# The Oxidation of D- and L-Glycerate by Rat Liver

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1. The interconversion of hydroxypyruvate and L-glycerate in the presence of NAD and rat-liver L-lactate dehydrogenase has been demonstrated. Michaelis constants for these substrates together with an equilibrium constant have been determined and compared with those for pyruvate and L-lactate. 2. The presence of D-glycerate dehydrogenase in rat liver has been confirmed and the enzyme has been purified 16–20-fold from the supernatant fraction of a homogenate, when it is free of L-lactate dehydrogenase, with a 23–29% recovery. The enzyme catalyses the interconversion of hydroxypyruvate and D-glycerate in the presence of either NAD or NADP with almost equal efficiency. D-Glycerate dehydrogenase also catalyses the reduction of glyoxylate, but is distinct from L-lactate dehydrogenase in that it fails to act on pyruvate, D-lactate or L-lactate. The enzyme is strongly dependent on free thiol groups, as shown by inhibition with *p*-chloromercuribenzoate, and in the presence of sodium chloride the reduction of hydroxypyruvate is activated. Michaelis constants for these substrates of D-glycerate dehydrogenase and an equilibrium constant for the NAD-catalysed reaction have been calculated. 3. An explanation for the lowered $V_{max.}$ with D-glycerate as compared with DL-glycerate for the rabbit-kidney D-α-hydroxy acid dehydrogenase has been proposed.

Both optical isomers of [3-$^{14}$C]glycerate are consumed to an equal extent by respiring rat-liver slices (Dickens & Williamson, 1960) and contribute nearly identically to the $^{14}$C appearing in the respiratory carbon dioxide and in the glucose formed. The pattern of labelling of the carbon atoms within the glucose molecule from either isomer is essentially the same. The degree of randomization of radioactivity among the carbon atoms within the glucose molecule was small and indicated a fairly direct route for the incorporation of glycerate as intact $C_3$ units, without passing through the Krebs cycle.

Labelled hydroxypyruvate is also incorporated into glucose by rat-liver slices and into liver glycogen by the intact rat (Dickens & Williamson, 1959). Under comparable conditions, hydroxypyruvate was shown to be incorporated into glucose to about the same extent as glycerate and there was less randomization of the labelled carbon atoms than that reported for pyruvate or lactate, thus excluding its extensive involvement in the Krebs cycle and transketolase reactions before being incorporated.

As suggested by Dickens & Williamson (1960) the most obvious explanation for these results would be for these three compounds to be incorporated into glucose via a common pathway, and for this their interconversion must be established. Mammalian L-lactate dehydrogenases have been obtained in crystalline form from various tissues (Gibson, Davisson, Bachhawat, Ray & Vestling, 1953; Racker, 1952; Kubowitz & Ott, 1943; Straub, 1940), and hydroxypyruvate is as effective a substrate as pyruvate for these enzymes from skeletal and heart muscle (Meister, 1952; Stafford, Magaldi & Vennesland, 1954). The reduction product of hydroxypyruvate in the presence of NADH is L-glycerate, and the reverse reaction involving the oxidation of L-glycerate by NAD with muscle lactate dehydrogenase has been demonstrated (Franke & Holz, 1959; Stafford *et al.* 1954). It therefore seemed necessary to confirm the ability of the liver lactate dehydrogenase to catalyse the interconversion of L-glycerate and hydroxypyruvate. This has been achieved by using partially purified preparations from rat liver.

The oxidation of D-glycerate as well as D-lactate by a soluble D-α-hydroxy acid dehydrogenase from rabbit-kidney mitochondria in the absence of added cofactors has been described by Tubbs & Greville (1961). A positive naphtharesorcinol test indicated that hydroxypyruvate is the probable oxidation product of glycerate. These workers found that D-glycerate gave a lower $V_{max.}$ than DL-glycerate although $K_m$ was the same for both. A similar enzyme was also found in rat-liver mito-

chondria, but the reversibility of the reaction was not demonstrated. Another mitochondrial enzyme preparation from ox liver with wider specificity oxidizes both isomers of lactate and glycerate as well as glycollate (Schäfer & Lamprecht, 1961).

While the present work was in progress a soluble D-glycerate dehydrogenase was demonstrated in extracts of ox liver and shown to catalyse the reversible oxidation of D-glycerate to hydroxypyruvate in the presence of either NAD or NADP with equal efficiency (Willis & Sallach, 1961, 1962*a*; Heinz & Lamprecht, 1961; Heinz, Bartelsen & Lamprecht, 1962). This enzyme has also been detected in rat liver (Willis & Sallach, 1962*a*). In the present paper a further investigation of the properties of D-glycerate dehydrogenase from rat liver is described.

## MATERIALS

Water was redistilled in glass apparatus and, when possible, A.R. grade reagents were used throughout.

*Amino acids.* Glycine and DL-serine were from British Drug Houses Ltd., Poole, Dorest, L-serine was from Aldrich Chemical Co. Inc., Wis. U.S.A., and L-alanine was from the California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A.

*Other chemicals.* Tris, 2,7-dihydroxynaphthalene, 2,4-dinitrophenylhydrazine, hydrazine hydrate and chromotropic acid, which was purified by ethanol precipitation from aqueous solution before use, were from British Drug Houses Ltd. Naphtharesorcinol, twice recrystallized from benzene before use, was from Roche Products Ltd., Welwyn Garden City, Herts. L-Cysteine and glycolaldehyde were from L. Light and Co. Ltd., Colnbrook, Bucks. D-Glyceraldehyde was from Fluka A.-G., Buchs, Switzerland. Bovine serum albumin was from Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex. DEAE-cellulose was from Serva, Heidelberg, Germany. Oxalic acid, oxamic acid, *p*-chloromercuribenzoic acid, phenylmercuric acetate and EDTA (disodium salt) were from British Drug Houses Ltd. Sodium iodoacetate was from L. Light and Co. Ltd.

*Coenzymes and hydrogen acceptors.* ATP and the oxidized and reduced forms of NAD and NADP were purchased from Boehringer und Soehne G.m.b.H., Mannheim, Germany. Horse-liver cytochrome *c* was from Sigma Chemical Co., St Louis, Mo., U.S.A. 2,6-Dichlorophenol-indophenol, purified by the method of Savage (1957), and methylene blue were from British Drug Houses Ltd.

*Mitochondrial preparations.* Mitochondria from rat liver and rabbit kidney were prepared as described by Tubbs & Greville (1961). Mitochondrial respiration was measured in the Warburg apparatus under similar conditions to those described by these authors except that citrate was used as 'sparker'.

*Enzymes.* Crystalline rabbit-muscle lactate dehydrogenase and also glucose 6-phosphate dehydrogenase as suspensions in ammonium sulphate were obtained from Boehringer und Soehne G.m.b.H. Rat-liver lactate dehydrogenase was partially purified by ethanol and ammonium sulphate fractionation as described by Vestling, Gibson, Davisson & Ray (1951). Parsley-leaf D-glycerate dehydrogenase was prepared as described by Dickens & Williamson (1958*b*). D-Glycerate kinase was prepared from acetone-dried powders of rat liver by the method described for horse liver by Ichihara & Greenberg (1957*b*). Rabbit-kidney D-α-hydroxy acid dehydrogenase was extracted at 4°, with 0·02 M-tris buffer, pH 7·8 (500 ml.), from the acetone-dried powder (25 g.) prepared from frozen rabbit kidneys (kindly provided in quantity by J. Sainsbury) by a method described in a personal communication from Dr P. K. Tubbs. After removal from the tris extract of much inert protein by the addition of calcium phosphate gel (25 mg./ml.) (Keilin & Hartree, 1938), the enzyme was precipitated at 0° by the addition of solid ammonium sulphate (fraction 0–35% saturation). The ammonium sulphate precipitate was dissolved in 20 ml. of 0·02 M-tris buffer, pH 7·8, and dialysed against the same buffer for 16 hr. at 4°. After being centrifuged from the copious precipitate, the supernatant had most of the activity of the original extract. Further purification was effected by adsorption on a column (1 cm. diam.) of DEAE-cellulose (1 g./100 mg. of protein in the supernatant) that had previously been equilibrated with 0·02 M-tris buffer, pH 7·8. Gradient elution with increasing concentration of KCl in the same buffer caused elution of active material at 0·1–0·16 M-KCl. These combined fractions were again treated with ammonium sulphate (60% saturation). The precipitated enzyme was collected and kept as the suspension in ammonium sulphate solution. Before use, portions were dissolved in a small volume of the tris buffer and dialysed against the same buffer to remove ammonium sulphate.

