Assay, Purification and Properties of Mammalian D-2-Hydroxy Acid Dehydrogenase

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1. A new method is described for the measurement of D-2-hydroxy acid dehydrogenase in samples of animal tissues. 2. The distribution of the enzyme in a number of animals was determined. Of the animal tissues tested, the most active source of the enzyme was found to be rabbit kidney cortex. 3. The enzyme was purified from rabbit kidney to a stage at which it appears to be homogeneous in the analytical ultracentrifuge and on polyacrylamide-gel electrophoresis. 4. The molecular weight was estimated by gel filtration to be approx. 102 000; combination of gel-filtration data and the sedimentation coefficient gave a value of 95 000. 5. The purified enzyme has a spectrum typical of a flavoprotein. The change induced in the spectrum on addition of D-malate or D-lactate suggests the formation of a flavin semiquinone. 6. Flavin can be removed by treatment with acid ammonium sulphate, and activity can be restored to the inactive apoenzyme by addition of FAD, but not of FMN or riboflavin. 7. Studies of acceptor specificity showed that the enzyme has a relatively weak D-2-hydroxy acid oxidase activity.

D-2-Hydroxy acid dehydrogenase [D-2-hydroxy acid-(acceptor) oxidoreductase, EC 1.1.99.6] was first extracted in soluble form from rabbit kidney and from rat liver by P. K. Tubbs and G. D. Greville (Tubbs & Greville, 1959, 1961; Tubbs, 1965) during investigations of the oxidation of the 'unnatural' D(-)-isomer of lactate by these organs. The enzyme was shown to oxidize D-lactate and a number of longer-chain D-2-hydroxy acids, D-malate, D-glycerate and *meso*-tartrate, to the corresponding keto acids. A number of artificial acceptors were used, including phenazine methosulphate, DCIP,† ferrieyanide, methylene blue and cytochrome *c*. No activity was detected with NAD⁺, NADP⁺ or oxygen as acceptors, though a weak oxidase activity has now been detected.

Partly purified preparations of the enzyme have also been reported by Dawkins & Dickens (1965) and by Britten (1968). Only a limited degree of purification was achieved by these workers, partly because of the small quantities of the source, rabbit kidney, that were used; it appears from the present work that each kilogram of rabbit kidneys probably contains only 15–30 mg. of the enzyme, and much of this must be lost during purification. In addition it has been found that the preparation of acetone-dried powders, which they used to obtain the enzyme

in soluble form, destroys a large proportion of the enzyme.

A new procedure is reported in this paper for the preparation of highly purified D-2-hydroxy acid dehydrogenase, and some physical properties of the enzyme are described. A preliminary report of these results has been published (Cammack, 1968a).

The capacity of animal tissues to oxidize D-lactate is of considerable interest in connexion with metabolic studies, e.g. the D-lactate acidosis of ruminants (Dunlop & Hammond, 1965). Several attempts have been made to measure the activity (e.g. van Eys *et al.* 1962), but with somewhat variable results owing to a number of special difficulties that could not be overcome until the properties of D-2-hydroxy acid dehydrogenase were better understood. Chief among these is the 'spontaneous activation' that the enzyme undergoes on extraction. The activity of the enzyme increases by up to 20-fold after its extraction in a soluble form (Tubbs & Greville, 1959). This process has been shown to be strongly dependent on pH (Cammack, 1968b). Extracts of rabbit kidney appear to contain a labile 'inactivating factor', which at pH 8.0 and 0° is capable of maintaining the enzyme in the inactive form for several days. However, below pH 7.0 the activation effect is much more rapid and takes place in a few minutes. D-2-Hydroxy acid dehydrogenase appears to be almost entirely responsible for the direct oxidation of D-lactate in most tissues, since animals lack an

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† Abbreviation: DCIP, 2,6-dichlorophenol-indophenol.

NAD-linked D-lactate dehydrogenase analogous to L-lactate dehydrogenase (EC 1.1.1.27). Glycollate oxidase (EC 1.1.3.1), a soluble enzyme present in some tissues, also oxidizes D-lactate at 3% of the rate with glycollate (Kun, Dechary & Pitot, 1954), but this is probably of negligible importance in most cases.

A fairly straightforward procedure is described by which animal tissues can be treated so that the D-2-hydroxy acid dehydrogenase can be measured by the sensitive spectrophotometric assay of Tubbs & Greville (1961). By this means the activity of the enzyme in various organs of a number of animals has been determined.

