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The Oxidation of D- α -Hydroxy Acids in Animal Tissues

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It was realized by 1913 that the lactate formed by mammalian tissues from various hexoses is entirely of L-configuration (Levene & Meyer, 1913). Dakin & Dudley (1913a) and Neuberger (1913), however, showed that animal tissues form lactate from methylglyoxal, which, until the work of Lohmann and others in 1931-33 (e.g. Meyerhof, 1933), was thereupon believed to be an intermediate in glycolysis. That methylglyoxal gives rise to the 'unnatural' D-lactate caused surprisingly few misgivings. This reaction is now thought to be part of an amino ketone cycle (see Discussion).

It has been almost universal practice to use DL-lactate in experiments with animal tissues, the D-isomer being ignored on the assumption that it is metabolically inert, despite various reports to the contrary (e.g. Dakin & Dudley, 1913b; Abramson & Eggleton, 1927; Cori & Cori, 1929; Brin, Olson & Stare, 1952).

Huennekens, Mahler & Nordmann (1951a, b) reported that fresh, crude mitochondrial suspensions from rabbit liver and kidney could oxidize D-lactate, but that the L-enantiomorph was not oxidized unless the particles were supplemented with a 'factor', supposed to be a lactate racemase, from the soluble cytoplasm. Mahler, Tomisek & Huennekens (1953) continued this work, using both manometric and dye reduction (Thunberg tube) techniques; no evidence was obtained for the involvement of coenzymes in the formation of pyruvate from D-lactate, and attempts to obtain D-lactate oxidation in non-particulate systems

were unsuccessful. Baker (1952) and Winitz *et al.* (1956) reported oxidation of D- α -hydroxy acids by rat-tissue preparations; again no coenzyme requirement was found. With kidney only the mitochondrial fraction was effective, and the slight activity in the soluble fraction of liver homogenates was perhaps due to glycollate oxidase (Kun, Dechary & Pitot, 1954), an enzyme virtually absent from kidney.

The present work has extended these various findings. Mitochondrial D- α -hydroxy acid dehydrogenase has been obtained in soluble form and partially purified. This communication is mainly devoted to the properties of this enzyme, which, it appears, should be added to the short list of mitochondrial 'cytochrome-reducing dehydrogenases' (Singer & Kearney, 1960).

Preliminary reports have appeared (Tubbs & Greville, 1959; Tubbs, 1960a), a detailed account has been given elsewhere (Tubbs, 1960b), and effects of metal-complex-forming agents on the dehydrogenase have been described (Tubbs, 1960c).

MATERIALS AND METHODS

Abbreviation. DEAE, Diethylaminoethylcellulose.

Water redistilled in Pyrex apparatus was used, and, when possible, AnalaR reagents were used. Solutions of substrates and labile compounds were stored at -10° unless prepared immediately before use. KCN solutions were neutralized with HCl.

Buffers. Tris buffers were prepared from the 'Sigma 121' product (Sigma Chemical Co., St Louis, Mo., U.S.A.) and HCl. Glycylglycine (Roche Products Ltd.) and KH_2PO_4 were brought to the required pH with KOH, as was $\text{Na}_4\text{P}_2\text{O}_7$ with HCl. Measurements of pH were made at room temperature with a Pye glass electrode and meter.

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Carboxylic acids. Calcium D- and L-lactate (California Corp. for Biochemical Research, Los Angeles 63, Calif., U.S.A.) were passed through columns of Amberlite IR-120 or Zeo-Karb 225 (used in the hydrogen form after previous washing with NaOH), and the resulting acids neutralized with KOH. DL- α -Hydroxy-*n*-butyric acid (L. Light and Co. Ltd.) was converted with BaCO₃ into the barium salt, which was recrystallized twice from aqueous ethanol and then converted into the potassium salt by the procedure used for lactate. The D-isomers of α -hydroxy-*n*-pentanoic, α -hydroxy-*n*-hexanoic and α -hydroxy- γ -methylpentanoic acids, and both diastereoisomers of D- α -hydroxy- β -methyl-*n*-pentanoic acid, were obtained, as sodium salts, through the kindness of Dr C. G. Baker, National Cancer Institute, Bethesda, Md., U.S.A.; they had been prepared in the laboratory of the late Dr J. P. Greenstein (see Winitz *et al.* 1956). DL- α -Hydroxy-*n*-octanoic acid was prepared from DL- α -amino-*n*-octanoic acid (Kodak Ltd.) (Winitz *et al.* 1956); the sodium salt was recrystallized twice from acetone. Barium DL- and L-glycerate were kindly given by Professor F. Dickens, F.R.S.; the barium salts were converted into potassium salts by a slight excess of K₂SO₄ or by the procedure described for lactate. D-Malic and D-tartaric acid (California Corp. for Biochemical Research) and glycollic acid (Fluka A. G., Buchs, Switzerland) were neutralized with KOH. Tests with purified L-malate dehydrogenase, kindly carried out by Mr C. J. R. Thorne, showed that the malic acid was free from the L-enantiomorph. Barium D-3-phosphoglycerate (C. F. Boehringer und Soehne GmbH., Mannheim, Germany) was dissolved in dil. HCl and the potassium salt prepared as described for lactate. Lithium pyruvate was prepared from freshly distilled pyruvic acid [b.p. 71–73° (19 mm. Hg)] (Wendel, 1932). Sodium D- β -hydroxybutyrate had been made by Lehninger & Greville (1953).