Rat-liver D-glycerate dehydrogenase was prepared essentially as described for the ox-liver enzyme (Willis & Sallach, 1962*a*), with slight modification as follows. Rat livers were homogenized in 0·154 M-KCl in an MSE blender as a 25% (w/v) homogenate. After centrifugation for 30 min. at 5000 rev./min. at 0° the turbid supernatant was decanted and stirred with one-twentieth of its volume of 1 M-$MnCl_2$. The suspension was then dialysed for 2 hr. at 4° against 10 vol. of 0·05 M-sodium acetate, pH 6, and, after centrifugation as above, the enzyme was precipitated by the addition of saturated ammonium sulphate solution in an ice bath. The protein fraction precipitating between 30 and 60% ammonium sulphate saturation was washed with 60% saturated ammonium sulphate, dissolved in a volume of 0·02 M-sodium phosphate buffer, pH 6·2, containing cysteine (1 mM) equivalent to one-tenth of the original supernatant and dialysed against the same buffer overnight. Any precipitate formed during dialysis was spun down and the supernatant treated with an equal volume of calcium phosphate gel (25 mg./ml.). After standing 10 min. in ice and centrifuging, the supernatant was again treated with calcium phosphate gel (1 mg. of gel/mg. of protein). After removal of the gel the supernatant was brought to 60% saturation with ammonium sulphate. The protein precipitate was dissolved in a volume of the same sodium phosphate buffer equivalent to one-twentieth of the original supernatant and dialysed against the same buffer overnight.

At this stage of purification the preparation usually failed to reduce pyruvate in the presence of NADH, but to ensure complete removal of lactate dehydrogenase one further treatment with calcium phosphate gel (1 mg. of gel/mg. of protein) was carried out as a routine. After removal of the gel the resulting supernatant was dialysed overnight against

0·01 M-sodium phosphate buffer, pH 5·8, containing cysteine (1 mM) and then freeze-dried. The white powder was stored at −15° for months with only slight loss of activity.

*Substrates.* DL-Glyceric acid, both as the free acid and as its calcium salt, were from L. Light and Co. Ltd. Barium D-glycerate was prepared by the bromine oxidation of D-glyceraldehyde (Baer, Grosheintz & Fischer, 1939) and recrystallized as described by Dickens & Williamson (1960); $[\alpha]_D^{21}$ for the (anhydrous) barium salt (*c* 2·75 in water) was +10·7°; Dickens & Williamson (1960) gave +9·9° and Meyerhof & Schulz (1938) give +11·5°. The addition of an equal volume of 20% ammonium molybdate to the neutral sodium salt, prepared quantitatively from the above barium salt by the addition of $Na_2SO_4$, gave $[\alpha]_D^{22}$ +108·5° for sodium glycerate (+80·0° calc. as barium glycerate); Meyerhof & Schulz (1938) give $[\alpha]_D$ +104° (basis of calculation not stated), Fager & Rosenberg (1950) found $[\alpha]_D$ +83°, molybdate-enhanced, for the sodium salt, Stafford *et al.* (1954) give +75° and +82°, and Dickens & Williamson (1960) give +120° for the sodium salt and +88·5° for the barium salt. Chromatography of the free acid in ethanol–aq. ammonia (sp.gr. 0·88)–water (8:1:1, by vol.) produced the typical pattern of a major acid spot, $R_F$ 0·4, and a minor acid spot, $R_F$ 0·15, and in ether–acetic acid–water (13:3:1, by vol.) a single acid spot, $R_F$ 0·39. Chromotropic acid assay (see the Analytical Methods section), with twice-recrystallized barium DL-glycerate as standard, showed 98% purity. After 75 min. incubation with D-glycerate kinase 93% of the periodate-oxidizable material was phosphorylated. Fig. 1 shows the behaviour of D-glycerate towards rabbit-muscle L-lactate dehydrogenase. Tested with parsley-leaf D-glycerate dehydrogenase in the presence of NAD, D-glycerate and (with twice the amount) DL-glycerate were equally effective substrates, whereas L-glycerate (see below) was not measurably attacked.

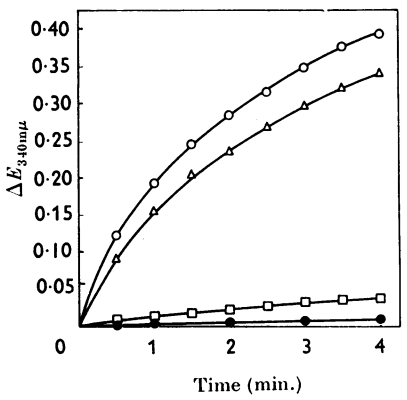


Fig. 1. Demonstration of optical purity of glycerate, determined with rabbit-muscle L-lactate dehydrogenase. All incubation mixtures (3·0 ml.) contained: tris buffer, pH 9 (500 μmoles); hydrazine chloride, pH 9 (400 μmoles); NAD (2 μmoles); enzyme (0·125 mg. of protein); substrate. The substrates used were: ○, DL-glycerate (75 μmoles); △, L-glycerate (37·5 μmoles); □, D-glycerate (37·5 μmoles); ●, none.

Barium L-glycerate was prepared by deamination of L-serine with nitrous acid (Fischer & Jacobs, 1907) and recrystallized as for the D-isomer; $[\alpha]_D^{21}$ for the barium salt (*c* 2·5 in water) was −9·45°; values of −9·07° (Frankland & Appleyard, 1893) and −9·77° (Frankland & Done, 1905) have been reported. The $[\alpha]_D^{22}$, molybdate-enhanced (final molybdate concn. 10%), was −109° calculated for sodium glycerate (−80·4°, calc. as barium glycerate). Dickens & Williamson (1960) gave −116°, calc. as the sodium salt, for the molybdate-enhanced rotation. Chromatography of the free acid in ethanol–ammonia–water produced the same pattern of acid spots as the DL- and D-samples plus an extra acid spot running just ahead of the major glycerate spot. In ether–acetic acid–water two spots were produced, one corresponding to glycerate and the other fainter spot, $R_F$ 0·59, to glycollic acid. Samples (0·5 g.) of L-glycerate were purified by chromatography of the free acid on Whatman 3MM paper in ethanol–aq. ammonia (sp.gr. 0·88) (9:1, v/v) for 2 days. Good separation of the two acids was obtained in this solvent, with $R_F$ 0·18 for glycerate and $R_F$ 0·235 for glycollate. The acid bands were eluted with water and concentrated before making up to a known volume. The faster acid band was identified as glycollic acid by its cherry-red colour in the 2,7-dihydroxynaphthalene test. The complete absence of glycine in the L-serine used for preparation of L-glycerate was confirmed by chromatography in phenol–water (4:1, w/v), and it appears that glycollic acid is formed during the deamination process. The small radioactive spot reported by Dickens & Williamson (1960) as running slightly ahead of the L-glycerate prepared from L-[3-$^{14}$C]serine was undoubtedly glycollic acid. Polarimetry of the glycollate-free L-glycerate gave $[\alpha]_D^{21}$ −10·75°. The glycerate content, determined by chromotropic acid assay, was also improved from 85 to 95%. After 75 min. incubation with D-glycerate kinase there was a fall of only 3% in the periodate-oxidizable material. Fig. 1 shows the behaviour of L-glycerate towards rabbit-muscle L-lactate dehydrogenase.

Pyruvic acid from British Drug Houses Ltd. was purified by twice fractionally distilling and collecting the fraction coming over at 70°/15 mm. Hg. Lithium pyruvate was prepared as described by Dickens & Williamson (1958*a*). Lithium hydroxypyruvate was obtained by the hydrolysis of bromopyruvic acid (prepared as described by Sprinson & Chargaff, 1946) and recrystallized as described by Dickens (1962). Glyoxylic acid as the free acid was from L. Light and Co. Ltd. and was prepared as its sodium salt by periodate oxidation of tartaric acid (Radin & Metzler, 1955). Calcium D- and L-lactate were obtained from the California Corp. for Biochemical Research. Glycollic acid, tartaric acid, *meso*-tartaric acid and calcium D-gluconate were obtained from British Drug Houses Ltd. L-Malic acid and β-propiolactone were from L. Light and Co. Ltd. The recrystallized double calcium–zinc salt of β-hydroxypropionic acid was prepared from β-propiolactone as described by Den, Robinson & Coon (1959). The barium salts of glucose 6-phosphate, 2-phospho-D-glyceric acid and 3-phospho-D-glyceric acid were obtained from Boehringer und Soehne G.m.b.H. Soluble barium and calcium salts were dissolved in water, passed through a column of Amberlite IR-120 ($H^+$ form) and neutralized with standard KOH before making up to a known volume. Insoluble barium salts were shaken as a suspension with excess of Amberlite IR-120 ($H^+$ form) until all the material had dissolved. After removal of the resin the

filtrate was treated as above. Barium salts were also converted into potassium salts by the addition of the calculated amounts of $K_2SO_4$.

## ANALYTICAL METHODS

*Colorimetric estimation of glyceric acid.* Glyceric acid was determined either by colorimetric estimation of formaldehyde liberated from C-3 by periodate (Frisell, Meech & Mackenzie, 1954) or by the colour reaction with chromotropic acid in nearly conc. $H_2SO_4$ (Bartlett, 1959). In the latter assay, for a given amount of glyceric acid, maximum colour production occurred when the chromotropic acid concentration was 0·025%, and the method was modified accordingly. Glycolaldehyde, which also produced a green colour with identical absorption spectrum with a peak at 690 mμ, behaved similarly.

