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# Effects of Inhibitors on Mitochondrial D-α-Hydroxy Acid Dehydrogenase

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Previous papers (Tubbs & Greville, 1959; Tubbs, 1960*a*; Tubbs & Greville, 1961) have reported properties of a D-α-hydroxy acid dehydrogenase extracted in soluble form from acetone-dried rabbit-kidney mitochondria. An account of some studies on this enzyme has been presented elsewhere (Tubbs, 1960*b*), and various effects of metal-complex-forming agents on the activity of the enzyme have been the basis of a suggestion that the dehydrogenase contains a metal component near to, or actually at, the substrate-binding site (Tubbs, 1960*c*). This paper is devoted to the action of various types of inhibitors on the activity of the enzyme.

## MATERIALS AND METHODS

The preparation and assay of the enzyme were as described by Tubbs & Greville (1961). Materials used in the experiments here reported were obtained as described in that paper, except as mentioned below.

Sodium pyruvate was obtained from C. F. Boehringer und Söehne, Mannheim, Germany. α-Hydroxyisobutyric acid was given by Dr R. Brown, Department of Organic Chemistry, Cambridge University. Phenylmercuric acetate and *p*-chloromercuribenzoate were from Sigma Chemical Co., St Louis, Mo., U.S.A. β-Chlorovinylarsenious oxide (lewisite) was a gift from Sir Rudolph Peters, F.R.S., and ω-chloroacetophenone from Dr J. E. Varner. Iodoacetamide was recrystallized from water.

## RESULTS

*Competitive inhibitors.* The most potent inhibitor of this type yet found is oxalate; $K_i$ values of 5·6, 9·7 and 4·3 μM were obtained with three preparations of enzyme, the ratio $K_m$ (D-lactate)/$K_i$ (oxalate) being 325, 370 and 376 in the three experiments. Inhibition by oxalate is illustrated in Fig. 1. Boeri, Cremona & Singer (1960) have reported a $K_i$ for oxalate of 2·5 μM in a similar enzyme from yeast, which was assayed by ferricyanide reduction at pH 8·0.

Contrary to a preliminary report (Tubbs & Greville, 1959), L-lactate is a weak competitive inhibitor of the enzyme ($K_i$ 12·5 mM). α-Hydroxyisobutyrate inhibits in a similar manner ($K_i$ 7·5 mM), as does malonate ($K_i$ 1·2 mM).

Inhibition by L-lactate results in both $V_{max.}$ and $K_m$ being apparently decreased when DL-lactate is used as substrate; presumably this applies also to the use of other DL-α-hydroxy acids as substrates (Tubbs & Greville, 1961). The effect of a competitive inhibitor, when present as a contaminant of the substrate, of increasing the apparent affinity of an enzyme for its substrate is not generally appreciated, although the case must often arise experimentally. From the usual equations for competitive inhibition it may readily be shown that:

$$v = \frac{V_{max.}}{1+K_m\left(\frac{1}{[S]}+\frac{R}{K_i}\right)}$$

or

$$\frac{1}{v} = \frac{1}{V_{max.}}+\frac{K_m}{V_{max.}}\left(\frac{1}{[S]}+\frac{R}{K_i}\right),$$

where $v$ is the observed reaction velocity, $V_{max.}$ the velocity at infinite substrate concentration in

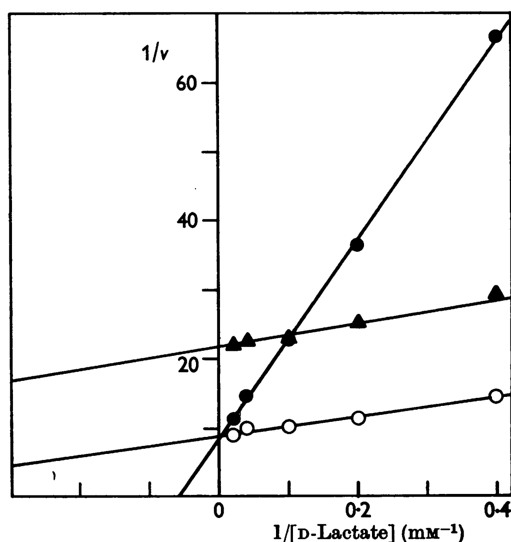


Fig. 1. Inhibition by oxalate. Except for addition of oxalate and variations of D-lactate concn. as indicated, the standard assay was used. Protein (1·4 mg.), purified to stage 2 (Tubbs & Greville, 1961), was present in each assay. The unit of velocity is $-\Delta E_{600\,m\mu}$/min. ○, No addition; ●, oxalate concn. 40 μM; ▲, D-lactate contained oxalate (molar ratio lactate to oxalate of 250:1).

