–75% saturated	20	15 000	100	7·4	55
5. Pooled selected fractions from DEAE-cellulose column	100	7 000	1·82	37	26
6. Crystals, crop I	1	2 100	5·5	385	7·8
7. Crop I, recrystallized three times	2	2 000	2·5	410*	7·4

* This corresponds to the oxidation of 160 μmoles of DPNH/min./mg. of protein in the presence of excess of tartronic semialdehyde.

Other methods

Estimation of protein. The protein content of crude sonic extracts, and of the material obtained in steps 1–4 of the purification procedure, was estimated by the biuret method of Gornall, Bardawill & David (1949). The protein content of materials obtained in steps 5–7 of the purification procedure was estimated by the spectrophotometric method of Warburg & Christian (1941).

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

Chromatographic and radioautographic procedures. The methods used for chromatography and radioautography have been previously described (Kornberg, 1958; Kornberg & Gotto, 1961).

Thermodynamic symbols. The symbols used are based on those given in the *Report of the Royal Society Committee on Symbols* (1951) (cf. Burton & Krebs, 1953).

RESULTS

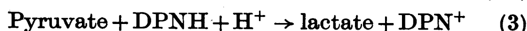
Enzymic reduction of tartronic semialdehyde. When tartronic semialdehyde, prepared enzymically or chemically, was added to cuvettes containing tartronic semialdehyde reductase and DPNH, $E_{340\,m\mu}$ of the solution decreased rapidly. This reaction occurred only when both enzyme and substrate were present, and did not take place if the enzyme was boiled before incubation. In the presence of excess of DPNH and enzyme, the total decrease of E was stoicheiometrically related to the quantity of tartronic semialdehyde added (reaction 2). In the presence of excess of tartronic semialdehyde, the initial rates of oxidation of DPNH were proportional to the quantities of crystalline enzyme added over the range at which such rates could be conveniently measured (0·1–1·0 μg. of enzyme protein/ml.).

A similar oxidation of DPNH, proportional in rate to the quantity of tartronic semialdehyde reductase added, was observed when the substrate was generated during the course of the incubation by an excess of glyoxylate carboligase and glyoxylate (reaction 1). DPNH was oxidized only in the complete system (reactions 1 and 2); no decrease in $E_{340\,m\mu}$ was detected when glyoxylate, glyoxylate carboligase or tartronic semialdehyde reductase was omitted or when the combined enzymes were boiled before incubation (Fig. 4).

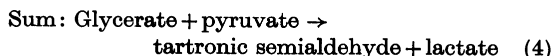
The net formation of glycerate from glyoxylate in this coupled enzyme system was demonstrated by using sodium [1-^{14}C]glyoxylate as the substrate. Under these conditions, half the isotope added was evolved as $^{14}CO_2$ (Kornberg & Gotto, 1961) and half appeared in labelled glycerate (Table 2). No such formation of [^{14}C]glycerate was detected when either of the constituent enzymes was omitted. This confirmed that glycerate was the product of the enzymic reduction of tartronic semialdehyde.

Enzymic oxidation of glycerate. The enzymic formation of tartronic semialdehyde and DPNH from glycerate and DPN^+ (reaction 2, from right to left, i.e. reaction 2*a* below) proved difficult to demonstrate directly as the equilibrium of the system greatly favoured glycerate formation (see later), but was readily demonstrated when the DPNH formed was reconverted into DPN. This was done in two ways. In the first, the oxidation of glycerate, catalysed by crystalline tartronic semialdehyde reductase, was coupled to the reduction of pyruvate, catalysed by crystalline lactic dehydrogenase (reaction 3) in the presence of DPN^+. The overall removal of pyruvate (reaction 4) depended on the presence of both enzymes, glycerate and DPN^+ (Table 3).

$$\text{Glycerate} + \text{DPN}^+ \rightarrow \text{tartronic semialdehyde} + \text{DPNH} + \text{H}^+ \quad (2a)$$



$$\text{Pyruvate} + \text{DPNH} + \text{H}^+ \rightarrow \text{lactate} + \text{DPN}^+ \quad (3)$$



$$\text{Sum: Glycerate} + \text{pyruvate} \rightarrow \text{tartronic semialdehyde} + \text{lactate} \quad (4)$$