Hydroxypyruvic acid was determined either colorimetrically or enzymically (Dickens & Williamson, 1958*b*). Glycollic acid was determined colorimetrically with 2,7-dihydroxynaphthalene in 32 N-$H_2SO_4$ (Eegriwe, 1932).

*Protein.* This was estimated by the methods of Warburg & Christian (1941) and Lowry, Rosebrough, Farr & Randall (1951).

*Preparation of dinitrophenylhydrazones.* Recrystallized derivatives of hydroxypyruvic acid (m.p. 160–161°), pyruvic acid (m.p. 218°) and glyoxylic acid (m.p. 194° from ethyl acetate–light petroleum, 204° from aq. ethanol) were prepared for use as chromatographic standards: these m.p. values are uncorrected. Three solvents were used for chromatography: butan-1-ol–ethanol–0·5 N-ammonia (7:1:2, by vol.) (El Hawary & Thompson, 1953); butan-1-ol–ethanol–water (7:1:2, by vol.) (Cavallini, Frontali & Toschi, 1949*a*,*b*); propan-2-ol–aq. ammonia (sp.gr. 0·88)–water (20:2:1, by vol.) (Smith & Smith, 1960*a*).

*Electrolytic reduction of dinitrophenylhydrazones.* Dinitrophenylhydrazones of oxo acids were reduced in the electrolytic desalter (Smith & Smith, 1960*b*) to the corresponding amino acids, which were identified chromatographically in phenol–water (4:1, w/v) and butan-1-ol–acetic acid–water (12:3:5, by vol.).

*Assay of* D-α-*hydroxy acid dehydrogenase.* The soluble enzyme was assayed in cells of 1 cm. light-path in the Unicam SP.500 spectrophotometer. The final amounts in a total volume of 3·0 ml. were: tris buffer, pH 8·5 (125 μmoles); substrate (75 μmoles); 2,6-dichlorophenol-indophenol (114 μmoles) or cytochrome *c* (155 μmoles). The reaction was started by addition of enzyme and followed by measuring the decrease in $E_{600m\mu}$ when dichlorophenol-indophenol was the oxidant, or the increase in $E_{550m\mu}$ when cytochrome *c* was the oxidant.

*Standard assay of* L-*lactate dehydrogenase or* D-*glycerate dehydrogenase.* Enzymes were assayed in silica cells of 1 cm. light-path, total volume 3·0 ml., containing: tris buffer, pH 9·0 (500 μmoles); hydrazine, pH 9·0 (400 μmoles); substrate (30 μmoles); NAD or NADP (0·9 μmole). The reaction was started by addition of enzyme and followed by measuring the increase in $E_{340m\mu}$.

The reduction of hydroxypyruvate, pyruvate or glyoxylate in the presence of lactate dehydrogenase or D-glycerate dehydrogenase was followed at 340 mμ in silica cells of 1 cm. light-path containing: sodium phosphate buffer, pH 6·0 (100 μmoles); substrate (0·6–3·0 μmoles); NADH (0·3 μmole) or NADPH (0·25 μmole); enzyme and water to give a total volume of 3·0 ml.

*Assay with* D-*glycerate kinase.* The optical configuration of glyceric acid was tested by means of its ability to undergo phosphorylation by ATP in the presence of D-glycerate kinase. The reaction was followed either manometrically in Warburg apparatus as a result of $CO_2$ evolution from bicarbonate by the extra acid liberated during phosphorylation, or by the loss of the ability to liberate formaldehyde on treatment with periodate. Manometric flasks were set up containing: $MgCl_2$ (10 μmoles); $NaHCO_3$ [saturated with $CO_2+N_2$ (5:95) before use] (33 μmoles); NaF (25 μmoles); ATP (neutralized with NaOH before use) (20 μmoles); glycerate (15 μmoles); D-glycerate kinase (0·3 ml.); water to give a final volume of 3·0 ml. For the colorimetric method, samples were taken from an incubation mixture containing: sodium phosphate buffer, pH 7·4 (150 μmoles); $MgCl_2$ (2 μmoles); ATP (sodium salt) (3 μmoles); glycerate (3 μmoles); D-glycerate kinase (0·5 ml.); water to give a final volume of 3·0 ml.

## RESULTS

*Oxidation of* D- *and* L-*glycerate in the presence of supernatant fraction from rat liver.* Dialysed rat-liver supernatant catalyses the oxidation of both D- and L-glycerate in the presence of NAD, but in the presence of NADP only D-glycerate is oxidized (Fig. 2). The activity with L-glycerate appears to be due to lactate dehydrogenase, which is present in rat-liver supernatant in much higher concentrations than is D-glycerate dehydrogenase (Fig. 3). The supernatant fraction also causes the reduction of pyruvate and hydroxypyruvate in the presence of either NADH or NADPH, but it is impossible to decide the relative activity of the two separate enzymes.

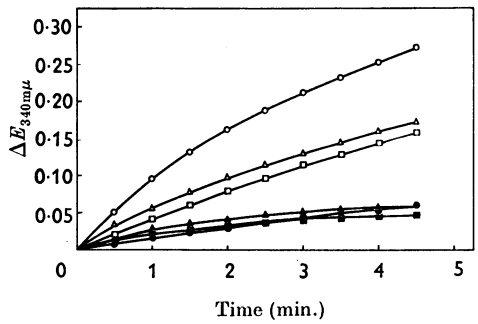


Fig. 2. Reduction of NAD or NADP by dialysed rat-liver supernatant in the presence of D- or L-glycerate. The standard assay system was used containing 1 ml. of 10% supernatant together with the following additions: ○, L-glycerate (30 μmoles)+NAD (2 μmoles); △, D-glycerate (30 μmoles)+NADP (1 μmole); □, D-glycerate (30 μmoles) +NAD (2 μmoles); ▲, L-glycerate (30 μmoles)+NADP (1 μmole); ●, NAD (2 μmoles) but no glycerate; ■, NADP (1 μmole) but no glycerate.

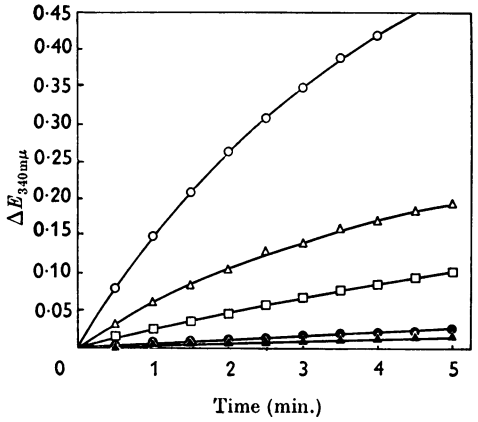


Fig. 3. Reduction of NAD by dialysed rat-liver supernatant (20%) in the presence of various substrates. The standard assay system was used containing the following additions: ○, L-lactate (30 $\mu$moles)+supernatant (0·01 ml.); △, L-glycerate (30 $\mu$moles)+supernatant (0·2 ml.); □, D-glycerate (30 $\mu$moles)+supernatant (0·2 ml.); ●, 3-phospho-D-glycerate (30 $\mu$moles)+supernatant (0·2 ml.); ▲, supernatant (0·2 ml.) but no added substrate.

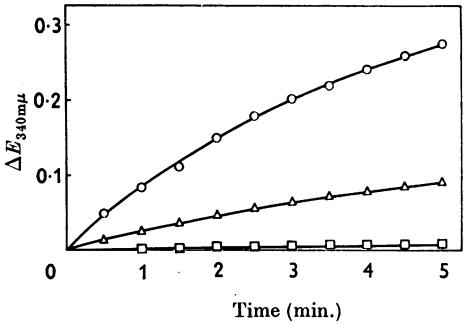


Fig. 4. Reduction of NAD by rat-liver L-lactate dehydrogenase in the presence of the L-lactate or L-glycerate. The standard assay system was used containing: ○, L-lactate (30 $\mu$moles)+enzyme (29·4 $\mu$g. of protein); △, L-glycerate (30 $\mu$moles)+enzyme (294 $\mu$g. of protein); □, enzyme (294 $\mu$g. of protein) but no substrate.

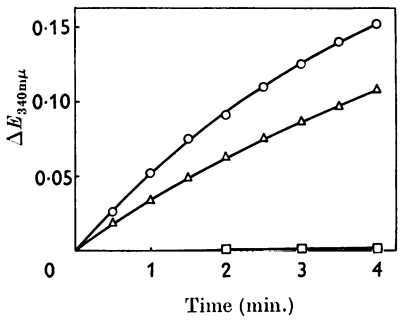


Fig. 5. Reduction of NAD by rabbit-muscle L-lactate dehydrogenase in the presence of the L-lactate or L-glycerate. The standard assay system was used containing: ○, L-lactate (30 $\mu$moles)+enzyme (1·25 $\mu$g. of protein); △, L-glycerate (30 $\mu$moles)+enzyme (25 $\mu$g. of protein); □, enzyme (25 $\mu$g. of protein) but no substrate.