absence of inhibitor, [S] the substrate concentration, $K_m$ and $K_i$ the usual Michaelis and inhibition constants, and $R$ the ratio of inhibitor concentration to substrate concentration. Thus the slope of a plot of $1/v$ against 1/[S] is unchanged; $K_m$ and $V_{max.}$, however, are each decreased by the factor $1/[1+(RK_m/K_i)]$. This effect is illustrated in Fig. 1, with oxalate as inhibitor. With a DL-mixture (when $R = 1$), the apparent $K_m$ is equal to $K_m K_i/(K_m+K_i)$; this has been pointed out by Huang & Niemann (1951).

Competitive inhibition by cyanide is illustrated in Fig. 2. The effect of pH variation on cyanide inhibition shows that cyanide ion, and not hydrocyanic acid, is the inhibitory species (Table 1). When included in the standard assay at a concentration of 1 mM sodium azide has no effect.

*Inhibition by pyruvate.* Inhibition by pyruvate is shown in Figs. 3 and 4. The inhibition is competitive with respect to indophenol; with respect to D-lactate the situation is more complex. The observations can be interpreted on the assumption that pyruvate in fact competes both with indophenol and with lactate; with respect to lactate, the competition with indophenol would result in 'uncompetitive' inhibition, and this effect, superimposed on the real competition with the lactate, could result in the mixed type of inhibition shown in Fig. 3. Under appropriate conditions the observed result might appear to be inhibition non-competitive with respect to lactate, as indeed reported by Molinari & Lara (1960) for D-lactate dehydrogenase from *Propionibacterium pentosaceum*. With respect to indophenol the observed overall effect could be simple competition, as found (Fig. 4); for even the competition with lactate could appear as competition with indophenol. Such effects of inhibitors acting at intermediate stages of sequential enzymic processes have been extensively discussed by Hearon, Bernhard, Friess, Botts & Morales (1959).

Table 1. *Cyanide inhibition at various* pH *values*

$K_i$ was determined from double-reciprocal plots (e.g. Fig. 4); $CN^-$ ion concentrations were calculated assuming a p$K$ of 9·21 for HCN (Ang, 1959). The standard indophenol assay was used, except (i) D-lactate concentration was varied between 2·5 and 25 mM, (ii) the pH was varied, and (iii) rates were determined in the absence and in the presence of cyanide (0·8 mM). The enzyme had been fully activated by storage at 4° for 6 days.

| pH | $K_i$ (μM) | [$CN^-$] (μM) corresponding to $K_i$ |
|---|---|---|
| 7·8 | 510 | 20 |
| 8·2 | 200 | 18 |
| 8·6 | 84 | 17 |
| 8·8 | 71 | 20 |

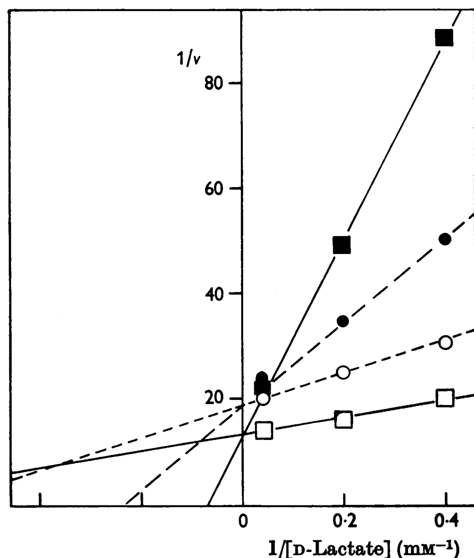


Fig. 2. Cyanide inhibition at different pH values. Experimental conditions were as given in Table 1 (1·1 mg. of enzyme, stage 2, per assay); ○, pH 7·8, no cyanide; ●, pH 7·8, 0·8 mM-cyanide; □, pH 8·8, no cyanide; ■, pH 8·8, 0·8 mM-cyanide.