In the second method, which also served as the basis of the convenient assay procedure (*b*) (see Materials and Methods section) for tartronic semialdehyde reductase, the DPNH formed from DPN^+ concomitantly with glycerate oxidation was reconverted into DPN^+ in the presence of DPNH-dehydrogenase and 2:6-dichlorophenol-indophenol as electron acceptor. Although the relatively crude enzyme obtained in steps 1–4 (Table 1) of the purification procedure catalysed the reduction of 2:6-dichlorophenol-indophenol in the presence of glycerate and DPN^+, and without addition of DPNH-dehydrogenase, the material obtained after DEAE-cellulose column chromatography (step 5, Table 1) did not catalyse the reduction of the dye unless DPNH-dehydrogenase was also present. The enzymic oxidation of glycerate in this system again required the presence of the enzyme, glycerate, catalytic amounts of DPN^+, DPNH-dehydrogenase and the electron acceptor: no glycerate oxidation was observed if any one of these constituents was omitted (Fig. 5). The initial rate of dye reduction was proportional to the amounts of enzyme added over the range at which such rates could be conveniently measured (0·1–1·0 μg. of protein/ml.). The material formed in the complete system formed a bis derivative with 2:4-dinitrophenylhydrazine which behaved identically with the derivative prepared from the enzymically or chemically prepared tartronic semialdehyde.

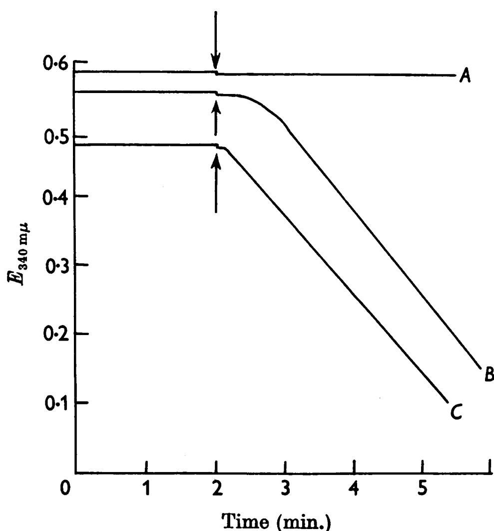


Fig. 4. Formation of glycerate from glyoxylate by purified enzymes. The complete system contained, in 1·0 ml.: 50 μmoles of potassium phosphate, pH 7·5; 5 μmoles of $MgCl_2$; 0·5 μmole of thiamine pyrophosphate; 0·1 μmole of DPNH; purified glyoxylate carboligase (0·25 mg.); tartronic semialdehyde reductase (0·05 mg.). Glycerate formation was measured as the rate of decrease in $E_{340\,m\mu}$. The reactions were initiated by the addition, at the times indicated by arrows, of glyoxylate carboligase to *A* and *B*, and of tartronic semialdehyde reductase to *C*. In curve *A*, glyoxylate was also omitted from the reaction mixture. (Taken from tracings made with a Cary recording spectrophotometer.)

Equilibrium of the reaction. The apparent and thermodynamic equilibrium constants, K and K_H respectively, for reaction 2*a* were calculated from the equations:

$$K = \frac{[\text{tartronic semialdehyde}^-]\,[\text{DPNH}]}{[\text{glycerate}^-]\,[\text{DPN}^+]}$$

and

$$K_H = \frac{[\text{tartronic semialdehyde}^-]\,[\text{DPNH}]\,[\text{H}^+]}{[\text{glycerate}^-]\,[\text{DPN}^+]}$$

The equilibrium concentrations were measured by adding various amounts of glycerate to DPN^+ and crystalline tartronic semialdehyde reductase, and

Table 2. *Conversion of* [1-^{14}C]*glyoxylate into* [^{14}C]*glycerate by purified enzymes*

The complete system contained, in a total volume of 3·0 ml.: 100 μmoles of potassium phosphate, pH 7·5; 10 μmoles of magnesium chloride; 0·5 μmole of thiamine pyrophosphate; 1 μmole of DPNH; 0·10 ml. of a solution of sodium [1-^{14}C]glyoxylate (giving 1×10^5 counts/min. under the conditions of radioassay used); 0·2 mg. of glyoxylate carboligase; 0·3 mg. of crystalline tartronic semialdehyde reductase. After incubation at 23° for 30 min., 0·30 ml. was pipetted into 0·10 ml. of saturated 2:4-dinitrophenylhydrazine in 2N-HCl, and the remainder was poured into 4·0 ml. of ethanol. These two fractions were analysed by chromatography and their radioactivity was measured as described by Kornberg (1958) and Kornberg & Gotto (1961).