MATERIALS AND METHODS

Chemicals. Tris (Trizma base) and phenazine methosulphate were from the Sigma (London) Chemical Co., London S.W.6; calcium D-lactate, D-malic acid and ferritin were from Calbiochem Ltd., London W.1; Nitro Blue Tetrazolium and Amido Black were from G. T. Gurr Ltd., London N.W.9; FAD, FMN, NAD⁺, NADP⁺, NADH, oxaloacetic acid, sodium pyruvate, L-lactate dehydrogenase, L-malate dehydrogenase, yeast alcohol dehydrogenase and catalase were from Boehringer Corp. (London) Ltd., London W.5; soya-bean trypsin inhibitor was from Worthington Biochemical Corp., Freehold, N.J., U.S.A.; bovine serum albumin was from Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex; DEAE-cellulose was from H. Reeve Angel and Co. Ltd., London E.C.4; hydroxyapatite (Bio-Gel HTP) was from Bio-Rad Laboratories, Richmond, Calif., U.S.A.; Celite 545 was from Johns-Mansville Products, New York, N.Y., U.S.A.; Sephadex G-25 and G-200 and Blue Dextran were from Pharmacia (G.B.) Ltd., London W.5. Ubiquinone with one isoprenoid unit in the side chain was a gift from Dr A. P. Dawson and ovalbumin was a gift from Dr D. S. Bendall. Other chemicals were the purest products of British Drug Houses Ltd., Poole, Dorset, or Hopkin and Williams Ltd., Chadwell Heath, Essex. Calcium D-lactate was converted into the potassium salt either as described by Tubbs & Greville (1961) or by addition of a slight excess of K₂CO₃ soln. to a solution of calcium D-lactate. DCIP was purified by the method of Savage (1957). Apoferritin was prepared from ferritin by the method of Granick & Michaelis (1943).

Phosphate buffers were made by adjusting the stated concentration of KH₂PO₄ to the required pH with KOH pellets, and tris buffers by adjusting the stated concentration of tris base to the required pH with conc. HCl.

Animals. Rats (Sprague-Dawley albino strain, about 120g.), rabbits (about 2kg.), guinea pigs (about 350g.), pigeons (about 450g.) and ferrets (about 1kg.) were obtained from laboratory animal suppliers. Samples of liver, kidney cortex and heart of ox, pig and lamb were obtained from local slaughterhouses. Experiments with rats showed no significant difference in the activity of D-2-hydroxy acid dehydrogenase in tissues of animals killed by decapitation, cervical dislocation or injection of Nembutal, though the use of ether resulted in considerable losses of activity. The large quantities of rabbit kidneys used for purification were

a gift from J. Sainsbury's Poultry Packing Station at Bury St Edmunds, Suffolk.

Determination of protein. Protein was normally assayed by the u.v.-absorption method of Warburg & Christian (1941). For crude tissue extracts and turbid suspensions the biuret method (Bailey, 1962) was used, with the addition of 0.2% sodium deoxycholate. Bovine serum albumin was used as standard.

Assay of enzyme. The standard enzyme assay used was a modification of the spectrophotometric assay of Tubbs & Greville (1961), which measured the reduction of DCIP with D-lactate as substrate. The assay mixture contained 50 μ moles of potassium D-lactate, 80 nmoles of DCIP, 100 μ moles of tris-chloride buffer, pH 8.6, and 1–500 μ l. of enzyme solution in a total volume of 2.0 ml. The assay temperature was 30°. The reaction was usually started by addition of the enzyme. The decrease of E_{600} with time was followed in a Beckman DK2A recording spectrophotometer, and the initial rate was measured.

Impure preparations of the enzyme often contained compounds, such as thiols, that reduce DCIP non-enzymically. To compensate for these a blank cell was used that contained DCIP and tris buffer but no D-lactate. An equal volume of enzyme preparation was added to each cell and the difference in extinction between the two cells was recorded.

Unit of activity. The unit of enzyme activity is the quantity catalysing the oxidation of 1 μ mole of D-lactate/min. under the above conditions. This corresponds to a rate of ΔE of 11.0/min., assuming a molar extinction for DCIP of 22000 (Armstrong, 1964).

Assay of the enzyme in samples of animal tissues. The problem was to process the tissue in such a way as to obtain all of the enzyme in a form in which it could be assayed with DCIP and D-lactate as described above. The difficulties were that the enzyme is present in a particulate fraction of the cell, and when solubilized it is in an apparently inactive form (Cammack, 1968b); in addition, tissue homogenates contain compounds that interfere with the assay.

Tissue samples (2–4g.) were removed shortly after the death of the animals. They were washed with ice-cold 50 mM-potassium phosphate buffer, pH 7.4, blotted, weighed and chopped finely with scissors in approx. 4 vol. of the same buffer. Most tissues were homogenized in a Perspex-in-glass hand-operated homogenizer. Tougher tissues were blended in an MSE homogenizer (Measuring and Scientific Equipment Ltd.) in a 10 ml. beaker at medium speed for 2 min. The final volume of the homogenate was measured.