Electron acceptors. 2:6-Dichlorophenol-indophenol (British Drug Houses Ltd.) was purified by the method of Savage (1957); the crystals were stored *in vacuo* in the dark, and stock solutions (0.57 mM in 10 mM-tris, pH 7.8) were kept at 4° for not more than 2 weeks. Methylene blue was a 'zinc-free' product (Merck, Germany). 5-Methylphenazinium methosulphate (phenazine methosulphate) was synthesized, with the kind help of Dr R. Hill, F.R.S., by the method of Singer & Kearney (1957) except that xylene was used as solvent; aqueous solutions (1%, w/v) were stored at -10° in darkness. Pyocyanine was prepared from phenazine methosulphate (McIlwain, 1937). Cytochrome c from horse heart (Keilin & Hartree, 1945) was further purified by the method of Margoliash (1954); solutions (0.5 mM) of the oxidized form were prepared by aeration at pH 2.5 and stored frozen at pH 7.8. FMN and FAD were obtained from Sigma Chemical Co., and NAD was obtained from Pabst Laboratories, Milwaukee, Wis., U.S.A.

Sources of other materials. Sodium 5-ethyl-5-isoamylbarbiturate (amytal) (Eli Lilly and Co. Ltd.), Mepacrine hydrochloride (Imperial Chemical Industries Ltd.), ATP (Sigma Chemical Co.) and DEAE (0.59 m-equiv./g. dry wt.) (California Corp. for Biochemical Research). Hexokinase (stage V; Berger, Slein, Colowick & Cori, 1946) was kindly given by Miss R. M. Carpenter.

Protein determination. Protein concentration was determined in soluble preparations by the method of Warburg & Christian (1941). It was expressed as mg./ml. by multi-

plying $E_{280\text{ m}\mu}$ by a factor (Layne, 1957) obtained from the ratio $E_{280\text{ m}\mu}/E_{260\text{ m}\mu}$. This ratio was never less than 0.85.

Mitochondrial respiration. This was measured in the Warburg apparatus, with air as the gas phase and KOH in the centre well.

Assay of D- α -hydroxy acid dehydrogenase. This was performed in a Beckman model DU spectrophotometer. Water from a thermostat was circulated through 'thermospacers' on either side of the cell compartment and kept the latter at 25°. In the standard assay the following were placed in a cell (light-path 1 cm.; final volume 3 ml.; final concentrations given): tris buffer, pH 8.5, 41.5 mM; D-lactate, 25 mM; 2:6-dichlorophenol-indophenol, 38 μ M. Enzyme (0.01–0.3 ml.; 0.05–3 mg. of protein) was added and $E_{600\text{ m}\mu}$ measured at 30 sec. intervals for 5 min. The mixture was brought to 25° before addition of enzyme and (when used) cyanide.

The unit is defined as that amount of enzyme which causes a decrease in $E_{600\text{ m}\mu}$ of 0.01/min. after subtraction of the rate in the absence of added substrate; with dialysed and purified preparations this blank was negligible. Since the activity of freshly prepared extracts is greatly stimulated by low concentrations of cyanide (see below), the effect of including 0.5 mM-cyanide in the system should always be tested.

Preparation of mitochondria. Kidneys removed from rabbits immediately after death were put in ice-cold 0.25 M-sucrose. The preparation was carried out in a room at 4° and materials were kept close to 0°. After removal of capsule, medulla and fibrous tissue, the cortex was rinsed with 0.25 M-sucrose and finely cut with scissors. It was homogenized in 9 vol. of 0.25 M-sucrose by eight to ten strokes in a Potter-Elvehjem homogenizer (internal diameter of tube 25.0 mm.), the Perspex plunger (diameter 24.5 mm.) being rotated at low speed by a geared motor. Fractionation was carried out in 50-ml. tubes in swing-out buckets in a Major refrigerated centrifuge (Measuring and Scientific Equipment Co. Ltd.). The homogenate was centrifuged for 9 min. at about 350g. The mitochondria were separated from the supernatant by a total of approx. 41 000g_{av.}-min. (de Duve & Berthet, 1953), the centrifuge being set to run at 2600g_{av.} for 15 min. They were then washed twice with 0.125 M-KCl, being sedimented at 41 000g_{av.}-min. as before, and suspended in a volume of 0.25 M-sucrose equal to that of the cortex used. This procedure was also applied to kidneys of other species, but with liver the homogenizer pestle was not mechanically rotated and the mitochondria were washed with 0.25 M-sucrose.

When the mitochondria were required for the preparation of acetone-dried powder the second washing was omitted and the pellet was resuspended in a minimal quantity (about one-third of the cortex volume) of 0.25 M-sucrose.

Preparation of soluble D- α -hydroxy acid dehydrogenase. Acetone-dried powders were obtained by slowly adding mitochondrial suspensions to 15 vol. of continuously stirred acetone at -10°. After 2–3 min. the acetone was removed by centrifuging at -10° (250g; 3 min.) and decanting, and the powder washed twice on the centrifuge with acetone at -10°. It was dried in a desiccator (0.05 mm. Hg) containing P₂O₅, KOH and paraffin-wax shavings. The yield was about 2 g. from 100 g. of fresh kidney cortex and about 4.5 g. from 100 g. of liver. Stored in a desiccator at 4°, the powders gave fully active extracts even after many weeks.

The dried mitochondria were extracted with 20 mM-tris, pH 7.8 (1 ml./30–50 mg.), for 60–90 min. at 0°; the mixture was continuously stirred and care taken to break up lumps. The clear yellow supernatant after centrifuging at 8000g for 30 min. contained the enzyme.