	10^{-3} × Radioactivity (counts/min.)			
	Glyoxylate		Glycerate	
Reaction mixture	(Initial)	(Final)	(Initial)	(Final)
Complete system	100	0·04	0	49·7
Glyoxylate carboligase omitted	100	100	0	0
Tartronic semialdehyde reductase omitted	100	98·6	0	0·43

following $E_{340\,m\mu}$ until this was constant. The equilibrium concentration of DPNH was calculated from $E_{340\,m\mu}$ (Horecker & Kornberg, 1948); that of tartronic semialdehyde was taken to be equal to the concentration of DPNH thus determined. Since the amounts of glycerate and of DPN^+ utilized in the reaction were less than 1 % of those added, the concentration of these reactants was assumed to be constant throughout. The pH was measured with a glass electrode.

The mean value obtained for K was 2×10^{-6} at pH 7·5 and $1{\cdot}6 \times 10^{-5}$ at pH 8·5. This tenfold increase in K accompanying a tenfold decrease in H^+-ion concentration was expected, as a decrease in H^+-ion concentration favours glycerate oxidation (reaction 2). In contrast, K_H was independent of pH, and was determined as $5{\cdot}8 \times 10^{-14}$ M at pH 7·5 and $5{\cdot}1 \times 10^{-14}$ M at pH 8·5 (Table 4). Taking the latter figure as the most reliable, $\Delta G'$ at pH 7 was calculated to be +8·6 kcal./mole. Similarly, from the mean value of $K_H = 5{\cdot}1 \times 10^{-14}$ M,

$$\Delta G^0 = +18 \text{ kcal./mole.}$$

Table 3. *Oxidation of glycerate coupled to reduction of pyruvate by crystalline enzymes*

The complete system contained, in 1 ml.: 50 μmoles of potassium phosphate, pH 8·5; 0·5 μmole of DPN^+; 0·05 mg. of crystalline lactic dehydrogenase (Boehringer); 0·3 mg. of crystalline tartronic semialdehyde reductase; 25 μmoles of sodium DL-glycerate; 1·0 μmole of potassium pyruvate. After incubation for 20 min. or 60 min. at 18°, samples (0·2 ml.) were removed and assayed for pyruvate content (Friedemann & Haugen, 1943).

Reaction mixture	Pyruvate removed (μmoles) In 20 min.	In 60 min.
Complete system	0·37	0·60
DPN^+ omitted	0·01	0·05
Lactic dehydrogenase omitted	0	0
Tartronic semialdehyde reductase omitted	0·01	0·05
Glycerate omitted	0	0