The enzyme was then obtained in a soluble form by ultrasonic treatment. This gave much higher and more consistent yields of soluble enzyme than other methods. About 10 ml. of homogenate was transferred to a polypropylene tube (diameter 2.4 cm., length 6 cm., wall thickness 0.16 cm.) and subjected to ultrasonic oscillation with a Soniprobe (Dawe Instruments Ltd.) with a $\frac{1}{2}$ in. tip. The output was adjusted to give a meter reading of 2.8 A. The sample was maintained below 10° by immersing the vessel in acetone-solid CO₂. Ultrasonic treatment was usually carried out for 1 min.; further treatment for 20 min. had little effect on release of soluble D-2-hydroxy acid dehydrogenase or of other proteins.

The samples were centrifuged at 126000g at 4° for 1 hr. in the 10 $\times$ 10 ml. angle rotor of an MSE Superspeed 50 refrigerated centrifuge. After centrifugation, the tubes were handled very gently to avoid disturbance of the fatty layers

Table 1. *Purification of D-2-hydroxy acid dehydrogenase from 2.4 kg. of rabbit kidneys*

For homogenate and mitochondria, enzyme was measured by the method described for animal tissues, and protein by the biuret method. At the other stages enzyme was measured in the standard assay and protein by u.v. absorption. Yield and purification are expressed relative to the original homogenate.

Stage	Volume (ml.)	Total activity (units)	Specific activity (units/mg.)	Purification	Yield (%)
Homogenate	8000	654	0.0011	(1)	(100)
Washed mitochondria	1800	333	0.0062	5.7	51
Ultrasonic treatment, pH 5.5 precipitation	1490	297	0.040	37	45
(NH ₄) ₂ SO ₄ precipitation, dialysis	97	251	0.162	148	38
DEAE-cellulose column	130	165	0.795	730	25
Polyethylene glycol fractionation	9.3	127	1.95	1780	19
Hydroxyapatite column	34	56	7.1	6500	8.5
(NH ₄) ₂ SO ₄ precipitation	2.0	54.5	7.4	6750	8.3

at the top and bottom; these contained an activity, probably cytochrome oxidase, that catalysed the reoxidation of reduced DCIP and thus interfered with the assay.

A 1 ml. sample of the clear supernatant was removed with a syringe and applied to the top of a 10 cm. $\times$ 1.1 cm. column of Sephadex G-25 equilibrated with 25 mM-potassium phosphate buffer, pH 6.3, at 20°. The protein fraction from the column was collected in a volume of 2.5 ml. This process served to remove inhibitors of the enzyme and compounds such as thiols that reduced DCIP non-enzymically and thus interfered with the assay. The enzyme, now at pH 6.3, was allowed to change to the active form (Cammack, 1968b) on standing at 20° for 15 min.

The extract was finally assayed as described above, with a blank cell without D-lactate; 0.250 ml. of the extract was added to each cell. To prevent a change in pH, the assay medium contained 300 μ moles of tris, pH 8.6.

Purification of D-2-hydroxy acid dehydrogenase from rabbit kidneys. It was found that most of the enzyme could be obtained in a crude mitochondrial preparation, which was made by homogenizing the rabbit kidneys in sucrose, followed by differential centrifugation. The enzyme was then in an insoluble form, and much unwanted protein could be removed by washing the mitochondria with phosphate buffer. The enzyme could then be extracted in a soluble form by freezing and thawing in 0.125 M-KCl soln., or by preparation of an acetone-dried powder. However, higher yields were obtained by exposure to ultrasonic oscillation, and this method was adopted for the purification. The resulting suspension of membrane fragments was clarified by adjusting to a slightly acid pH and centrifuging. The enzyme could then be purified by a series of conventional protein-fractionation techniques.

The purification procedure is summarized in Table 1. All steps were carried out at 0–5°, except the polyethylene glycol step, which was done at room temperature. The pH was raised and lowered with 1.0 M-tris-chloride buffer, pH 9.0, and 1.0 M-acetic acid-sodium acetate buffer, pH 4.0, respectively. Centrifugation was carried out in the MSE Highspeed 18 refrigerated centrifuge unless otherwise stated; for large volumes the 6 $\times$ 250 ml. angle rotor was used and for smaller volumes the 8 $\times$ 50 ml. angle rotor was used.