Fractionation with $(\text{NH}_4)_2\text{SO}_4$ was carried out at 4°. Solid $(\text{NH}_4)_2\text{SO}_4$ was added to 32% saturation (Dixon, 1953). After stirring for 30 min. and centrifuging (3000g; 20 min.), the precipitate was discarded, and the supernatant brought to 42% saturation with $(\text{NH}_4)_2\text{SO}_4$ and stirred and centrifuged as before. The precipitate was dissolved in 20 mM-tris, pH 7.8. The resulting clear solution contained most of the dehydrogenase, but usually the material precipitated at 50% saturation with $(\text{NH}_4)_2\text{SO}_4$ was added and the combined solution dialysed overnight against the above tris buffer. Most experiments have been performed with enzyme purified to this stage.

Further purification has been achieved by chromatography on columns of DEAE at 4°. The DEAE was equilibrated with 20 mM-tris, pH 7.8, protein (125 mg./g. dry wt. of DEAE) dissolved in the same buffer passed into the column, and an increasing KCl gradient applied (Sober & Peterson, 1958). The enzyme emerged from the column when the KCl concentration in the eluate was 0.12–0.15M. To concentrate fractions containing the enzyme, $(\text{NH}_4)_2\text{SO}_4$ was added to 60% saturation, the precipitate dissolved in a small quantity of the tris buffer, and the solution dialysed at 4° against the same buffer.

RESULTS

Oxidation of D-lactate by particulate preparations

Complete stereospecificity of liver and kidney particles for D-lactate, as reported by Huennekens *et al.* (1951a, b) and Mahler *et al.* (1953) was never observed; however, the D-enantiomorph was oxidized much more rapidly than the L. Typical experiments with rabbit-kidney and rat-liver particles are shown in Fig. 1. In other experiments citrate (0.33 mM) replaced fumarate as 'sparker'. It is not possible to compare the present results exactly with those of the authors mentioned above, since all oxygen uptakes reported by them were net values, the blank values, of unstated magnitude, having been subtracted.

In agreement with Huennekens *et al.* (1951b), pigeon-breast-muscle mitochondria, which were isolated and twice washed in 0.25M-sucrose or 0.125M-potassium chloride, both containing either 1 mM-EDTA, pH 7.4, or 1 mM-tris, pH 7.4, showed absolute stereospecificity for L-lactate.

Attempts to confirm the existence of the 'lactate racemase' of Huennekens *et al.* (1951a, b) were unsuccessful. Since liver and kidney particles had been found not to be absolutely specific for D-lactate, pigeon-breast-muscle mitochondria were used. Addition of 'racemase' preparations, made from rabbit-kidney cortex by the method of Huennekens *et al.* (1951b) did not enable D-lactate to act as a substrate (Table 1). Oxidation of

Fig. 1. *A*, Respiration of rabbit-kidney particles. Each vessel contained: phosphate, pH 7.5, 13 mM; MgCl_2 , 3.6 mM; ATP, 1 mM; KCl, 42 mM; particles from 0.9 g. of cortex. Final vol., 3 ml. Where added, fumarate 1 mM and lactates 3.3 mM. Temp., 30°; equilibration, 6 min. The particles were prepared and twice washed in 0.125M-KCl; N-NaOH was added during homogenization to maintain neutrality (cf. Huennekens *et al.* 1951a). *B*, Respiration of rat-liver mitochondria. Experimental conditions as in *A*, except that phosphate was 25 mM, lactates, where added, 13 mM, and temp. 38°. Each vessel contained mitochondria from 0.33 g. of tissue. In *A* and *B*: O, no added substrate; Δ, L-lactate; □, D-lactate; ●, fumarate; ▲, fumarate + L-lactate; ■, fumarate + D-lactate.

Table 1. *Respiration of pigeon-breast-muscle mitochondria: failure of rabbit-kidney preparation to stimulate D-lactate oxidation*

Each vessel contained: phosphate, pH 7.5, 13 mM; MgCl_2 , 3.3 mM; KCl, 110 mM; ATP, 1 mM; NAD, 0.15 mM; cytochrome *c*, 25 μM; fumarate, 0.33 mM; mitochondria from 1 g. of muscle. Lactates, when added, 14 mM. Final vol., 3 ml.; temp., 30°. Preparation from rabbit-kidney cortex was made by the procedure of Huennekens, Mahler & Nordmann (1951b); 0.5 ml. was added/vessel. Table shows O₂ uptake (μl.) in 30 min.

	Rabbit-kidney preparation	
	Heated*	Unheated
D-Lactate	16	37
L-Lactate	18	35
	96	270

* 10 min. at 100°.

Table 2. *Reduction of 2:6-dichlorophenol-indophenol by mitochondria in the presence of D-lactate*

Each Thunberg tube contained: mitochondria from 100 mg. of rabbit-kidney cortex; phosphate, pH 7.5, 17 mM; dye, 50 μ M; KCl, 10 mM. Final vol., 3 ml.; temp., 37°. Reaction was started by the addition of dye and substrate from hollow stopper after 3 min. thermal equilibration. Air pressure was lowered to 0.3 atm. before incubation to avoid leakage due to increase in pressure on warming.

Additions: cyanide (mM)	1.7	1.7	33	33	1.7	1.7
D-Lactate (mM)	17	—	17	—	17	—
EDTA (mM)	—	—	—	—	1.0	1.0
Reduction time (min.)	3.3	> 80	42	> 80	60	> 80

Table 3. *Inhibition of mitochondrial respiration by amytal*

Each vessel contained: phosphate, pH 7.5, 33 mM; tris, pH 7.6, 41 mM; MgCl₂, 7 mM; sucrose, 80 mM; substrates or KCl, 15 mM; glucose, 20 mM; ATP, 2 mM; excess of yeast hexokinase; mitochondria from 0.4 g. of rat liver. Sodium amytal or NaOH (final concn., 2 mM) added after 6 min. equilibration. Final vol., 3 ml.; temp., 30°. Rates of O₂ uptake were constant for 50 min. Rates in the absence of added substrate have been subtracted from the other rates.