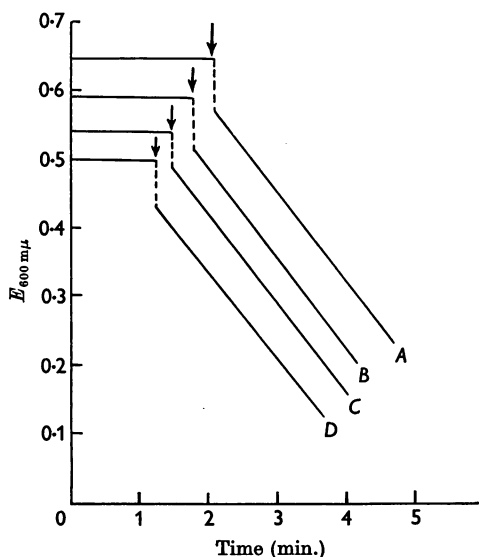


Fig. 5. Oxidation of D-glycerate, catalysed by crystalline tartronic semialdehyde reductase. The complete system contained, in 1 ml.: 50 μmoles of potassium phosphate, pH 8·5; 10 μmoles of KCN; 0·5 ml. of 0·01 % 2:6-dichlorophenol-indophenol; 0·3 μmole of DPN^+; 0·4 μg. of crystalline tartronic semialdehyde reductase; 0·01 ml. of DPNH-dehydrogenase; 5 μmoles of potassium D-glycerate. From each cuvette, one constituent had been omitted: this was added at the time indicated by the arrow. *A*, Glycerate omitted; *B*, DPN^+ omitted; *C*, tartronic semialdehyde reductase omitted; *D*, DPNH-dehydrogenase omitted. (Taken from a tracing made with a Cary recording spectrophotometer.)

Table 4. *Equilibrium constants for the oxidation of glycerate to tartronic semialdehyde*

The reaction mixture contained, in 1 ml.: 100 μmoles of potassium phosphate, pH 7·5 or 8·5 as specified; 0·50 mg. of crystalline tartronic semialdehyde reductase; 1·50 or 3·50 μmoles of DPN^+ as specified; various amounts of potassium D-glycerate. $E_{340\,m\mu}$ was measured until there was no further change. Concentrations of glycerate, DPN^+ and H^+ were assumed not to vary throughout the reaction and the concentration of tartronic semialdehyde formed was taken to be equal to that of the DPNH. K and K_H were calculated as described in the text.

Equilibrium concn. (M)				Calc. equilibrium constants	
$10^3[DPN^+]$	$10^5[DPNH]$	10^2[glycerate]	$10^9[H^+]$	10^5K	$10^{14}\text{M} \times K_H$
1·50	1·10	0·46	3·2	1·75	5·61
1·50	1·40	0·92	3·2	1·42	4·54
1·50	1·70	1·38	3·2	1·42	4·54
1·50	2·07	1·84	3·2	1·56	4·97
1·50	2·40	2·30	3·2	1·67	5·34
1·50	2·70	2·76	3·2	1·76	5·74
			Average	1·60	5·12
1·50	0·41	0·70	32	0·16	5·00
3·50	0·70	0·70	32	0·21	6·61
			Average	0·19	5·80

From the value of $\Delta G'$ obtained, the oxidation–reduction potential for reaction (2) is $-0{\cdot}228$ v. Since the oxidation–reduction potential of the system, $DPNH + H^+ \rightleftharpoons DPN^+ + 2H + 2e$, is $-0{\cdot}320$ v (Burton & Wilson, 1953), E_0' of the reaction

$$\text{Glycerate}^- \rightleftharpoons \text{tartronic semialdehyde}^- + 2H + 2e$$

at pH 7 and 25° is $-0{\cdot}092$ v. This places the reaction among the more electropositive of known DPN-linked systems.

Properties of the enzyme

pH *optimum*. The crystalline enzyme catalysed the oxidation of DPNH by enzymically prepared tartronic semialdehyde over a wide range of H^+-ion concentration. The reaction proceeded at maximal velocity between pH 6·2 and pH 8·7, but even at pH 5, over 60 %, and at pH 10 over 30 %, of this rate was observed. With chemically prepared tartronic semialdehyde, the pH optimum appeared to lie further to the acid side, and assays with this substrate were done at pH 7·2.

Effect of concentration of reactants on initial rate. (*a*) Tartronic semialdehyde. The Michaelis constant (K_m) for enzymically prepared tartronic semialdehyde was determined from the plot of reciprocal rate of DPNH oxidation against reciprocal substrate concentration (Lineweaver & Burk, 1934) when various amounts of tartronic semialdehyde were reduced in the presence of an excess of crystalline enzyme and of DPNH at pH 8·5. The K_m thus determined was 2×10^{-4} M.