Preparation of washed mitochondrial fraction. Kidneys (2.4 kg.) were removed from freshly killed 10-week-old rabbits and kept at 0° for up to 20 hr. before use. They were

then blended in eight batches in a total volume of 6 l. of 0.25 M-sucrose–10 mM-tris, pH 8.0; each batch was homogenized for 2 min. at maximum speed in a 1 l.-capacity Waring Blendor. The combined homogenates were centrifuged in an MSE Major refrigerated centrifuge at 1250 rev./min. in the 4 $\times$ 1 l.-capacity rotor (350 g_{av}) for 6 min. The supernatant was carefully poured off through muslin, and centrifuged at 22 000 g for 10 min. The cloudy red supernatant was discarded, and the precipitate resuspended, with the aid of a Perspex-in-glass hand-operated homogenizer, in 6 l. of 10 mM-potassium phosphate buffer, pH 7.5. The suspension was centrifuged at 22 000 g for 15 min. The supernatant was discarded and the buff-coloured precipitate resuspended in 10 mM-potassium phosphate buffer, pH 6.8, to a final volume of 1.8 l.

Ultrasonic treatment. The suspension of lysed mitochondria was divided into two 900 ml. batches, and each batch was subjected to ultrasonic oscillation in a 1 l. Pyrex beaker surrounded by ice-water by using a Dawe Soniprobe at full power (about 6.5 A) for 60 min. The temperature remained below 12°.

The suspension was then vigorously stirred, the pH adjusted to 5.5 and the thick white precipitate removed by centrifugation at 22 000 g for 10 min. The yellow, faintly cloudy, supernatant was readjusted to pH 7.0.

Ammonium sulphate precipitation. Solid (NH₄)₂SO₄ was added slowly to the solution with stirring to give 27% (w/v) (44% saturation). The suspension was stirred for 30 min. and centrifuged at 22 000 g for 10 min. The precipitate was redissolved in 10 mM-potassium phosphate buffer, pH 6.8, to a final volume of about 100 ml., and dialysed overnight against 10 l. of the same buffer. A precipitate formed during dialysis, which was removed by centrifugation at 37 000 g for 1 hr.

DEAE-cellulose column. A 20 cm. $\times$ 2 cm. column of Whatman DE52 was equilibrated with 25 mM-tris-chloride buffer, pH 8.0. The supernatant from the previous step was adjusted to pH 8.0 and applied to it. The column was then washed with 50 ml. of tris-chloride buffer, followed by 50 ml. of 60 mM-NaCl soln., then a linear gradient of NaCl soln., 400 ml. in all, from 60 mM to 0.2 M, in the same tris buffer. Fractions of volume 10 ml. were collected. Enzyme was assayed in the eluent fractions, and protein was estimated by measuring *E*₂₈₀ without correction for nucleic acid. The results of a typical run are shown in Fig. 1.

Fractional precipitation with polyethylene glycol. Use was

Fig. 1. Elution of partly purified enzyme from a DEAE-cellulose column with a gradient of NaCl soln. Buffer was 25 mM-tris-chloride, pH 8.0. The hatched area indicates the fractions that were retained for further purification.

Fig. 2. Elution of enzyme from a hydroxyapatite-Celite column with a gradient of phosphate buffer, pH 7.4. The hatched area indicates the fractions that were retained.

made in this step of the fact that the precipitation of proteins by polyethylene glycol is markedly dependent on pH (Polson, Potgieter, Largier, Mears & Joubert, 1964). The most active fractions from the DEAE-cellulose column were combined and a 50% (w/v) solution of polyethylene glycol 6000 in water was added with stirring to bring the polyethylene glycol concentration to 15%. The pH was carefully lowered to 6.8 and the solution stirred at room temperature for 20 min., during which time a white precipitate appeared. This precipitate contained most of the protein but little of

the enzyme, and was removed by centrifugation at 12000 *g* for 10 min. at 20°. The supernatant was decanted, adjusted to pH 5.5, stirred for 20 min. and centrifuged again. The precipitate from the second centrifugation was redissolved in 25 mM-tris-chloride buffer, pH 8.0, to a final volume of about 10 ml.

Hydroxyapatite column. Bio-Gel HTP was mixed with an equal weight of Celite 545 to permit a fairly rapid flow, and poured into a 20 cm. × 2 cm. column; the column was washed with 2 vol. of water before use. The enzyme was

Table 2. *Activity of D-2-hydroxy acid dehydrogenase in organs of various animals*

For details of assay and animals, see the Materials and Methods section. Each value is the average of determinations on at least two animals. The standard deviation of each determination when a number of determinations were made on samples of the same tissue was about $\pm 10\%$.

Animal	Organ ...	Activity (units /kg. wet wt. of tissue)					
		Kidney	Liver	Heart	Brain	Skeletal muscle	Spleen
Rat		106	155	55	29		11
Guinea pig		46	86	64			
Pigeon		107	55	32	15	25†	
Ferret		136	89	51	27	9	
Rabbit		264	24	22	9		
Lamb		51*	41	11			
Pig		63*	37				
Ox		40*	47				

* Kidney cortex.