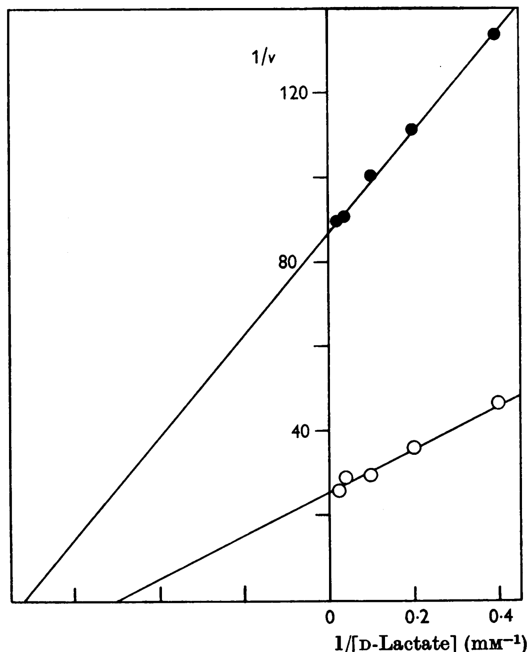


Fig. 3. Inhibition by pyruvate at various concentrations of D-lactate. Standard assay conditions (indophenol concn. 37·8 μM); 0·92 mg. of protein, stage 2, per assay. ○, No addition; ●, 10 mM-pyruvate present.

The interpretation described above would be applicable if the enzymic mechanism can be represented by the scheme:

(1) $E_{ox.} + \text{lactate} \rightleftharpoons E_{ox.}\text{–lactate} \rightleftharpoons E_{red.}\text{–pyruvate} \rightleftharpoons E_{red.} + \text{pyruvate}$,

(2) $E_{red.} + \text{indophenol} \rightarrow E_{ox.} + \text{reduced indophenol}$,

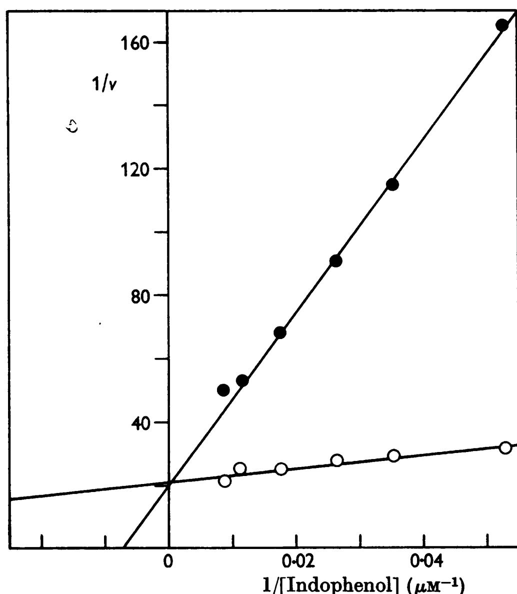


Fig. 4. Inhibition by pyruvate at various concentrations of indophenol. Conditions were as in Fig. 3, but D-lactate concn. was 25 mM throughout. ○, No addition; ●, 10 mM-pyruvate present.

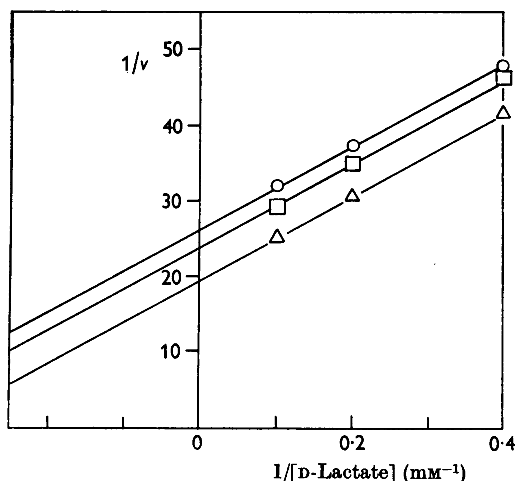


Fig. 5. Enzyme activity at various concentrations of D-lactate and indophenol. Conditions, except as below, were as for Fig. 3. ○, Indophenol 18·9 μM; □, indophenol 37·8 μM; △, indophenol 114 μM.

where $E_{ox.}$ and $E_{red.}$ are oxidized and reduced enzyme. As pointed out by Alberty (1956), this mechanism would result in the slope of plots of $1/v$ against $1/[S]$ being independent of the indophenol concentration; this is actually observed (Fig. 5). Similar results have recently been reported by Massey, Gibson & Veeger (1960) and Massey, Palmer & Bennett (1961), working respectively with lipoyl dehydrogenase and D-amino acid oxidase.