*Oxidation of* L-*glycerate and* L-*lactate by* L-*lactate dehydrogenases from liver and muscle.* The rat-liver L-lactate dehydrogenase has been compared with the crystalline rabbit-muscle lactate dehydrogenase, and it was found that pyruvate and hydroxypyruvate were reduced by both these enzymes in the presence of either NADH or NADPH, but the rate of reduction by NADH was always higher than that by NADPH. The magnitude of the difference in rates with the two coenzymes was pH-dependent. For example, with the rabbit-muscle enzyme, at pH 5 pyruvate was reduced 5 times and hydroxypyruvate 10 times as fast by NADH as by NADPH, and at pH 7 pyruvate was reduced 60 times and hydroxypyruvate 730 times as fast. At pH 6 the rat-liver enzyme reduced both substrates about 30 times as fast with NADH as with NADPH. These findings are in agreement with those of Mehler, Kornberg, Grisolia & Ochoa (1948), who reported pyruvate to be reduced by NADH 170 times as fast as by NADPH at pH 7·4, and Meister (1950) has found ratios between 100 and 380 at pH 7·2, depending on the coenzyme concentration.

In the reverse direction the oxidation of L-lactate and L-glycerate at pH 9 occurred only in the presence of NAD. Under identical conditions L-lactate is oxidized some 30 times as fast as L-glycerate by either lactate dehydrogenase (Figs. 4 and 5). The equilibria for the reaction of pyruvate or hydroxypyruvate in the presence of either enzyme are far towards their reduction. By using $\epsilon_{mM}^{340m\mu}$ 6·22 for the determination of the NADH concentration (Horecker & Kornberg, 1948) the following values were determined for the equilibrium constants:

$$K = \frac{[\text{Oxo acid}^-][\text{NADH}][\text{H}^+]}{[\text{Hydroxy acid}^-][\text{NAD}^+]}$$

Experiments in the presence of rat-liver enzyme gave for the oxidation of L-lactate and L-glycerate respectively $2{\cdot}38 \times 10^{-12}$ M ($\Delta G' = 10{\cdot}5$ kcal.) and $2{\cdot}59 \times 10^{-13}$ M ($\Delta G' = 11{\cdot}9$ kcal.); experiments in the presence of the rabbit-muscle enzyme gave $2{\cdot}47 \times 10^{-12}$ M ($\Delta G' = 10{\cdot}6$ kcal.) and $1{\cdot}08 \times 10^{-13}$ M ($\Delta G' = 12{\cdot}5$ kcal.) respectively ($\Delta G'$ denotes the free-energy change at pH 7 and 25°; Krebs & Kornberg, 1957). The values for the lactate–pyruvate system lie within the range of values

reported in the literature (Kubowitz & Ott, 1943; Racker, 1950; Neilands, 1952; Hakala, Glaid & Schwert, 1956). These results indicate that the reduction of hydroxypyruvate by lactate dehydrogenase is less readily reversible than the corresponding reduction of pyruvate, but in all cases the addition of oxidized substrate (pyruvate or hydroxypyruvate) produced an immediate and rapid re-

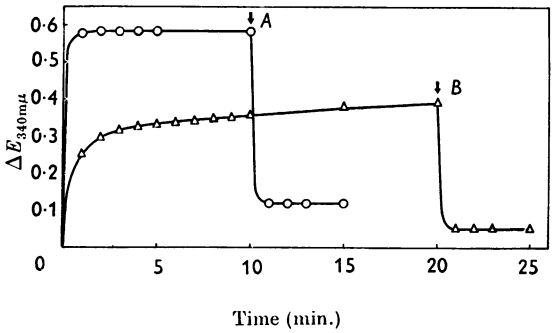


Fig. 6. Demonstration of the reversal of NAD reduction in the presence of rat-liver lactate dehydrogenase by the addition of the oxidized substrate. The initial incubation mixture (3·0 ml.) contained: tris buffer, pH 9 (500 $\mu$moles); NAD (3 $\mu$moles); enzyme (2·94 mg. of protein); either L-lactate (12 $\mu$moles) (○) or L-glycerate (60 $\mu$moles) (△). At *A*, 0·2 ml. of 6 mM-lithium pyruvate was added, and at *B*, 0·2 ml. of 6 mM-lithium hydroxypyruvate was added.

oxidation of enzyme (Fig. 6). The reduction of both oxidized substrates by NADH was maximal at pH 6·5 and that by NADPH at pH 5 in the presence of the rabbit-muscle enzyme. Table 1 summarizes the Michaelis constants for these substrates determined by the method of double-reciprocal plots (Lineweaver & Burk, 1934) with both lactate dehydrogenases: values of other workers are included for comparison.

*Purification and properties of* D-*glycerate dehydrogenase.* The enzyme was purified to a stage where it was free of L-lactate dehydrogenase, and of the substrates tested only hydroxypyruvate and glyoxylate were reduced (see Table 2). Reduction of both substrates occurred in the presence of either NADH or NADPH, but under comparable conditions hydroxypyruvate was reduced 5 times as fast as glyoxylate. Pyruvate and $\alpha$-oxoglutarate were not reduced. As shown in Table 3, of all the compounds tested, only D-glycerate was oxidized by the enzyme to any appreciable extent in the presence of either NAD or NADP. Reaction rates were studied over the range where they were proportional to protein concentration.

No separation of the activities with either coenzyme was achieved but variable ratios of the reduction of hydroxypyruvate by NADH and NADPH were produced by different enzyme preparations. However, it was noticed that, in common with the ox-liver enzyme (Willis & Sallach, 1962*b*; Heinz *et al.* 1962), inorganic anions, parti-

Table 1. *Michaelis constants for lactate dehydrogenases*

| Substrate | Coenzyme | Source of enzyme | pH | $K_m$ (mM) | Reference |
|---|---|---|---|---|---|
| Hydroxypyruvate | NADH | Rabbit muscle | 6.5 (phosphate) | 0·73 | This paper |
| | NADH | Rat liver | 6 (phosphate) | 0·4 | This paper |
| | NADPH | Rabbit muscle | 5 (acetate) | 1·4 | This paper |
| | NADPH | Rat liver | 6 (phosphate) | 1·0 | This paper |
| Pyruvate | NADH | Rabbit muscle | 6·5 (phosphate) | 0·06 | This paper |
| | NADH | Rabbit muscle | 7·2 | 0·052 | Meister (1950) |
| | NADH | Rat liver | 6 (phosphate) | 0·1 | This paper |
| | NADH | Rat skeletal muscle | 7·4 | 0·09 | Kubowitz & Ott (1943) |
| | NADH | Ox heart muscle | 7–10 | 0·01 | Neilands (1952) |
| | NADH | Ox heart muscle | 6·8 | 0·018 | Hakala *et al.* (1956) |
| | NADH | Ox heart muscle | 7·3 | 0·037 | Schwert & Hakala (1952) |
| | NADPH | Rabbit muscle | 5 (acetate) | 0·5 | This paper |
| | NADPH | Rat liver | 6 (phosphate) | 0·67 | This paper |
| L-Glycerate | NAD | Rabbit muscle | 9 (tris) | 20 | This paper |
| | NAD | Rabbit muscle | 7·2 | 36 | Franke & Holz (1959) |
| | NAD | Rabbit muscle | 7·2 | 21 | Franke & Holz (1959) |
| | NAD | Rat liver | 9 (tris) | 20 | This paper |
| L-Lactate | NAD | Rabbit muscle | 9 (tris) | 6·7 | This paper |
| | NAD | Rabbit muscle | 7·2 | 7·2 | Franke & Holz (1959) |
| | NAD | Rat liver | 9 (tris) | 4·0 | This paper |
| | NAD | Ox heart muscle | 7–10 | 1·0 | Neilands (1952) |

Table 2. *Purification of rat-liver D-glycerate dehydrogenase*

Enzyme activities are expressed in units/mg. of protein. One enzyme unit for the oxidation of 10 mM-D- or L-glycerate at pH 9 in the standard assay by 0·3 mM-NAD or -NADP is taken as $\Delta E_{340m\mu}$ 0·001/min. and for the reduction of pyruvate, hydroxypyruvate or glyoxylate at pH 6 in the standard assay by NADH or NADPH as $\Delta E_{340m\mu}$ 1·0/min., i.e. 1 reduction ≡ 1000 oxidation units.