Added substrate	O ₂ uptake (μ l./hr.)		Inhibition (%)
	NaOH	Amytal	
Succinate	420	438	-4
Pyruvate	93	2	98
D-Lactate	24	12	50
None	15	1	94

L-lactate, however, was enhanced by the addition of the kidney preparation. This was probably due to L-lactate dehydrogenase contained in it; Dr J. B. Chappell (personal communication) has found that addition of purified L-lactate dehydrogenase greatly stimulates the oxidation of L-lactate by pigeon-breast-muscle mitochondria prepared in potassium chloride-tris medium; with those prepared in sucrose-EDTA medium, however, addition of the dehydrogenase is not needed, and this has been confirmed in the present work.

Attempts were also made to prepare the 'racemase' from rat and rabbit liver. Despite variations in the method of preparation of 'racemase' and particles and in the conditions of the test, respiration in the presence of D-lactate and 'racemase' was never more than 10% greater than that in the presence of 'racemase' and absence of lactate, and always greatly less than that with L-lactate. The slight enhancement of activity in the presence of D-lactate was probably due to the glycollate oxidase (Kun *et al.* 1954) found to be present in the 'racemase' preparations. It is concluded that the existence of a mammalian α -hydroxy acid racemase must be regarded as doubtful.

The dye reduction (Thunberg tube) experiments of Mahler *et al.* (1953) have been repeated and extended. In agreement with these authors, NAD (0.15 mM) failed to stimulate indophenol reduction

by rabbit-kidney mitochondria with D-lactate as substrate, whereas with L-lactate NAD was strongly stimulatory, especially after prolonged storage (up to 99 hr.) of the mitochondria at 0°. Metal-complex-forming agents inhibited the dye reduction (Table 2). Mahler *et al.* (1953) used 33 mM-cyanide in experiments similar to that in Table 2; that the strong inhibitory effect was not noticed was presumably due to the removal of volatile hydrocyanic acid by the unnecessarily efficient evacuation procedure.

Support for the hypothesis that NAD is not involved in the oxidation of D-lactate came from experiments on barbiturate inhibition. Jowett & Quastel (1937) found that tissue-slice respiration was inhibited by 5-ethyl-5-phenylbarbiturate (Luminal) when the substrate was glucose, L-lactate, pyruvate or L-glutamate, whereas with succinate oxygen uptake was actually enhanced. Ernster, Jalling, Löw & Lindberg (1955) found amytal to be the most effective compound, and concluded that only the oxidation of intramitochondrial reduced NAD, and of substrates generating this, was inhibited. Table 3 shows the effect of amytal on the oxidation of succinate, pyruvate and D-lactate by rat-liver mitochondria; similar results were obtained with rabbit-kidney mitochondria with which the D-lactate oxidation was more rapid. The apparent partial inhibition of D-lactate oxidation is compatible with complete amytal insensitivity for the step D-lactate $\rightarrow$ pyruvate, since the further oxidation of pyruvate presumably contributed to the oxygen uptake observed with D-lactate in absence of amytal.

The experiments with particulate preparations described above suggested that (a) the initial oxidation of D-lactate does not involve NAD, and (b) a metal is involved in this process. These conclusions are strongly supported by findings reported below and elsewhere (Tubbs, 1960c).

Experiments with soluble D- α -hydroxy acid dehydrogenase

Purification. Typical results of the purification procedure as described under Materials and Methods are summarized in Table 4. Extensive further purification could no doubt be achieved; the

Table 4. *Purification of enzyme from 283 g. of rabbit-kidney cortex*

For description of purification procedure and definition of unit of activity see Materials and Methods. Standard assay with 0.5 mM-cyanide.

Stage	Volume (ml.)	$\frac{E_{280 \text{ m}\mu}}{E_{280 \text{ m}\mu}}$	Protein (mg.)	Units	Units/mg.	Purification factor	Yield (%)
(1) Extract	126	0.86	690	2020	2.9	[1]	[100]
(2) Combined $(\text{NH}_4)_2\text{SO}_4$ ppts.	74.5	1.16	242	1790	7.4	2.5	89
(3) DEAE eluate (concentrated)	6.7	1.58	39.5	1610	40.8	13.9	80

Fig. 2. Dependence of initial velocity on enzyme concentration. Enzyme (stage 2, Table 4) fully activated by storage for 2 days at 4°; the solution contained 4.3 mg. of protein/ml. Standard assay, without cyanide (see Materials and Methods).

Fig. 3. Dependence of initial velocity on substrate concentration. Enzyme (stage 2, Table 4) fully activated by storage for 4 days at 4°. Standard assay, except that pH was 7.9 and substrate was varied; cyanide absent. Unit of velocity, $-E_{600 \text{ m}\mu}/\text{min.}$ O, D-Lactate; Δ , D- α -hydroxy-n-pentanoate; $\square$, D- α -hydroxy-n-hexanoate.

Fig. 4. *A, B*, Increase in activity of extract of dried mitochondria in 10 mM-tris, pH 7.8, during storage at 4°. Standard assay, except that pH was 8.1; cyanide absent; 2.8 mg. of protein/assay. *A* (●), Extract dialysed for 4 hr. against the above buffer immediately after preparation; *B* (○), extract not dialysed. *C*, Effect of cyanide in assay of a different extract from that in *A, B*. The extract (2.2 mg. of protein/assay) was tested at pH 8.1 1 hr. after preparation. The horizontal line shows the activity, without cyanide, after storage at pH 7.8 and 4° for 5 days.