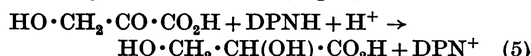
(*b*) Reduced pyridine nucleotides. At pH 8·5, the crystalline enzyme catalysed the reduction of tartronic semialdehyde approximately twice as rapidly with DPNH as with reduced TPN. The K_m values at this pH, determined from the Lineweaver–Burk plot, obtained when a constant excess of enzyme and of tartronic semialdehyde reacted with various amounts of DPNH or TPNH, were 2×10^{-5} M for DPNH and 5×10^{-5} M for TPNH. At pH 6·5, TPNH was oxidized more rapidly and at a rate similar to that observed with DPNH.

(*c*) Glycerate. From the effect of various concentrations of D-glycerate on the initial rate of reduction of 2:6-dichlorophenol-indophenol, in the presence of an excess of crystalline tartronic semialdehyde reductase, DPNH-dehydrogenase, electron-acceptor dye and DPN^+ at pH 8·5, K_m for D-glycerate was calculated to be 4×10^{-4} M.

Specificity for substrates. The crystalline tartronic semialdehyde reductase did not catalyse the oxidation of DPNH in the presence of glyoxylate, oxaloacetate, pyruvate, glycolaldehyde, DL-glyceraldehyde, glyoxal, formaldehyde, reductone [CHO·CH(OH)·CHO] or mesoxalate ($CO_2^- \cdot CO \cdot CO_2^-$).

Some enzymic oxidation of DPNH was observed in the presence of dihydroxyfumarate. However, this reaction was not accompanied by any decrease in $E_{290\,m\mu}$, as would be expected from the utilization of dihydroxyfumarate: it is therefore likely that the dihydroxyfumarate solution used had partially decomposed to tartronic semialdehyde (Chow & Vennesland, 1958).

The crystalline enzyme catalysed the oxidation of DPNH or TPNH in the presence of hydroxypyruvate but had a lower affinity for this substrate than for tartronic semialdehyde. At saturating concentrations of substrates, the rate of oxidation observed with hydroxypyruvate was less than 17 % of that noted with tartronic semialdehyde, and, at concentrations sufficient to saturate the enzyme with respect to tartronic semialdehyde, the rate observed with hydroxypyruvate at pH 8·5 was less than 3 % (Fig. 6). This low rate of enzymic reduction of hydroxypyruvate to glycerate contrasts sharply with the similar reaction (5) catalysed by the D-glyceric dehydrogenase of plants (Stafford *et al.* 1954; Holzer & Holldorf, 1957). Whereas hydroxypyruvate was rapidly reduced in the presence of DPNH and this enzyme, tartronic semialdehyde did not react (Fig. 6):



$$HO \cdot CH_2 \cdot CO \cdot CO_2H + DPNH + H^+ \rightarrow HO \cdot CH_2 \cdot CH(OH) \cdot CO_2H + DPN^+ \quad (5)$$

Malonic semialdehyde similarly reacted with the crystalline enzyme and reduced pyridine

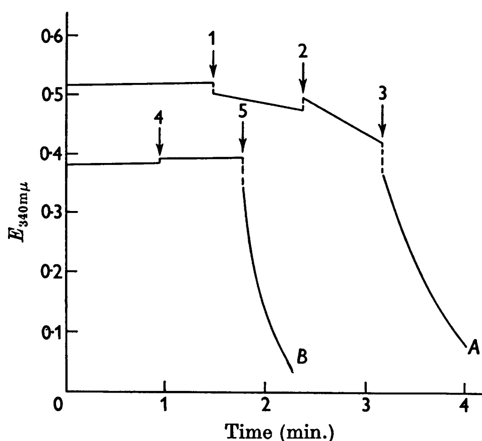


Fig. 6. Reaction of hydroxypyruvate and tartronic semialdehyde with tartronic semialdehyde reductase and D-glyceric dehydrogenase. The reaction mixtures contained, in 1·0 ml.: 50 μmoles of potassium phosphate, pH 7·5; 0·1 μmole of DPNH; either tartronic semialdehyde reductase (0·10 mg.) or D-glyceric dehydrogenase. *A*, Tartronic semialdehyde reductase used, 1·2 μmoles of hydroxypyruvate added at arrow 1, 90 μmoles of hydroxypyruvate at arrow 2 and 1·2 μmoles of tartronic semialdehyde at arrow 3; *B*, D-glyceric dehydrogenase used, 1·2 μmoles of tartronic semialdehyde added at arrow 4 and 1·2 μmoles of hydroxypyruvate at arrow 5. (Taken from a tracing made with a Cary recording spectrophotometer.)

nucleotides, but the observed oxidation proceeded 1·8 times as fast with DPNH as with TPNH. At saturating concentrations of substrates, the rate of DPNH oxidation at pH 8·5 was 13% of that observed with tartronic semialdehyde, and K_m for malonic semialdehyde was calculated to be of the order of 2×10^{-4} M.