| Coenzyme ... ... ... ... | | | NAD | | NADP | — | NADH | | | | | | NADPH | | | | | — |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Fraction | Total vol. (ml.) | Total protein (mg.) | L-Glycerate | D-Glycerate | D-Glycerate | D-Glycerate/NAD/NADP ratio | Pyruvate | Hydroxypyruvate | Glyoxylate | Hydroxypyruvate/Pyruvate ratio | D-Glycerate/L-Glycerate ratio | Pyruvate | Hydroxypyruvate | Glyoxylate | Hydroxypyruvate/Pyruvate ratio | Pyruvate/Hydroxypyruvate ratio | NADH/NADPH ratio | Glyoxylate/NADH/NADPH ratio |
| ... | | | | | | | | | | | | | | | | | | |
| Supernatant | 130 | 5106 | 2·55 | 0·65 | 3·2 | 0·2 | 4·28 | 3·42 | 0·342 | 0·8 | 0·255 | 0·12 | 0·103 | 0·017 | 0·86 | 33 | 20 | |
| 30–60% satd. $(NH_4)_2SO_4$ ppt. | 45 | 1880 | 10·8 | 9·58 | 4·77 | 2·0 | 8·36 | 6·57 | 0·479 | 0·785 | 0·886 | 0·239 | 0·407 | 0·0957 | 1·7 | 16·2 | 5 | |
| After first $Ca_3(PO_4)_2$-gel treatment | 80 | 1070 | 29·9 | 31·4 | 16·4 | 1·92 | 6·52 | 5·6 | 0·466 | 0·86 | 1·05 | 0·187 | 0·373 | 0·13 | 2·0 | 15 | 3·6 | |
| After second $Ca_3(PO_4)_2$-gel treatment | 115 | 274 | 2·1 | 29·4 | 14 | 17·85 | 1·65 | 0·23 | 0·504 | 11·5 | 14 | 0·0295 | 0·315 | 0·1135 | 10·7 | 8·4 | 4·4 | |
| 60% satd. $(NH_4)_2SO_4$ ppt. | 9 | 156 | 0·575 | 34·5 | 60 | 21·9 | 1·58 | 0·287 | 3·64 | 12·7 | ∞ | 0·115 | 0·725 | 0·23 | 6·3 | 5 | 2·5 | |
| After third $Ca_3(PO_4)_2$-gel treatment | 13 | 76 | 0 | 54·8 | ∞ | 34·3 | 1·6 | 0 | 4·12 | ∞ | 0 | 0 | 1·255 | 0·285 | ∞ | 3·3 | 2·6 | |

cularly $Cl^-$ and $SO_4^{2-}$, activated the reduction of hydroxypyruvate by the two reduced coenzymes to different degrees depending on pH and substrate concentration and could account for these variable ratios (see Table 4). D-Glycerate was always oxidized at a faster rate by NAD than by a comparable amount of NADP (Fig. 7). Studies with mixed coenzymes suggested that only one protein was responsible for both activities. Initial rates measured in the presence of equal amounts of either NAD plus NADP or NADH plus NADPH were always intermediate between the rates produced by the two coenzymes separately. Summation of activities in the presence of both coenzymes was never observed.

The enzyme was unstable when stored in solution either at 0° or at −15°. The presence of 1 mM-cysteine seemed to retard the rate of spontaneous inactivation. The enzyme remained active for months when stored as a freeze-dried preparation at −15°. Reactivation of the inactivated enzyme was not achieved by the addition of cysteine or reduced glutathione. The enzyme was sensitive to pH and was most stable at pH 5·8 in phosphate buffer. Enzymic activity was completely destroyed after heating in a boiling-water bath. The enzyme had a wide pH–activity range for the oxidation of D-glycerate, with a maximum at pH 10 for NAD as oxidant and somewhat higher for NADP. For the reverse reaction, reduction of hydroxypyruvate was optimum with NADPH in acetate buffer at pH 4·4 and with NADH below pH 4. Hydroxypyruvate concentrations varying from 0·2 to 1 mM had no effect on the pH optimum of either reaction. A freeze-dried enzyme preparation that had been stored for several months at −15° caused maximal oxidation at NADH at pH 6, but with NADPH rates were highest at pH 4 in acetate and pH 6 in phosphate. These values are nearer those reported for the ox-liver enzyme, whose pH optimum for the reduction of hydroxypyruvate by either coenzyme was between 6·8 and 7·0 (Heinz *et al.* 1962). Optimum hydroxypyruvate reduction by NADH in the presence of spinach-leaf D-glycerate dehydrogenase occurs at pH 6·2 (Holzer & Holldorf, 1957*b*).

The discrepancy between these results and those obtained previously may well have been due to the presence of higher concentrations of anions such as $SO_4^{2-}$ in the previous enzyme solutions. In agreement with results obtained with the leaf D-glycerate dehydrogenase and glyoxylate reductase (Holzer & Holldorf, 1957*b*; Zelitch, 1955) it has been found that small amounts of anion more strongly activate the reduction of hydroxypyruvate at lower pH values.

Michaelis constants for hydroxypyruvate, glyoxylate and D-glycerate are shown in Table 5. The equilibria for these reactions were strongly in

Table 3. *Activity of* D-*glycerate dehydrogenase towards various compounds*

Compounds were tested in the standard assay at pH 9. The following were not at all attacked in presence of either coenzyme: 1 mM-hydroxypyruvate and 10 mM concentrations of L-glycerate, D-lactate, L-lactate, glycollate, β-hydroxypropionate, ethanol, DL-glyceraldehyde, D-gluconate, DL-tartrate or *meso*-tartrate.

| Substrate | With 0·6 mM-NAD | With 0·3 mM-NADP |
|---|---|---|
| 10 mM-D-Glycerate | 100 (≡ 18·4 units/mg. of protein) | 100 (≡ 11·2 units/mg. of protein) |
| 8 mM-3-Phospho-D-glycerate | 0 | 0;2 |
| 4 mM-2-Phospho-D-glycerate | 0 | 4;7 |
| 10 mM-L-Malate | 5 | 0 |

Table 4. *Relative activation of hydroxypyruvate reduction by sodium chloride*

The values in parentheses are the actual activities expressed as $\Delta E_{340m\mu}$/min.; the remaining values denote activities relative to that in the absence of NaCl (= 1). The enzyme solution used for the experiments with 0·66 mM- and 1 mM-hydroxypyruvate was the same but different from that used for the experiment with 0·2 mM-hydroxypyruvate.

| Coenzyme ... ... ... | | NADH | | | NADPH | | |
|---|---|---|---|---|---|---|---|
| Concn. of NaCl (mM) ... | | 0 | 30 | 60 | 0 | 30 | 60 |
| Concn. of hydroxypyruvate (mM) | pH | | | | | | |
| 0·2 | 4 | 1 (0·03) | 3 | 2·5 | 1 (0·03) | 2·3 | 3·3 |
| | 6 | 1 (0·028) | 1·7 | 2 | 1 (0·02) | 1·4 | 1·85 |
| 0·66 | 4 | 1 (0·085) | 3 | 2·8 | 1 (0·085) | 1·65 | 2·24 |
| | 6 | 1 (0·08) | 1·9 | 1·7 | 1 (0·055) | 1·1 | 1·25 |
| 1·0 | 4 | 1 (0·07) | 3 | 3·3 | 1 (0·085) | 1·3 | 2 |
| | 6 | 1 (0·07) | 1·65 | 1·8 | 1 (0·06) | 1·03 | 1·08 |

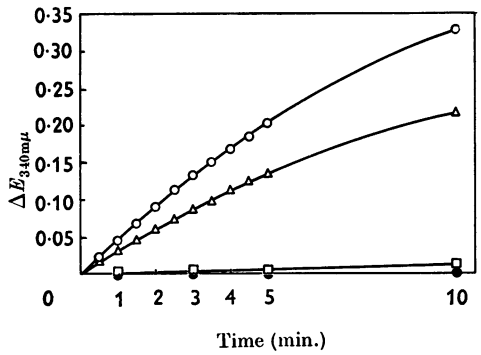


Fig. 7. Relative rates of coenzyme reduction in the presence of glycerate and rat-liver D-glycerate dehydrogenase. The standard assay system was used containing: enzyme (2·5 mg. of protein); glycerate (as indicated); coenzyme (as indicated). The substrates and coenzymes used were: ○, D-glycerate (30 μmoles)+NAD; △, D-glycerate (30 μmoles)+NADP; ●, L-glycerate (30 μmoles)+NAD; □, L-glycerate (30 μmoles)+NADP.