Fig. 5. Double-reciprocal plots showing that the increase in activity of the enzyme on storage was not due to change in K_m . Standard assay, except for variation in substrate concentration; 0.87 mg. of protein (stage 2, Table 4)/assay. Unit of velocity, $-E_{600 \text{ m}\mu}/\text{min.}$ O, 2 Days' storage at pH 7.8, 4°; ●, 7 days' storage.

limiting factor has been the small quantity of rabbit kidney available, for other tissues did not yield sufficiently active extracts by the method described.

Effect of enzyme and substrate concentrations. Progress curves were linear until about 50% of the indophenol had been reduced. The initial rate was proportional to the amount of added enzyme between 1 and 10 units (Fig. 2). The dependence of the initial velocity on substrate concentration is shown by double-reciprocal plots in Fig. 3. The Michaelis constant (K_m) for D-lactate has varied between 0.9 and 3.3 mM, but has usually been between 1 and 2 mM. No inhibition has been observed with excess of substrate up to at least 50 mM.

Activation on storage, or in the presence of cyanide. In preliminary work considerable difficulty was found in correlating results of experiments with the same extract on different days. This was because the enzyme undergoes 'spontaneous' activation. Fig. 4 (curves A, B) shows the rapid increase in enzyme activity which occurs on storing the crude extract at 4°; this increase is not due to a change in the affinity of the enzyme for the substrate (Fig. 5). Dialysis and partial purification of the enzyme, even through the DEAE stage, do not affect the activation process. No such 'spontaneous' activation in the cold seems to have been reported with other soluble oxidative enzymes, although superficially similar effects are shown by some hydrolytic enzymes (Gjessing, Emery & Clements, 1959).

Inclusion of dilute cyanide in the assay system resulted in immediate appearance of full activity (Fig. 4, curve C, and Fig. 6). EDTA, *o*-phenanthroline, oxalate, thiocyanate or azide did not activate; this renders unlikely the most obvious explanation for this effect of cyanide, namely the

forming of complexes with contaminating metal ions. It may well be that the cyanide activation is related to the other effects of this compound on the enzyme (Tubbs, 1960c). Preincubation with substrate, a procedure effective with succinate dehydrogenase (Kearney, 1957), did not result in activation.

Substrate specificity. Table 5 shows the values of K_m and of velocity at infinite substrate concentration (V_{max}) for various D- α -hydroxy acids, derived from double-reciprocal plots of results obtained in the indophenol assay. That the same enzyme was responsible for the oxidation of all these substrates was checked by mixed-substrate experiments, i.e. rates were determined with each substrate and D-lactate separately and also with equimolar mixtures of D-lactate with each other substrate. Insertion of the results into equation IV.69 of Dixon & Webb (1958) showed that only one dehydrogenase was involved, the calculated and observed velocities of the mixtures never differing by more than 12% and in most cases by much less.

The following hydroxy acids were not substrates: glycollate, L-lactate, D- α -hydroxy- β -methyl-*n*-pentanoate (both diastereoisomers), D- α -hydroxy- γ -methylpentanoate, L-tartrate, DL-mandelate, α -hydroxyisobutyrate, D- β -hydroxybutyrate and 3-phospho-D-glycerate. The limited evidence of these findings and those in Table 5 suggests that the structural features of a substrate include: (i) an α -hydroxy group and α -hydrogen, and D-configuration at the α -carbon atom; (ii) no branching at the β -carbon atom, although hydroxyl and carboxyl groups are permitted here; (iii) a side chain other than hydrogen, since glycollate is not a substrate.

Table 5 shows that K_m increases with chain length in the lactate homologues, and that V_{max} also varies with chain length. Other cases of

Fig. 6. Effect of cyanide in assay before and after storage of enzyme (stage 2, Table 4) at pH 7.9, 4°. Standard assay except for variation in pH; 0.65 mg. of protein/assay. O, pH 8.6; ●, pH 7.6; —, enzyme stored 1 day; ---, stored 8 days. (This preparation was unusual in that the activity under optimum conditions after 8 days' storage was less than that after 1 day.)

Table 5. Substrate specificity of D- α -hydroxy acid dehydrogenase

Standard assay except for D-malate and tartrates, when the buffer was phosphate, pH 6.6; with these substrates V_{max} is compared with that for D-lactate at pH 8.6, after allowance for the difference in the extinction of the indophenol at the two pH values.

	K_m (mM)	V_{max} (D-Lactate = 100)
D-Lactate	0.9-1.8	[100]
DL- α -Hydroxy- <i>n</i> -butyrate	2.9*	150
D- α -Hydroxy- <i>n</i> -pentanoate	2.9	29
D- α -Hydroxy- <i>n</i> -hexanoate	11.1	73
DL- α -Hydroxy- <i>n</i> -octanoate	12.5*	225
DL-Glycerate	10.5*	142
D-Malate	1.0	70
D-Tartrate	—	2-3
meso-Tartrate	0.6	37

* Concn. of D-isomer.

variation of $V_{\max}$ with chain length have been discussed by Lumry (1959). Fig. 3 shows that the slopes of the double-reciprocal plots for these substrates, representing the ratio $K_m/V_{\max}$, increase with chain length, and Fig. 7 shows that this ratio is in fact directly proportional to the number of carbon atoms in the substrate. The interpretation of this curious finding is not at present clear.