*Effects of thiol reagents.* Phenylmercuric acetate and *p*-mercuribenzoate cause gradual inhibition: for example, preincubation with the former (0·1 mM) at pH 7·9 and 0° for 30 min. resulted in 45% inhibition, from which D-lactate (25 mM) afforded no protection. Alkylating reagents (iodoacetamide, ω-chloroacetophenone) cause very little inhibition at a concentration of 0·1 mM, even after preincubation as above. β-Chlorovinyl-arsenious oxide is also only a weak inhibitor; it inhibits by about 5% when included (at 0·1 mM) in the standard assay.

*Inhibition by chelating agents.* EDTA inhibition of D-lactate oxidation by particulate preparations has been described by Tubbs & Greville (1961); the effects of this and other chelating agents on the soluble dehydrogenase have been briefly discussed elsewhere (Tubbs, 1960*c*). Preincubation of the enzyme with 0·5 mM-EDTA for 2 hr. at 0° causes complete inhibition, with either indophenol or cytochrome *c* as acceptor; with 1,10-phenanthroline (0·5 mM) inhibition proceeds rather more slowly. Inhibition by these reagents is not reversed by dialysis. However, protection from inhibition by 1,10-phenanthroline is afforded by including D-lactate in the preincubation system (Fig. 7). Competitive inhibitors also protect: L-lactate is slightly less effective than its enantiomorph, but oxalate (Fig. 6) protects at far lower concentrations. Pyruvate is not nearly as effective as lactate (Table 2).

The gradual inhibition by chelating agents obeys first-order kinetics (Fig. 7). Assuming that at infinite D-lactate concentration the enzyme is completely protected (Fig. 8), then the velocity constant for inhibition is given by

$$k = \frac{2 \cdot 303}{t} \log \frac{e_0}{e_t},$$

where $t$ is the time of incubation, and $e_0$ and $e_t$ are the measured enzyme activities at time zero and time $t$. Fig. 9 shows the decrease in $k$ resulting from increasing concentrations of D-lactate in the preincubation mixture. Burton (1951) has shown that when the concentration of a protective reagent is equal to the dissociation constant of the enzyme–protector complex then the velocity constant for inhibition (or denaturation) will be given by

$$k = \tfrac{1}{2}(k_0 + k_\infty),$$

where $k_0$ is the velocity constant for inhibition of the free (unprotected) enzyme, and $k_\infty$ that for inhibition in presence of infinite protector concentration. In the present case $k_\infty$ is zero, and from Fig. 9 the dissociation constant of the protected enzyme–D-lactate complex is found to be about 1 mM. In the assay, $K_m$ for D-lactate was 1·6 mM; at infinite indophenol concentration and 0° (the temperature of the protection experiment) a somewhat different $K_m$ would no doubt be observed, but the agreement between the protection and Michaelis constants for D-lactate is clearly close. As pointed out earlier (Tubbs, 1960*c*), this suggests that the metal component of the enzyme is at or near the substrate-binding site. Stachiewicz, Labeyrie & Slonimski (1961) have studied the similar inactivation by EDTA which occurs with yeast D-hydroxy acid dehydrogenase, and, as in the present case, it is concluded that a metal is situated near the substrate-binding site. These workers report that 7 μM-D-lactate halves the rate of inactivation by EDTA; this concentration, which is some 300 times smaller than the $K_m$ for the yeast (or kidney) enzyme, affords negligible protection to the mammalian dehydrogenase, and would suggest a remarkably high affinity for D-lactate. Also in contrast with the present case is the report that L-lactate affords no protection. As previously mentioned (Tubbs, 1960*c*), the relative potencies of oxalate and L-lactate as inhibitors of the mammalian enzyme closely correspond with their protective powers, and this, together with the fact that oxalate forms much more stable chelates than lactate, further supports the suggestion that the metal may be concerned with substrate binding.