favour of hydroxypyruvate reduction. A mean value of $0·61 \times 10^{-13}$ M for the apparent equilibrium constant:

$$K = \frac{[\text{Hydroxypyruvate}^-][\text{NADH}][\text{H}^+]}{[\text{D-Glycerate}^-][\text{NAD}^+]}$$

for the NAD system has been obtained. This value is lower than the equilibrium constant for the L-glycerate–NAD system catalysed by the rabbit-muscle lactate dehydrogenase by a factor of 1·5 and by the rat-liver lactate dehydrogenase by a factor of 4. It is also about one-fifth of the values obtained for the same reaction catalysed by spinach-leaf D-glycerate dehydrogenase ($3·26 \times 10^{-13}$ M; $\Delta G' = 11·8$ kcal.; Holzer & Holldorf, 1957*b*) and by tobacco-leaf glyoxylate reductase ($3·55 \times 10^{-13}$ M; $\Delta G' = 11·8$ kcal.; Zelitch, 1955). The discrepancy may be due to almost complete inactivation of the enzyme over the longer periods of these experiments, and also to the relative instability of hydroxypyruvate compared with that of pyruvate, especially at pH 9. The fall in extinction on the addition of hydroxypyruvate would require a much smaller amount of enzyme remaining active than would be necessary to drive the oxidation of D-glycerate to its true equilibrium point. A satisfactory equilibrium constant for the D-glycerate–NADP system was not obtained, since most enzyme preparations seemed to catalyse a slow reduction of NADP in the absence of hydrazine and D-glycerate and in the presence of D-glycerate a true equilibrium point was not reached. However, in the presence of hydrazine there was no reduction of NADP unless D-glycerate was also present. The high blank values in the

Table 5. *Michaelis constants for* D*-glycerate dehydrogenase*

| Substrate | Coenzyme | Source of enzyme | pH | $K_m$ (mM) | Reference |
|---|---|---|---|---|---|
| Hydroxypyruvate | NADH | Rat liver | 4 (acetate) | 0·031 | This paper |
| | NADH | Rat liver | 6 (phosphate) | 0·062 | This paper |
| | NADH | Rat liver | 7·4 (phosphate) | 0·025 | This paper |
| | NADH | Ox liver | 6·8 (phosphate) | 0·045 | Heinz *et al.* (1962) |
| | NADPH | Rat liver | 4 (acetate) | 0·017 | This paper |
| | NADPH | Rat liver | 7·4 (phosphate) | 0·25 | This paper |
| | NADPH | Ox liver | 6·8 (phosphate) | 0·02 | Heinz *et al.* (1962) |
| Glyoxylate | NADH | Rat liver | 6 (phosphate) | 0·25 | This paper |
| | NADH | Ox liver | 6·8 (phosphate) | 0·14 | Heinz *et al.* (1962) |
| | NADPH | Rat liver | 6 (phosphate) | 0·5 | This paper |
| | NADPH | Ox liver | 6·8 (phosphate) | 0·25 | Heinz *et al.* (1962) |
| D-Glycerate | NAD | Rat liver | 9 (tris) | 3·1–6·7 | This paper |
| | NADP | Rat liver | 9 (tris) | 1·7–3·3 | This paper |

Table 6. *Incubation conditions for the oxidation of* D *and* L*-glycerate*

The reagents indicated were contained in a total volume of 10 ml.

| Flask no. | Tris buffer, pH 9 (μmoles) | Hydrazine, pH 9 (μmoles) | Glycerate (μmoles) | NAD (mg.) | NADP (mg.) | Freeze-dried D-glycerate dehydrogenase (mg.) | Rat-liver lactate dehydrogenase (mg. of protein) | Rabbit-muscle lactate dehydrogenase (mg. of protein) |
|---|---|---|---|---|---|---|---|---|
| 1 | 500 | 400 | 300 (L-isomer) | 40 | 0 | 0 | 120 | 0 |
| 2 | 500 | 400 | 300 (L-isomer) | 40 | 0 | 0 | 0 | 1 |
| 3 | 500 | 400 | 300 (D-isomer) | 40 | 0 | 20 | 0 | 0 |
| 4 | 500 | 400 | 300 (D-isomer) | 0 | 20 | 20 | 0 | 0 |

absence of hydrazine may have been due to contamination of the D-glycerate dehydrogenase preparation by an NADP-specific glycerol dehydrogenase, which has been found (Moore, 1959) to catalyse the reduction of NADP in the presence of tris, the buffer used in these experiments.

Of the metabolic inhibitors tested *p*-chloromercuribenzoate was by far the most potent, suggesting a dependence on free thiol groups for activity: at 0·1 mM it completely inhibited hydroxypyruvate reduction by NADH and diminished its reduction rate by NADPH by 55%; at 1 μM it caused 80% inhibition in the presence of NADH and had no effect in the presence of NADPH. D-Glycerate oxidation by NAD and NADP was inhibited 95 and 87% respectively by 0·1 mM-*p*-chloromercuribenzoate, and 0·5 μM-inhibitor inhibited the NAD reaction by 55% but had no effect on that in the presence of NADP. D-Glycerate oxidation was considerably inhibited by 1 mM-hydroxypyruvate, and 10 mM-L-glycerate caused a 24% fall in D-glycerate oxidation by NAD but had no effect when NADP was oxidant. The reason for the different degree of inhibition with the two coenzymes is not clear, but it was regularly observed. Concentrations of oxalate down to 1 μM and of oxamate down to 0·1 mM also produced considerable inhibition of hydroxypyruvate reduction by either coenzyme; 1 mM-cyanide, 10 mM-fluoride and 10 mM-iodoacetate were weakly inhibitory. EDTA (10 mM) had little effect on hydroxypyruvate reduction, suggesting independence of metal ions. In all cases, comparable concentrations of inhibitor had greater effect on the NADH–NAD system than on the NADP–NADPH system. Similarly, excess of hydroxypyruvate had a greater inhibitory effect on its own reduction by NADH than by NADPH.

*Identification of enzymic reaction products.* (*a*) Oxidation of D- and L-glycerate to hydroxypyruvate. Flasks were set up as shown in Table 6 and incubated at room temperature for 3 hr. Protein was precipitated with trichloroacetic acid to a final concentration of 10% (w/v). A slight excess of 2,4-dinitrophenylhydrazine in 2 N-hydrochloric acid was added to the supernatant and washings, and after 1 hr. they were extracted with ethyl acetate (3 × 10 ml.). After being washed with water the combined ethyl acetate extracts were extracted with 10% (w/v) sodium carbonate (2 × 20 ml.). The alkaline extracts were immediately cooled in ice and

acidified to pH 2 with conc. hydrochloric acid. The oxo acid derivatives were re-extracted with ethyl acetate (2 × 10 ml.) and, after being washed with water to remove much of the trichloroacetic acid, were concentrated to yellow oils, which were stored in an evacuated desiccator for 24 hr. over moist sodium hydroxide flakes. The oils were taken up in ethyl acetate and streaked along the origin of Whatman no. 3MM papers, and the chromatograms were developed in propan-2-ol–aq. ammonia (sp.gr. 0·88)–water in the dark for 16 hr. together with a hydroxypyruvic dinitrophenylhydrazone marker. In all cases a major fast band and a minor slower band, corresponding to the two spots of authentic hydroxypyruvic dinitrophenylhydrazone, were produced. Each band was cut from the air-dried paper and eluted with 0·1 M-sodium phosphate buffer, pH 7·4. The acidified extracts were extracted with ethyl acetate, washed, concentrated and dried as above. Rechromatography of the fast-band extracts produced two spots corresponding to those given by authentic hydroxypyruvic dinitrophenylhydrazone in butan-1-ol–ethanol–ammonia ($R_F$ values 0·53 and 0·47) and propan-2-ol–ammonia–water ($R_F$ values 0·42 and 0·28). When the chromatograms were dipped in ethanolic sodium hydroxide the faster spot was green–brown and the slower spot orange–brown, distinct from pyruvic dinitrophenylhydrazone (deep brown) and glyoxylic dinitrophenylhydrazone (bright red–brown). Rechromatography of the slow-band extracts again produced two spots with the same $R_F$ values as those from the fast band, but in this case the main spot was the slower with less hydrazone in the fast area. These two bands are undoubtedly the *syn* and *anti* isomers. Solutions of the oxo acid and authentic hydroxypyruvic acid derivatives in 0·1 M-phosphate buffer, pH 7·4, had identical absorption spectra with peaks at 375–377 mμ (Fig. 8).

When the compounds in the two bands produced by chromatography of authentic hydroxypyruvic dinitrophenylhydrazone in propan-2-ol–ammonia–water were separately eluted and reduced both extracts yielded serine and alanine, which were identified by paper chromatography. Meister & Abendshein (1956) and Willis & Sallach (1962*a*) have also observed the production of alanine as well as serine after hydrogenation of hydroxypyruvic dinitrophenylhydrazone in the presence of platinum oxide. The fast bands of the derivatives obtained from the incubation mixtures of D- and L-glycerate all produced serine and alanine after reduction. Thus hydroxypyruvate is the oxidation product of L-glycerate in the presence of lactate dehydrogenase from rat liver as well as from rabbit muscle and of D-glycerate in the presence of D-glycerate dehydrogenase with either NAD or NADP as oxidant.