The crystalline enzyme also catalysed the reduction of mesoxalic semialdehyde with concomitant oxidation of DPNH or TPNH. The product of this reaction was shown to be hydroxypyruvate:

$$CHO{\cdot}CO{\cdot}CO_2H + DPNH \text{ (or TPNH)} + H^+ \rightarrow HO{\cdot}CH_2{\cdot}CO{\cdot}CO_2H + DPN^+ \text{ (or } TPN^+) \quad (6)$$

by the use of D-glyceric dehydrogenase (Fig. 7). Addition of this enzyme, which did not catalyse the reduction of tartronic semialdehyde (Fig. 6), to a reaction mixture in which DPNH oxidation in the presence of crystalline tartronic semialdehyde reductase and a limited quantity of mesoxalic semialdehyde had proceeded almost to completion, caused a further oxidation of DPNH. This effect was not observed when TPNH was used: it is known (Stafford *et al.* 1954) that the reduction of hydroxypyruvate to glycerate by D-glyceric dehydrogenase is specific for DPNH. Similarly, the rate of DPNH oxidation observed when mesoxalic semialdehyde was incubated with a mixture of crystalline tartronic semialdehyde reductase and D-glyceric dehydrogenase was greater than with tartronic semialdehyde reductase alone, as expected from the sum of reactions (5) and (6).

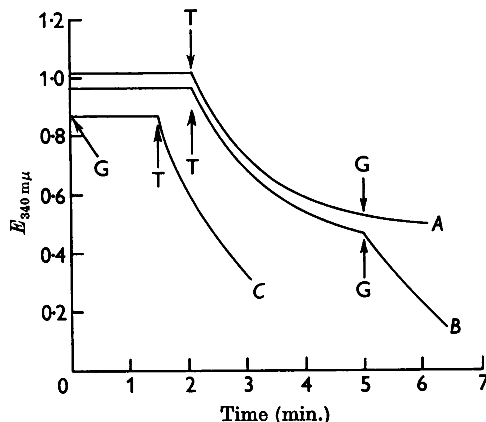


Fig. 7. Reduction of mesoxalic semialdehyde to hydroxypyruvate. The complete system contained, in 1·0 ml.: 50 μmoles of potassium phosphate, pH 8·5; 0·1 μmole of DPNH or TPNH as indicated; 0·01 ml. of tartronic semialdehyde reductase (0·1 mg. of protein); 0·01 ml. of D-glyceric dehydrogenase; 0·08 μmole of mesoxalic semialdehyde. Tartronic semialdehyde reductase was added at arrow T and D-glyceric dehydrogenase at arrow G. *A*, TPNH used; *B* and *C*, DPNH used. (Taken from a tracing made with a Cary recording spectrophotometer.)

The crystalline tartronic semialdehyde reductase did not catalyse the reduction of 2:6-dichlorophenol-indophenol in the presence of DPNH-dehydrogenase and DPN^+ when incubated with L-malate, L-lactate, 3-phosphoglycerate, tartrate, glycollate or β-hydroxypropionate, but, apart from glycerate, reacted only with hydroxypyruvate (presumably by reversal of reaction 6).

Inhibition of enzyme action. The rate of reduction of tartronic semialdehyde by DPNH, catalysed by the crystalline enzyme, was not decreased in the presence of 1 mM-iodoacetate, of 10 mM-glycolaldehyde, DL-glyceraldehyde, sodium fluoride, 3-phosphoglycerate or of 4 mM-phosphoenolpyruvate. The rate of DPNH oxidation was slightly decreased in the presence of 10 mM-pyruvate, L-malate, formaldehyde, glyoxal, oxalate or tartronate, and was strongly inhibited by glyoxylate, glycollate or fluoroacetate (Table 5).