† Breast muscle.

adjusted to pH 7.0 and loaded on to the column, which was then washed with 50ml. of water. A linear gradient of potassium phosphate buffer, pH 7.4, from 0 to 60 mM was applied, with a total volume of 400 ml. The result is shown in Fig. 2; the enzyme was eluted first from the column in a sharp peak. The fractions of highest specific activity were combined.

Second ammonium sulphate precipitation. Solid $(\text{NH}_4)_2\text{SO}_4$ to give 31.5% (w/v) (50% saturation) was added with stirring at 0°. Little purification was gained by this step, but it afforded a simple means of concentrating the enzyme.

RESULTS

Distribution of the enzyme in tissues of various animals. Table 2 shows the activity of D-2-hydroxy acid dehydrogenase in a variety of animal organs, expressed in units/kg. of tissue. No attempt was made to subdivide these organs, though it was found in rabbit that there was three times as much of the enzyme in kidney cortex as in kidney medulla. Rabbit kidney cortex, containing 400 units/kg., is the most active source of the enzyme so far found. The enzyme was detected in all the tissues tested, including pigeon breast muscle, in which it was previously thought to be absent (Tubbs & Greville, 1961). No great difference was observed in enzyme content between herbivores (rabbit, guinea pig) and carnivores (ferret). As might be expected, larger animals (ox, pig) have less of the enzyme/g. of tissue than the smaller ones.

Properties of the purified enzyme

Homogeneity in the analytical ultracentrifuge. Samples of enzyme purified from rabbit kidney were tested for homogeneity in a Beckman-Spinco

model E analytical ultracentrifuge. The boundary of sedimenting protein was observed with u.v. and schlieren optics, with samples of enzyme containing 0.65 and 1.2 mg. of protein/ml. respectively. In each case a single boundary was observed. With schlieren optics, a single symmetrical peak was observed, but it was too small to determine whether it accounted for all the protein. The results suggest that the enzyme preparation contains one major protein component. The average sedimentation coefficient obtained was $S_{20,w} 5.8\text{S}$.

Homogeneity by polyacrylamide-gel electrophoresis. Disc electrophoresis was carried out by the method of Davis (1964). Samples of D-2-hydroxy acid dehydrogenase containing 2.5–50 μg . of protein were applied to the gels. Electrophoresis was carried out at a current of 3 mA/tube for 35 min. at room temperature. Some of the gels were then fixed and stained for protein by immersion in 1% (w/v) Amido Black in 7% (v/v) acetic acid. The rest were stained for enzyme activity by immersion in a solution containing 25 mM-D-lactate, 0.03 mg. of phenazine methosulphate/ml., 0.3 mg. of Nitro Blue Tetrazolium/ml. and 40 mM-tris, pH 8.6, at 20° for at least 1 hr. The patterns observed in a typical run are illustrated in Fig. 3.

Three bands of enzyme activity were detected, which corresponded with the major protein bands. The bands did not stain for enzyme activity if D-lactate was omitted from the staining mixture. Most of the enzyme was present in the most rapidly moving band. The presence of more than one band of enzyme suggests that it may exist in different isoenzyme forms. However, other explanations are possible; for example, the less-mobile bands might be polymerized forms of the enzyme.

It appears from these experiments that the major

Fig. 3. Polyacrylamide-gel electrophoresis of purified D-2-hydroxy acid dehydrogenase. In gel 1, 5 μ g. of protein was run and stained for enzyme activity. In gel 2, 25 μ g. was run and stained for protein. For conditions see the text. The arrow shows the direction of migration of material.

protein component in the purified enzyme preparation is D-2-hydroxy acid dehydrogenase.

Stability. The enzyme could be stored in a concentrated solution in 50mM-tris-chloride buffer, pH 8.0, in the dark at 4° for several weeks without appreciable loss of activity. It was normally stored for longer periods as a precipitate in 32% (w/v) ammonium sulphate solution in 50mM-tris-chloride buffer, pH 8.0 at 4°. In this form it was stable for many months.

The enzyme could be kept for 30 min. at 20° at any pH between 5.5 and 10 without appreciable loss of activity, but below pH 4.8 or above pH 10.5 it was very unstable. At pH 7.3 the enzyme was unstable above 55°. Reversible inhibitors of the enzyme protected the enzyme against inactivation by heat or low pH. For example, at 62° and pH 7.3 the enzyme activity decreased by 79% in 10 min.; in the presence of 1 mM-oxalate the decrease was only 5%.