(*b*) Reduction of hydroxypyruvate. Incubation mixtures were set up, containing lithium hydroxypyruvate (154 μmoles), coenzyme (10 mg. of NAD or NADP) and dehydrogenase, together with a second system (shown in Table 7) that regenerates the

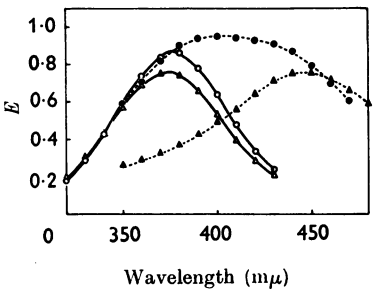


Fig. 8. Absorption spectra of hydroxypyruvic 2,4-dinitrophenylhydrazones, after chromatography in propan-2-ol–aq. ammonia (sp.gr. 0.88)–water and elution of the two isomers. Fast-running isomer in 0·1 M-phosphate buffer, pH 7·4 (○), and 1 N-NaOH (●). Slow-running isomer in 0·1 M-phosphate buffer, pH 7·4 (△), and 1 N-NaOH (▲).

Table 7. *Incubation conditions for the reduction of hydroxypyruvate*

The reagents indicated were contained in a total volume of 50 ml.

| Flask no. | Enzyme system | Coenzyme | Ethanol (ml.) | Alcohol dehydrogenase (ml.) | 2 N-$NH_3$ to bring pH to 8 (ml.) | Tris buffer, pH 7·3 (m-mole) | Glucose 6-phosphate (μmoles) | Glucose 6 phosphate dehydrogenase (mg.) |
|---|---|---|---|---|---|---|---|---|
| 1 | Rat-liver lactate dehydrogenase (30 mg.) | NAD | 1 | 0·1 | 0·1 | 0 | 0 | 0 |
| 2 | Rabbit-muscle lactate dehydrogenase (0·5 mg.) | NAD | 1 | 0·1 | 0·1 | 0 | 0 | 0 |
| 3 | Rat-liver D-glycerate dehydrogenase (100 mg.) | NAD | 1 | 0·2 | 0·1 | 0 | 0 | 0 |
| 4 | Rat-liver D-glycerate dehydrogenase (100 mg.) | NADP | 0 | 0 | 0 | 1 | 575 | 0·1 |

reduced coenzyme and allows the reduction of hydroxypyruvate to go to completion.

After 6 hr. at room temperature, when the hydroxypyruvate concentration had fallen to a very low level, the solutions were acidified with acetic acid and boiled. After centrifuging and shaking the cooled solutions with activated charcoal to remove nucleotides, the filtrates from flasks 1–3 were passed directly through columns of Amberlite IR-120 ($H^+$ form). Unchanged glucose 6-phosphate and its oxidation product 6-phosphogluconic acid were removed from the filtrate of flask 4 as their barium salts by adding 5 ml. of 0·4 N-barium hydroxide and dilute ammonia solution, until pink to phenolphthalein, and filtering. The filtrate was then passed through a column of the same cation-exchange resin. The elutates from each column were concentrated to syrups *in vacuo*. Solutions of the syrups were streaked along the origins of Whatman 3MM papers and developed in ethanol–aq. ammonia (sp.gr. 0·88)–water together with a glyceric acid marker. The air-dried papers were dipped in chlorophenol red indicator and the bands corresponding to glyceric acid were cut from the paper and eluted with water. After concentration the ammonium glycerate (chromotropic acid assay) amounted to 68 μmoles (44%) from the liver lactate dehydrogenase, 65·5 μmoles (42·5%) from the rabbit-muscle lactate dehydrogenase, 127 μmoles (82%) from the D-glycerate dehydrogenase (with NAD) and 120 μmoles (76%) from the D-glycerate dehydrogenase (with NADP). Rechromatography in ethanol–ammonia–water produced the pattern of spots typical of glyceric acid (major acid $R_F$ 0·4 and minor acid $R_F$ 0·17) and in diethyl ether–acetic acid–water a single spot ($R_F$ 0·39).

When assayed manometrically with D-glycerate kinase, high rates of carbon dioxide evolution were produced from both glycerate samples formed from hydroxypyruvate incubated with D-glycerate dehydrogenase, but those formed by reduction of hydroxypyruvate in the presence of either of the lactate dehydrogenases failed to increase the rate of carbon dioxide evolution above that of the water control. It appears that, whereas only L-glycerate is formed from hydroxypyruvate in the presence of lactate dehydrogenase, the glycerate formed in the presence of D-glycerate dehydrogenase has the D-configuration. Periodate assay confirmed that at least 90% of the periodate-formaldehydeogenic material formed from hydroxypyruvate in the presence of D-glyceric dehydrogenase was D-glycerate, whereas of the glycerate formed in the presence of lactate dehydrogenase there was a decrease of not more than 6% of the total formaldehydogenic material.

*Respiration of mitochondria in the presence of glycerate.* In agreement with Tubbs & Greville (1961) we find that D-lactate is rapidly oxidized by rabbit-kidney mitochondria. The initial rate of oxidation of D-glycerate was greater than that of L-glycerate and of citrate alone and was almost as high as that of D-lactate (Fig. 9). However, the rate soon fell off and after complete oxidation of citrate the rates of oxygen uptake with D- and L-glycerate were identical with those of the citrate control. Oxygen uptake with L-lactate was higher than with citrate alone, but this may have been due to contamination with traces of the D-isomer. With rat-liver mitochondria D-lactate was oxidized at a much greater rate than was the L-isomer. When D- and L-glycerate were tested for their ability to be oxidized by rat-liver mitochondria, however, the oxygen uptake with either isomer was never significantly higher than that produced by the citrate 'sparker' alone.

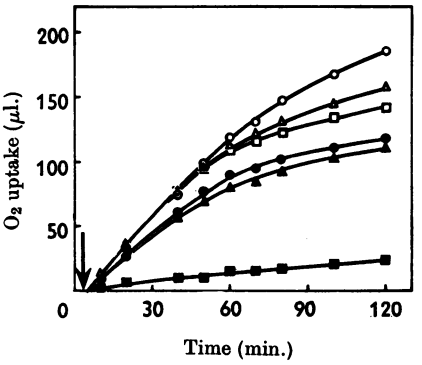


Fig. 9. Respiration of rabbit-kidney mitochondria in the presence of lactate and glycerate. Warburg flasks (2·0 ml. of liquid), set up as described in the Analytical Methods section, contained glycerate or lactate (10 μmoles) and citrate (1 μmole). Flasks were tipped after 5 min. equilibration at 30° (arrow). The gas phase was air. The substrates used were: ○, D-lactate+citrate; △, L-lactate+citrate; □, D-glycerate+citrate; ●, citrate alone; ▲, L-glycerate+citrate; ■, none.

*Soluble D-α-hydroxy acid dehydrogenase.* The enzyme preparation oxidized L-lactate at about 18% of the rate at which D-lactate was oxidized, but this activity may have been due to contamination with the D-isomer since both rates were decreased equally after preincubating the enzyme with EDTA. Tubbs & Greville (1961) have reported that enzyme freshly prepared from acetone-dried powders of mitochondria has low activity but undergoes activation on storage at 4° or on the addition of cyanide. However, the enzyme that we obtained from acetone-dried powders of whole kidneys was not activated by storage at 4°, and low concentrations of cyanide partially inhibited the enzymic oxidation of both D-lactate and DL-glycerate.

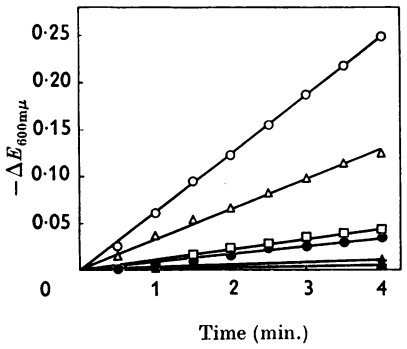


Fig. 10. Reduction of 2,6-dichlorophenol-indophenol at pH 8·5 by various substrates in the presence of rabbit-kidney D-α-hydroxy acid dehydrogenase (0·895 mg. of protein). The standard assay (3·0 ml.) for this enzyme contained the following substrates: ○, D-lactate (75 μmoles); △, DL-glycerate (75 μmoles); □, L-lactate (75 μmoles); ●, D-glycerate (75 μmoles); ■, L-glycerate (75 μmoles); ▲, none.

The rate of dichlorophenol-indophenol reduction by DL-glycerate was higher than by comparable amounts of D-glycerate (Fig. 10). L-Glycerate and glycollate failed to reduce the dye in the presence of the enzyme and therefore distinguish it from the dehydrogenase studied by Schäfer & Lamprecht (1961). Various samples of DL- and D-glycerate produced different rates of dye reduction and it seems that some unknown impurity may act as an inhibitor. The results suggest that this inhibitor, which may be a polymer or anhydride, is particularly liable to arise when glyceric acid is prepared by evaporation of a solution under strongly acid conditions. The inhibitor, once formed, is stable to alkali. Chromatography of the D-isomer in ethanol–ammonia–water and elution of the main (ammonium glycerate) band improved the substrate activity, but initial rates of dye reduction were still higher with the DL-acid. More nearly linear double-reciprocal plots were obtained when cytochrome *c* was used as the electron acceptor, but DL-glycerate was again oxidized at a greater rate than D-glycerate and a reason for this has not been found. In a few instances when straight-line double-reciprocal plots were obtained with dichlorophenol-indophenol, values for $K_m$ calculated for D-glycerate were approx. 14 mM. With cytochrome *c* as acceptor, values calculated for the D-isomer, with samples of DL- and D-glycerate, were 2·86 and 0·66 mM respectively.