Curdel & Labeyrie (1961) found that $Zn^{2+}$ and $Co^{2+}$ ions (and no other metal ions tested) reactivated the yeast enzyme after EDTA treatment and

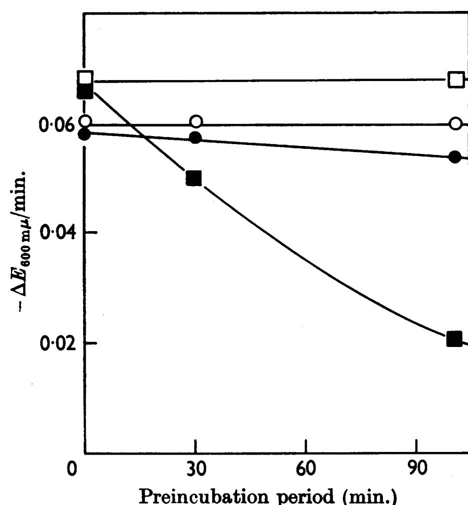


Fig. 6. Protection by oxalate from inhibition by phenanthroline. Enzyme (stage 2; 4·5 mg./ml.) was preincubated at 0° and pH 7·8 for the periods shown. After incubation activity was assayed in the standard system, by using 0·9 mg. of protein in each test. ○, Preincubation system contained 0·25 mM-oxalate ●, preincubation system contained oxalate and 0·5 mM-phenanthroline; □, neither oxalate nor phenanthroline present; ■, phenanthroline, but no oxalate, in preincubation mixture.

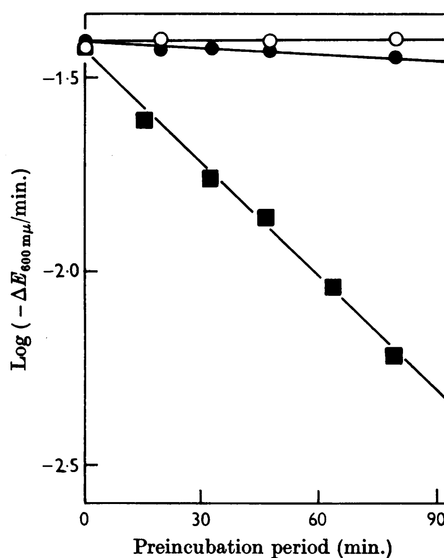


Fig. 7. Kinetics of inhibition by phenanthroline. Enzyme (stage 3; 440 μg./ml.) was preincubated at pH 8·1 and 0° for the periods shown. After incubation activity was assayed at pH 8·1, by using 132 μg. of protein in each test; concn. of substrate was always 25 mM. Preincubation system contained: ■, 0·5 mM-phenanthroline; ●, phenanthroline and 50 mM-D-lactate; ○, no phenanthroline, D-lactate present or absent.

Table 2. *Protection from inhibition by* 1,10-*phenanthroline*

Enzyme was preincubated for 110 min. at 0° in a system containing: 1,10-phenanthroline, 0·5 mM; tris, pH 7·9, 37·5 mM; protective compounds, 50 mM; protein, 0·44 mg./ml. The standard assay was used, except that the D-lactate concentration was 50 mM; controls (no preincubation) were used to correct for inhibition by pyruvate and α-hydroxyisobutyrate.

| Protective compound | None | D-Lactate | L-Lactate | α-Hydroxyisobutyrate | Pyruvate |
|---|---|---|---|---|---|
| Inhibition (%) | 80 | 0 | 0 | 12 | 50 |

dialysis, and have suggested that the metal may be involved in electron transfer; it is, however, reported that $Mg^{2+}$ ions can to some extent reactivate both the yeast hydroxy acid dehydrogenase and D-lactate–cytochrome reductase (Boeri *et al.* 1960; Gregolin & Singer, 1961). These ions and $Ca^{2+}$ ions (Tables 3 and 4) can apparently produce some reactivation of the similarly treated kidney enzyme, but, as previously reported (Tubbs, 1960*c*), 1,10-phenanthroline still inhibits the enzyme reactivated with calcium chloride, suggesting either that a trace contaminant (e.g. $Zn^{2+}$ ions) is the true reactivator or that the calcium acts by removing bound EDTA from the enzyme. For the yeast dehydrogenase Curdel & Labeyrie (1961) produced evidence lending support to the suggestion by themselves and by Boeri *et al.* (1960) that the native metal of the enzyme may be removed by EDTA and be functionally replaced by added metal ions. Further studies with the different enzymes are obviously necessary.