Molecular weight. The molecular weight of the enzyme was estimated by the gel-filtration method of Andrews (1964, 1965). A 40 cm. $\times$ 3.5 cm. column of Sephadex G-200 was equilibrated for several weeks with 0.1 M-potassium chloride-50mM-tris buffer, pH 7.6, at 20°. A sample of each protein was applied to the column in a small volume of buffer, and 4.4 ml. fractions were collected at a flow rate of 0.5 ml./min. The volume, V , at which the peak of each protein was eluted was determined by assaying the fractions. The distributions of the various materials in the fractions were determined as follows: by measuring E_{280} for soya-bean trypsin inhibitor, bovine serum albumin, ovalbumin, fibrinogen and apoferritin, E_{407} for horse-heart cytochrome *c* and E_{625} for Blue Dextran. Yeast alcohol dehydrogenase was assayed spectrophotometrically at 340 nm. with acetaldehyde and NADH. D-2-Hydroxy acid dehydrogenase was assayed with DL-lactate and DCIP. Fig. 4 shows a plot of V/V_0 against the logarithm of molecular weight of the proteins, where V_0 is the void volume of the column, obtained from the elution volume of Blue Dextran. From this,

Fig. 4. Estimation of the molecular weight of D-2-hydroxy acid dehydrogenase by gel filtration on Sephadex G-200. For details see the text. The standard proteins are: *A*, horse heart cytochrome *c*; *B*, soya-bean trypsin inhibitor; *C*, ovalbumin; *D*, bovine serum albumin; *E*, yeast alcohol dehydrogenase; *F*, apoferritin; *G*, fibrinogen. The elution volume of D-2-hydroxy acid dehydrogenase is indicated by the arrow.

the molecular weight of D-2-hydroxy acid dehydrogenase was estimated to be 102 000. Andrews (1965) suggests that the error in such determinations is about $\pm 10\%$.

Siegel & Monty (1966) have stated that this method gives a measure of the Stokes radius of a protein rather than of its molecular weight. Accordingly the data of Ackers (1964) relating the elution volume to the ratio a/r (a is Stokes radius of molecule and r is the effective pore radius of gel) were applied to five of the standard proteins for which the Stokes radius was known. This gave a value for r of about 170 Å, from which the Stokes radius of D-2-hydroxy acid dehydrogenase was estimated to be 38 Å. Combining this with the value of the sedimentation coefficient obtained from the ultracentrifuge and assuming a partial specific volume of 0.725 gave, by the formulae of Siegel & Monty (1966), a value for the molecular weight of 95 000. This is in fairly good agreement with the value obtained from the Andrews method, considering the approximations involved.

Flavin spectrum. Spectral measurements of purified D-2-hydroxy acid dehydrogenase were made in a Cary 15 recording spectrophotometer at 15°. Fig. 5 (curve *A*) shows the spectrum of the purified enzyme. It is a typical flavoprotein spectrum.

Effect of substrates on the flavin spectrum. On

Fig. 5. Spectrum of D-2-hydroxy acid dehydrogenase in 25 mM-phosphate buffer, pH 7.4. A, Oxidized enzyme; B, enzyme after addition of 1 mM-D-malate.

addition of 1 mM-D-malate, a substrate of the enzyme, the spectrum was bleached (Fig. 5, curve B), with an increase in E between 500 and 700 nm. A very similar spectrum was observed when 25 mM-D-lactate was added to the enzyme at pH 8.5, and this was almost unaffected by the addition of 25 mM-pyruvate.

This species may be a semiquinone form, from its absorption of wavelengths above 500 nm., though it does not closely resemble either the blue or the red type observed with other flavoproteins (Massey & Palmer, 1966). However, the spectra were measured in aerobic conditions, and since the enzyme is an oxidase it would be expected to use up the dissolved oxygen with the formation of oxaloacetate or pyruvate and presumably hydrogen peroxide. A similar effect is seen with D-amino acid oxidase (EC 1.4.3.3) of pig kidney. When D-alanine is added to a solution of this enzyme in aerobic conditions the spectrum changes as the dissolved oxygen is exhausted to a rather featureless spectrum similar to Fig. 5 (curve B) (Nakamura, Nakamura & Ogura, 1963). It was proposed by Massey & Gibson (1964) that this might be due to a complex between a pyruvate radical and a flavin semiquinone radical. By analogy, the spectrum shown in Fig. 5 (curve B) might be due to a complex between an oxaloacetate radical and a flavin semiquinone radical. With D-amino acid oxidase, prolonged incubation with D-alanine results in further reduction of the flavin to the fully reduced form; such an effect has not been observed with D-2-hydroxy acid dehydrogenase.