Turnover number of the enzyme. With DPNH as electron donor in the enzymic reduction of tartronic semialdehyde and at pH 8·5, the turnover number of the crystalline enzyme was 16 000 moles of DPNH oxidized/min./100 000 g. of protein. From

Table 5. *Inhibition of tartronic semialdehyde reductase*

Tartronic semialdehyde reductase was assayed as described in the Materials and Methods section (*a*), by measurement of the rate of oxidation of DPNH in the presence of tartronic semialdehyde and of the compounds listed below. The concentration of these added compounds in the reaction mixture was 10 mM unless indicated otherwise. Inhibition is expressed as:

$$\frac{\text{rate observed with tartronic semialdehyde and added compound}}{\text{rate observed with tartronic semialdehyde}} \times 100.$$

Added compound	Rate (% of uninhibited rate) of oxidation
None	100*
DL-Glyceraldehyde	100
Glycolaldehyde	100
Phosphoenolpyruvate (4 mM)	100
Sodium fluoride	98
3-Phosphoglycerate	95
Iodoacetate (1 mM)	95
Glyoxal	86
L-Malate	85
Pyruvate	85
Formaldehyde	79
Oxalate	78
Tartronate	74
Glyoxylate	52
Fluoroacetate	19
Glycollate	14

* This corresponded to a change in $E_{340\,m\mu}$ of 0·38 unit/min.

the estimated weight-average molecular weight (M_W) of 91 000 (see Appendix), this corresponds to a turnover number of 14 600 moles of DPNH oxidized/min./mole of enzyme.

Stability. The crystalline enzyme, stored as a suspension in ammonium sulphate, pH 7·5, at 2°, lost less than 10% of its activity over a month.

DISCUSSION

The metabolic routes whereby *Pseudomonas* spp. grow on glycollate or on other precursors of glyoxylate have been shown to involve the formation of glycerate from glyoxylate (Kornberg & Gotto, 1959, 1961). This synthesis of a C_3 acid from the C_2 substrate is a process involving two enzymic steps. In the first of these (reaction 1), 2 molecular units of glyoxylate undergo a decarboxylative condensation to yield a molecular unit each of carbon dioxide and of a C_3 compound which was postulated to be hydroxypyruvate (Krakow & Barkulis, 1956) or its isomer, tartronic semialdehyde. In the second enzymic step, the C_3 compound is reduced to glycerate.

Although authentic tartronic semialdehyde was not then available for comparison, the properties of the C_3 compound enzymically formed in reaction (1), which were listed by Kornberg & Gotto (1959, 1961), showed that it was not hydroxypyruvate and indicated that it was tartronic semialdehyde, which was supported by the findings of Krakow *et al.* (1959, 1961). This view is strengthened by the isolation of the crystalline enzyme catalysing reaction (2), and by the study of its properties, as reported in this paper.

In particular, both crude extracts of glycollate-grown *Pseudomonas* and the crystalline enzyme have been found to react with equal facility with the C_3 compound enzymically formed and with authentic tartronic semialdehyde, prepared by Professor D. B. Sprinson. Neither crude bacterial extracts nor the crystalline enzyme were found to catalyse the rapid reduction of hydroxypyruvate which would be expected were hydroxypyruvate an intermediate in growth on glycollate.

The crystalline enzyme, which catalyses the reduction of tartronic semialdehyde to glycerate and its reversal, may be distinguished from the other known glycerate-forming enzyme, the D-glyceric dehydrogenase of plants (Stafford *et al.* 1954; Holzer & Holldorf, 1957), on the basis of specificity for substrate and for coenzyme. Whereas the bacterial enzyme reacts rapidly with tartronic semialdehyde but only slowly with hydroxypyruvate, the plant enzyme reacts rapidly with hydroxypyruvate and does not react with tartronic semialdehyde. Moreover, unlike the bacterial enzyme, the plant enzyme also catalyses the reduction of glyoxylate. The crystalline bacterial enzyme reacts with TPNH at half the rate at which it reacts with DPNH, and the K_m values for those two coenzymes are of comparable magnitude (5×10^{-5} M and 2×10^{-5} M respectively): the plant enzyme reacts only with DPNH. On the basis of these findings, it was proposed (Gotto & Kornberg, 1961*a*) to name the bacterial glycerate-forming enzyme 'tartronic semialdehyde reductase'.