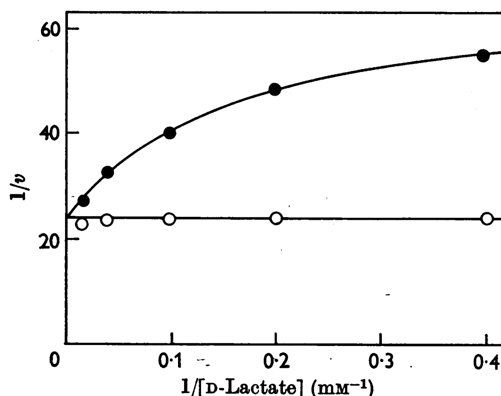


Fig. 8. Complete protection from phenanthroline at infinite concentration of D-lactate. Enzyme (stage 3; 295 µg./ml.) was preincubated for 115 min. at 0° (pH 8·1); the preincubation mixtures contained the concn. of D-lactate indicated. After incubation the enzyme activity was assayed as for Fig. 7, by using 89 µg. of protein in each test. ●, 0·5 mM-Phenanthroline in preincubation system; ○, phenanthroline absent.

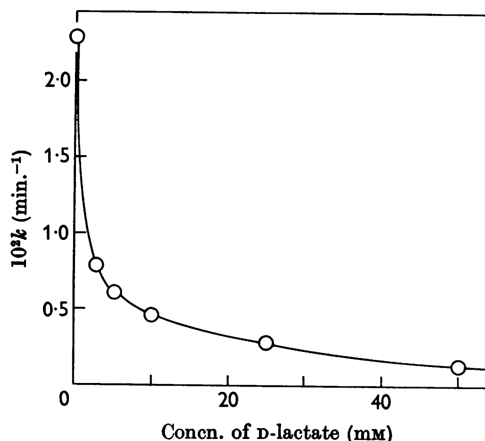


Fig. 9. Effect of substrate on the velocity constant for phenanthroline inhibition. Experimental conditions as for Fig. 8; *k* was calculated by using the equation given in the text.

*Inhibitor potentiation by cyanide.* The fact that cyanide, unlike any other substances tested, potentiates inhibition by 1,10-phenanthroline and (to a lesser degree) EDTA has already been reported and tentatively attributed to a ligand-exchange process (Tubbs, 1960*c*). At the time of writing the earlier communication it was not known that Davison (1957) had shown cyanide potentiation of inhibition of monoamine-oxidase preparations by iproniazid (1-isonicotinoyl-2-isopropylhydrazine); further work on both D-hydroxy acid dehydrogenase and monoamine oxidase is necessary before the common factors, if any, between these two cyanide potentiations become evident. For the dehydrogenase the relationship between (i) the

Table 3. *Reactivation by metal ions after inhibition by ethylenediaminetetra-acetate*

Enzyme (stage 2; 4·5 mg./ml.) was completely inhibited by preincubation with 0·5 mM-EDTA for 150 min. at 0° and pH 7·8. No reactivation was obtained by subsequent dialysis for 17 hr. at 4° against 200 vol. of 20 mM-tris buffer, pH 7·8; the buffer was changed after 4 hr. Samples of enzyme were then incubated for 30 min. at 0° with the metal salts (0·5 mM) shown, before being tested in the standard system with or without cyanide. (Uninhibited enzyme gave a decrease of $E_{600\,m\mu}$/min. of 0·074, with or without cyanide.)

| | Decrease of $E_{600\,m\mu}$ | |
|---|---|---|
| Metal salt | No cyanide | 0·4 mM-Cyanide |
| $ZnSO_4$ | 0·067 | 0·072 |
| $FeSO_4$ | 0·012 | 0·034 |
| $MgCl_2$ | 0·000 | 0·020 |
| None | 0·000 | 0·000 |

Table 4. *Reactivation by calcium ions after inhibition by ethylenediaminetetra-acetate*

Enzyme was completely inhibited by EDTA and subsequently dialysed, as described in Table 3. Samples were then incubated at 4° for 24 hr. without or with $CaCl_2$ (approx. 2 mM), and were then tested in the standard assay without cyanide; the system contained 0·72 mg. of protein and, where present, approx. 130 µM-$Ca^{2+}$.

| | Decrease of $E_{600\,m\mu}$/10 min. |
|---|---|
| Incubated with $CaCl_2$ | 0·036 |
| Incubated without $CaCl_2$ | 0·000 |
| Enzyme not exposed to EDTA | 0·330 |

potentiation of inhibition, (ii) the enhancement of reactivation by metals (Table 3), and (iii) the activation of fresh enzyme (Tubbs & Greville, 1961) by cyanide likewise remains to be clarified.