Contrary to an earlier report (Tubbs & Greville, 1959), it has now been found that L-lactate is a weak competitive inhibitor of D-lactate oxidation, K_i being about four times the K_m for D-lactate. Assuming, as seems reasonable, that the L-enantiomorphs of other substrates also competitively inhibit, the K_m and $V_{\max}$ values shown in Table 5 will be too low in cases where the DL-compound was used. $K_m/V_{\max}$ (Fig. 7) will, however, be correct, as may be shown from the usual competitive-inhibition equation (Tubbs, 1961).

The one sample of D-glycerate tested gave a lower $V_{\max}$ than that with DL-glycerate, although K_m was the same with both. The reason for this is not known. A competitive inhibitor contaminating the D-glycerate would have decreased K_m as well as $V_{\max}$; a non-competitive inhibitor would have caused a non-linear double-reciprocal plot, and this was not observed.

The observation (Table 5) that mesotartarate is oxidized far more rapidly than D-tartrate, although the latter offers twice as many centres with the 'correct' configuration, may imply that the group not bound at the catalytic site causes inhibition when it has the D-configuration.

Influence of pH and buffer composition on reaction rate. Fig. 8 shows effects of different buffers on the rate of reduction of the indophenol by D-lactate and D-malate. The second negative charge on the latter substrate lowers the pH optimum by over

2 units. Pyrophosphate inhibits somewhat, perhaps by virtue of its chelating ability. A few measurements have been made of the variation of K_m and $V_{\max}$ with pH; K_m appears to increase, and $V_{\max}$ to decrease, on the acid side of the pH optimum (Fig. 9). Several factors influence the pH-activity curve when cyanide is present in the assay system [Fig. 6 and also Tubbs (1960c)]: (i) the activation of fresh enzyme by cyanide is more marked at the higher pH values; (ii) cyanide ion inhibits competitively, so inhibition by a given concentration of cyanide increases with pH, due to increasing dissociation; (iii) 'spontaneous' activation of the enzyme does not alter the K_m for substrate or the inhibition by cyanide, whereas it

Fig. 8. Variation of enzyme activity with pH. Buffers 33 mM, substrates 25 mM. $\circ$, Tris; $\square$, glycylglycine; Δ , pyrophosphate; ∇ , orthophosphate; —, D-lactate; ---, D-malate. Enzyme (stage 2, Table 4) fully activated by storage; 0.43 mg. of protein/assay; cyanide absent. Allowances were made for change in extinction of the indophenol below pH 7.

Fig. 7. Variation of $K_m/V_{\max}$ with chain length of α -hydroxy *n*-fatty acids. $V_{\max}$ for each substrate is expressed as the maximal velocity relative to that for D-lactate. In the first experiment ($\circ$) all substrates had D-configuration (assay at pH 7.9); in the second ($\bullet$), with a different enzyme preparation, the C_4 and C_5 acids were DL (assay at pH 8.6). Enzyme (stage 2, Table 4) fully activated by storage; cyanide absent.

Fig. 9. Variation of $V_{\max}$ and K_m with pH. Enzyme (stage 2, Table 4) fully activated by storage; 0.78 mg. of protein/assay. Standard assay, except for variation of pH and substrate concentration; cyanide absent. $V_{\max}$ ($\circ$) and K_m ($\bullet$) were obtained from double-reciprocal plots.

abolishes the acceleration by cyanide and so reduces the optimum concentration of this compound eventually to zero. The observed enzymic activity is thus dependent, in a complicated manner, on the 'age' of the enzyme, the pH, and the concentrations of substrate and of cyanide.

Reaction with various oxidants. In the presence of the dehydrogenase, the following substances have been found to be reduced by D-lactate: 2:6-dichlorophenol-indophenol, methylene blue, pyocyanine, phenazine methosulphate, cytochrome *c* and ferricyanide. There was no appreciable direct reaction with oxygen. No separation of indophenol- and cytochrome *c*-reducing activities was obtained by the partial purification of the enzyme, and, so far as tested, chelating agents were found to inhibit regardless of the acceptor employed.

The rate of reduction of the indophenol was little affected by change in dye concentration between 20 and 100 μ M, under otherwise standard assay conditions; the apparent K_m for this dye is about 10 μ M. Nevertheless, the indophenol assay does not measure the full activity of the enzyme, for the rate of dye reduction was increased by 35–40% when phenazine methosulphate (11 μ M) was present as a mediator. The corresponding increase in the rate of reduction of cytochrome *c* (50 μ M) was 440%. The optimum phenazine methosulphate concentration has not been determined, but with the above concentration the overall rate of dehydrogenation was the same whether the indophenol (38 μ M) or cytochrome *c* (50 μ M) was the final acceptor. NAD (0.15 mM) was not reduced by the enzyme and had no effect on the rate with the indophenol or cytochrome *c*; these observations were made with the salt-fractionated preparation.

The optimum pH has not yet been determined with acceptors other than the indophenol.

Stoichiometry of D-lactate oxidation. The oxidation of D-lactate by oxygen has been followed with methylene blue as carrier. The partially purified extract used contained catalase. Table 6 shows that

under these conditions the oxidation can be represented by the equations:

Overall:

The formation of pyruvate in a similar experiment to that in Table 6 has been confirmed by paper chromatography of the 2:4-dinitrophenylhydrazones, with butan-1-ol-aqueous ammonia as solvent (Cavallini, Frontali & Toschi, 1949). The enzymic reaction was stopped after 90 min., when 6.1 μ moles of oxygen had been taken up, by addition of trichloroacetic acid (to 3.3%, w/v), which precipitated nearly all the methylene blue. The main spot had R_f 0.35 (cf. Cavallini *et al.* 1949); a fainter spot at R_f 0.51 was due presumably to the second isomer of the dinitrophenylhydrazone (Stewart, 1953). Neither spot was obtained when either pyruvate or incubation was omitted.