Effect of reversible inhibitors on the flavin spectrum. The spectrum of the oxidized enzyme was altered on addition of inhibitors of the enzyme. The most pronounced effects were seen in the presence of oxalate and oxaloacetate (Fig. 6). The effects, though small, were observed reproducibly with several preparations of enzyme. Both inhibitors induce a red shift in the spectrum, but whereas the peak heights are increased with oxalate, they are decreased with oxaloacetate.

Fig. 6. Effect of inhibitors on the spectrum. —, Oxidized enzyme in 25 mM-phosphate buffer, pH 7.4; ---, enzyme + 0.1 mM-oxaloacetate; ·····, enzyme + 0.2 mM-oxalate.

Changes in the spectrum in the presence of inhibitors (Fig. 6) have been observed with a number of flavoproteins (Veeger, DerVartanian, Kalse, DeKok & Koster, 1965). For example, the spectrum of succinate dehydrogenase (EC 1.3.99.1) undergoes a red shift in the presence of a number of dicarboxylic acids, but a much more pronounced effect is seen with oxaloacetate that is different from that observed in the present case (DerVartanian & Veeger, 1964).

Preparation of the apoenzyme and recombination with flavin. Flavin was removed from the enzyme by treatment with acid ammonium sulphate by a modification of the method of Warburg & Christian (1938). A sample of the enzyme was run into 10 vol. of 0.1 M-hydrochloric acid saturated with ammonium sulphate at 0°, with vigorous stirring. The resulting suspension was stirred for 20 min., then centrifuged at 10000g for 10 min. at 0°. The supernatant was discarded and the precipitate resuspended in 0.2 M-potassium phosphate buffer, pH 7.4. Traces of free flavin were removed from the solution by passing it through a column of Sephadex G-25. In a typical experiment, the resulting solution had only 2.7% of the activity of the original enzyme sample. On adding 10 μM-FAD to a sample of the apoenzyme preparation and leaving overnight at 0°, the activity was increased to 41.5% of the original. Thus the yield of apoenzyme was at least 39%.

Fig. 7 shows the effect of incubation of the apoenzyme with FAD, FMN and riboflavin on enzymic activity. Only FAD caused reconstitution of the holoenzyme. The rate and extent of reactivation of the apoenzyme depended on the concentration of FAD present. This was probably due, not to

Fig. 7. Effect of flavin derivatives in restoring activity to apo-D-2-hydroxy acid dehydrogenase. Apoenzyme, prepared as described in the text, was incubated at 30° in 25mM-phosphate buffer, pH 7.4, in the presence of the following: Δ , 0.1 mM-FAD; $\square$, 0.01 mM-FAD; $\circ$, 0.001 mM-FAD; $\blacktriangle$, 0.01 mM-FMN; $\bullet$, 0.01 mM-riboflavin; $\blacksquare$, no addition. At the point indicated by the arrow, 0.1 mM-FAD was added to a sample of apoenzyme that had been incubated alone at 30°.

an equilibrium between apoenzyme and holo-enzyme, but to the instability of the apoenzyme at 30°. If the apoenzyme was incubated alone at 30° for 60 min., no activity was restored by addition of FAD. Hence, if lower concentrations of FAD were present, the rate of reconstitution was lower, and more apoenzyme was destroyed before it could combine with FAD. For this reason it was not possible to determine the equilibrium constant for association of FAD.

Flavin fluorescence. Attempts were made to detect fluorescence of the flavin in the enzyme. An Aminco-Bowman spectrophotofluorimeter was used, exciting at 450nm. and measuring emission at 530nm. Fresh samples of purified enzyme showed only a very small fluorescence, corresponding to 3% of that of a solution of FAD with the same extinction at 450nm. The fluorescence increased if the enzyme was stored for several days, and this was probably due to release of free flavin as the enzyme denatured.

Acceptor specificity. A systematic study of the acceptor specificity of the enzyme has not yet been undertaken, but a few observations have been made that supplement the previous work (Tubbs & Greville, 1961).

(a) Ubiquinone. The activity of the enzyme was tested with a sample of ubiquinone with one isoprenoid unit in the side chain. This was estimated by measuring E_{275} , taking the absolute molar extinction of the oxidized form as $15\,140\text{ cm}^{-1}\text{ M}^{-1}$ (Morton, 1965). With D-lactate as substrate at 30° and pH 8.6, the rate with 0.06mM-ubiquinone was 72% of the rate with DCIP.