Incubation mixtures (total vol. 3·5 ml.) set up in tris buffer, pH 8·5 (125 μmoles), with D- or DL-glycerate (150 μmoles), enzyme (9 mg. of protein) and methylene blue (0·05 μmole) in the absence of hydrazine, resulted in the formation of a substance giving an orange dinitrophenylhydrazone that had chromatographic properties similar to those of the bisdinitrophenylhydrazone of mesoxalic semi-aldehyde, a probable product of the autoxidation of hydroxypyruvate in alkaline solution. However, in the presence of hydrazine the autoxidation of hydroxypyruvate is prevented and this compound did not appear. Aerobic incubation in the presence of hydrazine, pH 8·5 (100 μmoles), as trapping agent allowed the identification of hydroxypyruvate by paper chromatography of its dinitrophenylhydrazone and by the grass-green colour produced in the naphtharesorcinol assay (Dickens & Williamson, 1958*b*). The same methods were used for the detection of hydroxypyruvate resulting from pooled enzymic assays with cytochrome *c* as oxidant.

## DISCUSSION

The NAD-linked L-lactate dehydrogenases of rat liver and rabbit muscle are capable of catalysing the reversible interconversion of L-glycerate and hydroxypyruvate. The $K_m$ for the oxidized substrate is low whereas that for L-glycerate is considerably higher, as might be expected since L-glycerate is not thought to be a natural substrate for these enzymes in animal tissues. There are no reports of L-glycerate occurring naturally and the only known precursor of this acid, apart from hydroxypyruvate, is L-glyceraldehyde, which is oxidized by aldehyde dehydrogenase (Holldorf, Holldorf, Schneider & Holzer, 1959). The equilibrium of the lactate-dehydrogenase reaction is strongly in favour of L-glycerate formation, but under physiological conditions the relatively higher concentration of the oxidized NAD (Glock & McLean, 1955) might favour L-glycerate oxidation. The hydroxypyruvate, once formed, could be removed by several reactions that would prevent its accumulation and therefore favour L-glycerate disappearance. Reactions contributing to the removal of hydroxypyruvate include transamination with L-alanine to L-serine, as shown for mammalian liver by Sallach (1956). A similar enzyme system from rat liver with L-glutamine as amino donor has been described by Meister, Fraser & Tice (1954) Hydroxypyruvate is also a substrate for transketolase reactions in which decarboxylation accompanies the formation of higher ketoses in the presence of suitable aldehyde acceptors (de la Haba, Leder & Racker, 1955; Dickens & Williamson, 1958*c*; Horecker, Smyrniotis & Klenow, 1953). Hydroxypyruvate is also both oxidized and decarboxylated by purified pigeon-breast-muscle pyruvate oxidase (Dickens, 1957; Jagannathan & Schweet, 1952; cf. Hedrick & Sallach, 1961). L-Glycerate is also attacked by L-amino acid oxidase

from rat kidney and liver (Blanchard, Green, Nocito-Carroll & Ratner 1946), but the reaction is very slow, with a turnover number of 6, which is far below the normal range for other flavoproteins. However, L-amino acid oxidase could be responsible for at least part of the conversion of L-glycerate into hydroxypyruvate in rat liver.

The demonstration of the presence in rat liver of D-glycerate dehydrogenase catalysing the reversible interconversion of hydroxypyruvate and D-glycerate completes the reaction sequence for the conversion of L-glycerate into glycogen. Lactate dehydrogenase coupled to D-glycerate dehydrogenase could act as a racemase system for the conversion of L-glycerate into the D-isomer, with NAD acting as the shuttling coenzyme. Kinases catalysing the phosphorylation of D-glycerate by ATP in liver have been described by Holzer & Holldorf (1957*a*) and by Ichihara & Greenberg (1957*b*), who considered the product to be 3-phosphoglycerate. However, there is considerable doubt whether the 3-phosphate is the primary product in this reaction, and Lamprecht, Diamanstein, Heinz & Balde (1959) have stated that it is in fact 2-phosphoglycerate, from which the 3-phosphate is derived by the action of phosphoglycerate mutase contaminating these kinase preparations. The previous chromatographic methods failed to distinguish the two phosphate esters, but, with a paper-chromatographic technique introduced by Cowgill (1955), Lamprecht, Heinz & Diamanstein (1962) have confirmed that 2-phosphoglycerate is the initial product in the phosphorylation of D-glycerate by their mutase-free kinase preparations from liver mitochondria. Once D-glycerate is phosphorylated, it is very easy to understand its conversion into hexose and glycogen via a reversal of reactions of the well-known Embden–Meyerhof glycolytic pathway.

Under the conditions used by Dickens & Williamson (1960) in their rat-liver-slice experiments, a maximum of 13% of 5–10 $\mu$moles of L-glycerate contained in 10 ml. of incubation medium was converted into glucose in 90 min. by 1g. of liver. According to our results, there appears to be amply sufficient L-lactate-dehydrogenase activity in rat liver to account for the conversion of this amount of L-glycerate into hydroxypyruvate. D-Glycerate-dehydrogenase activity cannot be measured by following the reduction of hydroxypyruvate by either NADH or NADPH in rat-liver supernatants, owing to the relatively high concentration of L-lactate dehydrogenase, but after removal of this enzyme it was possible to demonstrate a reasonably active D-glycerate dehydrogenase.

The $K_m$ for hydroxypyruvate for the rat-liver L-lactate dehydrogenase in the presence of NADH was 0·4 mM and in the presence of NADPH was 1·0 mM at pH 6. Comparable values for hydroxypyruvate with D-glycerate dehydrogenase at pH 6 are approx. 0·07 mM with either reduced coenzyme, i.e. 6–14-fold lower than that for the lactate dehydrogenase. At low concentrations of hydroxypyruvate, which are those existing in the tissues (Holzer & Holldorf, 1957*b*), D-glycerate dehydrogenase will therefore have a considerably higher affinity for this substrate than will lactate dehydrogenase, and this relationship may help to counteract the effect of the considerably higher concentration of the latter enzyme.

D-Glycerate dehydrogenase may well play a role in the synthesis of serine from carbohydrate (Sallach, 1955, 1956), but since 3-phosphoglycerate is not oxidized by this enzyme the latter cannot be involved in the pathway via the phosphorylated intermediates, as proposed by Ichihara & Greenberg (1957*a*). The oxidation of 3-phosphoglycerate by NAD in the presence of rat-liver supernatants was extremely low, in agreement with the results of Willis & Sallach (1964), who have purified an NAD-dependent 3-phosphoglycerate dehydrogenase from chicken liver and found the enzyme to be virtually absent from rat liver. With this fact in mind Willis & Sallach (1964) have criticized and re-interpreted the results of Ichihara & Greenberg (1957) for the incorporation of radioactivity from DL-[3-$^{14}$C]-glycerate into phosphohydroxypyruvate. Since D-glycerate dehydrogenase works equally well with either NAD or NADP in both directions, one of its functions might well be that of transhydrogenase catalysing the transfer of reducing units from NADPH to NAD with hydroxypyruvate and D-glycerate acting in catalytic amounts. Both these substances have been reported to be present in animal tissues (Holzer & Holldorf, 1957*b*; Kattermann, Dold & Holzer, 1961). Another function of the soluble D-glycerate dehydrogenase might be to act in conjunction with the mitochondrial D-$\alpha$-hydroxy acid dehydrogenase as a shuttle system for the transfer of reducing units from the cytoplasm to the respiratory chain with the regeneration of oxidized NAD. In rat liver, however, the activity of this mitochondrial enzyme is so low as to make this improbable.

Although the D-$\alpha$-hydroxy acid dehydrogenase from rabbit kidney is that which has been mainly studied, the enzyme is also present, though in low activity, in rat liver (Tubbs & Greville, 1961), and it may play a part in the biosynthesis of L-serine from carbohydrate sources. The rabbit-kidney enzyme appears to be distinct from the optically non-specific 2-hydroxy acid dehydrogenase prepared from ox liver by Schäfer & Lamprecht (1961), since no activity was detected with glycollate, L-lactate or L-glycerate. The flavoprotein-linked D-$\alpha$-hydroxy acid dehydrogenase of yeast reduces pyruvate to

D-lactate (Boeri, Cremona & Singer, 1960; Labeyrie, Naslin, Curdel & Wurmser, 1960). The reversibility of the reaction catalysed by the similar animal enzyme has not been demonstrated, however, but if it were capable of reducing hydroxypyruvate to D-glycerate it could also be involved in gluconeogenesis from L-glycerate and hydroxypyruvate.

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