The crystalline tartronic semialdehyde reductase differed in its substrate specificity also from other enzymes catalysing the reduction of :C:O linkages. For example, alcohol dehydrogenases are known to exhibit low substrate specificity (Dixon & Webb, 1958), commercial crystalline lactic dehydrogenase of muscle was found readily to reduce hydroxypyruvate and glyoxylate as well as pyruvate, and glyoxylate reductase of tobacco leaves (Zelitch, 1955) reduced hydroxypyruvate as well as glyoxylate. None of these enzymes reacts with tartronic semialdehyde. Furthermore, the crystalline tartronic semialdehyde reductase does not catalyse the reduction of pyruvate or glyoxylate, and indeed is inhibited by this latter substrate, but was found to react rapidly only with C_3 compounds of general structure $CHO{\cdot}R{\cdot}CO_2H$. Thus besides tartronic semialdehyde (R = CH·OH), only malonic semialdehyde (R = CH_2) and mesoxalic semialdehyde (R = CO) were found to be enzymically reduced. It is possible that the slow reduction of hydroxypyruvate at pH 8·5 is a result of its slow isomerization to tartronic semialdehyde (cf. Chow & Vennesland, 1958).

SUMMARY

1. Tartronic semialdehyde reductase was purified 200-fold and crystallized from glycollate-grown *Pseudomonas ovalis* Chester.

2. The enzyme catalyzed the reduction of tartronic semialdehyde to glyceric acid with reduced di- or tri-phosphopyridine nucleotide as electron donor. K_m for tartronic semialdehyde at pH 8·5 was measured to be 2×10^{-4} M. K_m for reduced diphosphopyridine nucleotide under these conditions was 2×10^{-5} M and for reduced triphosphopyridine nucleotide 5×10^{-5} M.

3. The crystalline enzyme oxidized 160 μmoles of reduced diphosphopyridine nucleotide/min./mg. of protein at pH 8·5, 23°, in the presence of tartronic semialdehyde. On the basis of the measured weight-average molecular weight M_w of 91 000, this gave a turnover number of 14 600 moles of reduced diphosphopyridine nucleotide oxidized/min./mole of enzyme.

4. The enzyme was shown also to catalyse the oxidation of glycerate with concomitant reduction of oxidized diphosphopyridine nucleotide. The

equilibrium constant K at 23° was 2×10^{-6} at pH 7·5 and $1{\cdot}6\times10^{-5}$ at pH 8·5; K_H was calculated to be $5{\cdot}1\times10^{-14}$ M. From these data, $\Delta G'$ at pH 7, 25°, was determined as 8·6 kcal./mole and ΔG° as 18 kcal./mole. Similarly, the oxidation–reduction potential for the reaction

$$\text{Glycerate}^{-} \rightleftharpoons \text{tartronic semialdehyde}^{-} + 2\text{H} + 2e$$

was found to be $E_0' = -0{\cdot}092$ v at 25° and pH 7.

5. The enzymic oxidation of glycerate to tartronic semialdehyde was also demonstrated by coupling it to the reduction of pyruvate to lactate, in the presence of oxidized diphosphopyridine nucleotide and crystalline lactic dehydrogenase, and to the reduction of 2:6-dichlorophenol-indophenol in the presence of oxidized diphosphopyridine nucleotide and reduced diphosphopyridine nucleotide dehydrogenase. The latter system served as a convenient assay of the enzyme.

6. The enzyme catalysed the reduction only of C_3 compounds of structure $CHO{\cdot}R{\cdot}CO_2H$, where R = CH·OH, CH_2 or CO. Tartronic semialdehyde (R = CH·OH) reacted more than five times as fast as any other substrate.

7. The enzyme catalysed the oxidation only of glyceric acid and of hydroxypyruvate.

8. The significance of these findings to the pathways of biosynthesis from glyoxylate is discussed.

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