(b) Oxygen. The purified enzyme was found to have a weak oxidase activity. This was measured in two ways. (1) A Clark-type oxygen electrode (Yellow Springs Instrument Co. Inc.) was used in an

apparatus similar to that described by Dixon & Kleppe (1965). The electrode was calibrated with air-saturated water at 30°, which contains 0.23mM-oxygen. D-Lactate oxidation was calculated on the assumption that 1 mole was oxidized by 1 mole of oxygen, with the formation of hydrogen peroxide. The rate of oxygen uptake was roughly halved by addition of catalase. (2) The pyruvate produced by oxidation of D-lactate was measured continuously, by using L-lactate dehydrogenase (EC 1.1.1.27) and NADH. This assay was more sensitive than the oxygen-electrode method. The reaction mixture contained 50 μ moles of potassium D-lactate, 0.33 μ -mole of NADH, 100 μ moles of tris-chloride buffer, pH 8.1, 0.01ml. of a suspension of rabbit muscle L-lactate dehydrogenase containing 360 units/ml., and 0.1–0.5 unit of D-2-hydroxy acid dehydrogenase in a total volume of 2.0ml. The oxidation of NADH was followed spectrophotometrically at 340nm. The rate was proportional to the quantity of D-2-hydroxy acid dehydrogenase added, and addition of greater quantities of L-lactate dehydrogenase had no effect. Similarly, the oxidase activity of the enzyme with D-malate as substrate was demonstrated by using L-malate dehydrogenase (EC 1.1.1.37). The reaction mixture contained 10 μ -moles of potassium D-malate, 0.33 μ mole of NADH, 0.01ml. of a suspension of pig heart L-malate dehydrogenase containing 720 units/ml., 100 μ moles of potassium phosphate buffer, pH 7.3, and 0.1–0.5 unit of D-2-hydroxy acid dehydrogenase.

The rate of oxidation of D-lactate by the enzyme with 0.23mM-oxygen as acceptor at pH 8.1 was determined by both of these methods to be approx. 13% of the corresponding rate with 0.040mM-DCIP as acceptor.

(c) NAD^+ and NADP^+ . In agreement with

previous results (Tubbs & Greville, 1961), no activity of the purified enzyme was detected with NAD⁺ or NADP⁺ as acceptor. The sensitivity of the assay was such that an activity equivalent to 0.1% of that towards DCIP would have been detected.

DISCUSSION

D-2-Hydroxy acid dehydrogenase has now been shown to be present in various mammals and one bird. A similar enzyme has been obtained from yeast, though the various preparations of this enzyme appear to differ in specificity towards substrates and acceptors (Cremona, 1964; Rytka & Tysarowski, 1965; Iwatsubo, 1963; Boeri, Cremona & Singer, 1960). The yeast enzyme appears to be very unstable. It is reported to be a flavoprotein, possibly containing FAD and zinc (Iwatsubo & Curdel, 1961). Its molecular weight was estimated by gel filtration to be 103000 (Iwatsubo & Curdel, 1963). D-Lactate dehydrogenases that do not use NAD⁺ or NADP⁺ as acceptor have been isolated from bacteria (Snoswell, 1963; Kline & Mahler, 1965; Wittenberger & Haaf, 1966), though none of these has a substrate specificity as wide as that of the mammalian enzyme.

The substrate oxidized by D-2-hydroxy acid dehydrogenase *in vivo* is not known. Several possibilities were suggested by Tubbs & Greville (1961). These included D-lactate, D-glycerate and long-chain D-2-hydroxy fatty acids found in cerebrospinal fluid. It has subsequently been proposed that the enzyme may be responsible for the oxidation of D-malate formed by the action of maleate hydratase on maleate (Englard, Britten & Listowski, 1967) and of D-2-hydroxyglutarate produced by the breakdown of hydroxylysine (Lindahl, Lindstedt & Lindstedt, 1967).

D-Lactate is a component of the 'aminoacetone cycle', proposed by Elliott (1959), which is involved with the metabolism of glycine and threonine. In addition, D-lactate is produced during fermentation by intestinal bacteria; a dramatic instance of this is D-lactate acidosis, a pathological condition of ruminants (Dunlop & Hammond, 1965). In this connexion it is perhaps significant that the activity of D-2-hydroxy acid dehydrogenase in normal ruminants is comparatively low (Table 2). Though this enzyme appears to be the only one that can oxidize D-lactate directly, D-lactate could also be metabolized by conversion into methylglyoxal by the glyoxalase system, followed by oxidation by keto aldehyde dehydrogenase, an enzyme found in sheep liver (Monder, 1967).

A possible alternative means of oxidizing D-glycerate is provided by a specific nicotinamide nucleotide-linked D-glycerate dehydrogenase (Dawkins & Dickens, 1965); however, the reaction of this

enzyme is reversible, and it is not known whether its action *in vivo* is to form D-glycerate or to oxidize it.

It may therefore be that the wide substrate specificity of D-2-hydroxy acid dehydrogenase is no accident, and its action may be to oxidize the hydroxy acids formed in a number of metabolic pathways.

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