In these experiments methylene blue was used in preference to phenazine methosulphate because the latter strongly inhibits catalase, and hydrogen peroxide decomposes pyruvate yielding acetate and carbon dioxide. With phenazine methosulphate it was therefore necessary to put alkali in the centre well of the manometer vessel before full oxygen uptake could be observed.

Possible flavoprotein nature of the enzyme. No stimulation of indophenol reduction by added FAD or FMN (10 μ M) has been observed, even after repeated precipitation of the dehydrogenase with ammonium sulphate or partial inactivation by ultraviolet irradiation. Indeed, preincubation for 45 min. at 0° with a high concentration (1 mM) of FMN caused inhibition (50%), perhaps due to chelation, from which D-lactate protected. Preincubation with Mepacrine (5 mM) caused no inhibition; the corresponding yeast enzyme is inhibited by such treatment (Baudras, Iwatsubo & Labeyrie, 1960), but such inhibition is unreliable evidence of involvement of flavin (see Hemker & Hülsmann, 1960).

The absorption spectrum of the purest preparation obtained, determined in a Beckman recording spectrophotometer, showed no appreciable change in the 450 m μ region on addition of D-lactate, although yeast D- α -hydroxy acid dehydrogenase shows a flavin difference spectrum on reduction (Boeri, Cremona & Singer, 1960; Nygaard, 1960*a*). However, addition of D-lactate appeared to lower slightly an absorption peak at 405 m μ , and to

Table 6. *Stoichiometry of oxidation of D-lactate by extract of rabbit-kidney mitochondria*

Each Warburg vessel contained, in a final vol. of 3 ml.: tris, pH 7.9, 42 mM; D-lactate, 25 mM; methylene blue, 2 mM; mitochondrial extract. Appropriate blanks and pyruvate standards were set up; these showed no O₂ uptake. After incubation for 77 min. at 30°, trichloroacetic acid was added and pyruvate determined by the method of Corwin (1959).

Enzyme (units)	O ₂ uptake (μ moles)	Pyruvate formed (μ moles)	Pyruvate/O ₂
15	2.20	4.7	2.14
30	3.95	8.5	2.15

increase the absorption below 380 $m\mu$ and between 425 and 350 $m\mu$; a similar difference spectrum has been reported on reduction of chicken-kidney hypoxanthine dehydrogenase (Landon & Carter, 1960).

Analogy with other enzymes makes it probable that the present dehydrogenase is a flavoprotein; confirmation, however, must await further purification.

Distribution of the dehydrogenase. The reduction of indophenol by D-lactate is catalysed by mitochondria, and extracts of acetone-dried mitochondria, from kidneys of the rat, rabbit, sheep, ox and pig, and from livers of the rat, rabbit and ox. Of the tissues tested only pigeon breast muscle showed no activity. Hellerman, Reiss, Parmar, Wein & Lasser (1960) recently reported slow oxidation of D-malate by pig-heart extracts. Soluble preparations with activity adequate for detailed studies have so far been obtained only from rabbit kidneys; the rat-liver enzyme, however, is similar with respect to pH optimum, cyanide activation and inhibition by chelating agents.

DISCUSSION

Dehydrogenases resembling the one described in this paper have been found in yeast (Labeyrie, Slonimski & Naslin, 1959; Nygaard, 1959*a, b*, 1960*a, b*; Boeri *et al.* 1960), *Escherichia coli* (Haugaard, 1959) and other bacteria (de Ley & Schel, 1959; Snoswell, 1959; de Ley & Dochy, 1960; Molinari & Lara, 1960). In yeast (Nygaard, 1960*a*), *Acetobacter* (de Ley & Dochy, 1960) and perhaps in other organisms studied, D-lactate dehydrogenase activity is attached to insoluble material; the enzyme in *E. coli* seems to be associated with the cytochrome-containing particles described by Asnis, Vely & Glick (1956). No detailed study has been made of the intracellular distribution of the mammalian enzyme; however, it has been found to be absent from the soluble fraction of homogenates. It thus appears that NAD-independent D-lactate dehydrogenases are widely distributed and characteristically particle-bound; NAD-dependent enzymes are known, however, from *Chlorella* (Warburg, Gewitz & Völker, 1957) and lactobacilli (see Dennis & Kaplan, 1960).

There are no reports that D-lactate is a normal constituent of animal tissues, but it accumulates in *Chlorella* under anaerobic conditions (Warburg *et al.* 1957) and is present in considerable amounts in yeast (Singer, Kearney, Gregolin, Boeri & Rippa, 1960).

The metabolic significance of D- α -hydroxy acid dehydrogenase has still to be determined, but the presence of such an enzyme in widely different

organisms suggests important functions. A few possibilities will be put forward, with special reference to the animal enzyme.

The putative succinate-glycine cycle (Shemin & Russell, 1953; Nemeth, Russell & Shemin, 1957) involves the conversion of δ -aminolaevulate into γ - δ -dioxovalerate, perhaps by transamination (Braunstein, 1960, p. 184). This keto-aldehyde is transformed into D- α -hydroxyglutarate by glyoxalases from various animal tissues (for references see Dalgleish, Johnson, Long & Tyler, 1952, p. 1114). It has not yet been possible to test the last-named substance with D- α -hydroxy acid dehydrogenase, but in view of the results with D-malate it is likely to be a substrate; if so, the enzyme could provide the link which allows completion of the cycle.

It has been suggested (Shemin, 1955; Gibson, Laver & Neuburger, 1958) that the reactions of the succinate-glycine cycle may be extended to form an acetate-glycine cycle; this has been formulated by Elliott (1959). Here, aminacetone, derived from glycine and acetyl-coenzyme A or from threonine (cf. Urata & Granick, 1961), is deaminated yielding methylglyoxal, which is converted into D-lactate by glyoxalase. The next step offers a role for D- α -hydroxy acid dehydrogenase, and oxidative decarboxylation of the pyruvate formed completes the cycle. As to whether this cycle takes place in animal tissues, it may be noted that aminoacetone is formed from acetyl-coenzyme A and glycine by particles from avian erythrocytes (Gibson *et al.* 1958) and from guinea-pig liver (Urata & Granick, 1961), and is converted into carbon dioxide in the rat (Nemeth *et al.* 1957) and to methylglyoxal by an enzyme from ox plasma (Elliott, 1960).

D-Glycerate was oxidized by the present dehydrogenase, and a positive naphthalene-1:3-diol test (Dickens & Williamson, 1958) indicated that the product was hydroxypyruvate. The enzyme may therefore be involved in the biosynthesis of L-serine, since one possible route for the formation of this amino acid from carbohydrate in animals is via D-glycerate and hydroxypyruvate, the latter yielding serine through the action of a reversible alanine-hydroxypyruvate transaminase (Sallach, 1955). In the opposite direction, hydroxypyruvate and D-glycerate are both converted into carbohydrate by animal tissues (Dickens & Williamson, 1959, 1960); hence if the action of D- α -hydroxy acid dehydrogenase on D-glycerate is reversible, the enzyme may also be involved in glyconeogenesis. It may be remarked that the yeast enzyme can reduce pyruvate to D-lactate (Labeyrie, Naslin, Curdel & Wurmser, 1960) and that the redox potential of the glycerate-hydroxypyruvate system is close to that of lactate-pyruvate (Zelitch, 1955).

Finally, the dehydrogenase may possibly play some part in the metabolism of the long-chain

D- α -hydroxy fatty acids found in cerebrosides (Skipski, Arfin & Rappoport, 1959; Kishimoto & Radin, 1959).

SUMMARY

1. Liver and kidney mitochondria oxidize D-lactate much faster than L-lactate, whereas pigeon-breast-muscle mitochondria oxidize only the L-isomer. This is in agreement with Huennekens *et al.* (1951*a, b*), but their finding that liver and kidney contain a 'racemase' enabling pigeon-muscle particles to oxidize D-lactate could not be confirmed.

2. Reduction of dyes by kidney mitochondria in the presence of D-lactate is inhibited by metal-complex-forming agents and not stimulated by nicotinamide-adenine dinucleotide.

3. A soluble D- α -hydroxy acid dehydrogenase has been extracted from rabbit-kidney-cortex mitochondria and partially purified.

4. The enzyme, which occurs in liver and kidney in several animal species, catalyses oxidation of lactate and straight-chain homologues, glycerate, malate and tartrate; it was specific for the D-isomer whenever this could be tested. The product of D-lactate oxidation is pyruvate.

5. With lactate and homologues, $K_m/V_{\max}$ is proportional to chain length.

6. Electron acceptors include various dyes, ferricyanide and cytochrome *c*, but not oxygen. Nicotinamide-adenine dinucleotide is not needed.

7. The enzyme when extracted has low activity, but undergoes activation during storage at 4°. Inclusion of cyanide in the assay system causes immediate full activation.

8. Possible metabolic roles of the enzyme are discussed.

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Thymidine Diphosphate Mannose and Thymidine Diphosphate Rhamnose in *Streptomyces griseus*

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Streptomyces griseus is grown in large amounts for the commercial production of streptomycin. Our interest in the mechanism of biosynthesis of the antibiotic led to a study of nucleotides in this organism, as it seemed possible that the carbohydrate residues in streptomycin, *N*-methyl-L-glucosamine and streptose, would occur in combination with nucleotides at some stage in the biosynthetic route.

Although nucleotides containing streptose or *N*-methyl-L-glucosamine have not yet been found in the complex mixture obtained from the micro-organism, several new nucleotides were detected. Two of these have been identified as thymidine diphosphate mannose and thymidine diphosphate rhamnose. The isolation and structure of these compounds is described, and their possible function as streptomycin precursors is discussed. A mechanism for the biosynthesis of rhamnose through

thymidine derivatives is suggested. A preliminary account of some of this work has been published (Baddiley & Blumsom, 1960).

MATERIALS AND METHODS

Abbreviations. TDP-mannose, thymidine diphosphate mannose; TDP-rhamnose, thymidine diphosphate rhamnose; TDP-glucose, thymidine diphosphate glucose.

Thymidine was purchased from the California Corp. for Biochemical Research, Los Angeles 63. Thymidine 5'-phosphate was kindly supplied by Dr A. M. Michelson. Uridine diphosphate glucose was obtained from the Nutritional Biochemicals Corp., Cleveland, Ohio. Guanosine diphosphate mannose and uridine diphosphate *N*-acetylglucosamine were kindly supplied by Dr E. Cabib. DL-Glyceric acid was purchased from L. Light and Co. Ltd., Colnbrook, Bucks. D-Glyceric acid was obtained by hydrolysis of its 3-phosphate with a prostate-phosphatase preparation. DPNH was purchased from C. F. Boehringer,