## DISCUSSION

From the results of inhibition experiments reported here and previously (Tubbs, 1960*c*) it appears that kidney D-α-hydroxy acid dehydrogenase contains a functional metal component; it further seems likely that the metal is involved in substrate binding, presumably by forming a chelate with the hydroxyl and carboxyl groups. Similar experiments have been carried out independently by two groups of workers with a very similar enzyme from yeast (see Curdel & Labeyrie, 1961; Boeri *et al.* 1960).

The yeast dehydrogenase is a flavoprotein (Boeri *et al.* 1960; Nygaard, 1960) and the general properties of the mammalian enzyme make it appear probable that the same is true in this case (Tubbs & Greville, 1961). Further experiments are necessary to shed light on the possible involvement of the metal component of either enzyme in electron transport, as has been suggested by Curdel & Labeyrie (1961); the oxidation of alcohols by metal ions may proceed by formation of a metal–substrate complex, followed by hydrogen-atom transfer (see, for example, Littler & Waters, 1959). Such a mechanism might well occur in an enzymic oxidation; however, the role of metals in flavoproteins is poorly understood (Singer & Massey, 1957), and if the native metal of the D-hydroxy acid dehydrogenase is in fact zinc (Curdel & Labeyrie, 1961) an electron-transfer function would seem unlikely.

If the metal is indeed the substrate-binding site the protein would of course greatly affect the binding, as well as the catalytic, specificity. Even simple optically-active ligands, such as 1,2-diaminopropane (pn), can confer very marked stereospecificity of metal–complex formation: for example DL-lactate is more readily resolved by treatment with $[Co(l\text{-pn})_2CO_3]^+$, which results in preferential co-ordination of D-lactate, than via the usual alkaloid salts (Gott & Bailar, 1952). The facts that D-α-hydroxy acids with branched hydrocarbon side chains are not substrates and that the reaction rate, even at infinite substrate concentration, is affected by the chain length of lactate homologues (Tubbs & Greville, 1961) indicate that the dehydrogenase protein interacts with the substrate side chain in a subtle manner.

Comparative lack of sensitivity to thiol reagents implies that no freely accessible thiol groups are involved in the action of the enzyme. Thiol groups could of course be important indirectly, for example by binding the metal component. It is also possible that some of the effects of cyanide may be due to rupture of disulphide linkages.

Further studies are necessary for an understanding of the mechanism (and indeed physiological role) of the D-α-hydroxy acid dehydrogenases; it is already evident that these, whether from animal tissues, yeast or bacteria, are very different enzymes from the well-known dehydrogenases with the opposite stereospecificity.

## SUMMARY

1. The action of inhibitors on D-α-hydroxy acid dehydrogenase from rabbit-kidney mitochondria is examined.

2. Oxalate, malonate, L-lactate, α-hydroxyisobutyrate and cyanide inhibit competitively with respect to the substrate; the first ($K_i$ 5–10 $\mu$M) is the most potent.

3. It is pointed out, in connexion with experiments with DL-hydroxy acids as substrates, that competitive inhibitors, when present as contaminants of the substrate, cause a decrease in the observed Michaelis constant, and thereby increase the apparent enzyme–substrate affinity.

4. Pyruvate inhibition with respect to substrate (D-lactate) is of mixed type, but is competitive with respect to acceptor (2,6-dichlorophenol-indophenol). The implications of this are discussed in connexion with the enzymic mechanism.

5. Inhibition by reagents attacking thiol groups is relatively weak.

6. Chelating agents inhibit the enzyme gradually; the reaction obeys first-order kinetics. Substrates and competitive inhibitors (except cyanide) protect the enzyme from such inhibition. There is a correlation between the apparent affinity for the enzyme as measured by $K_m$ or $K_i$ and as measured by the protection afforded from chelating agents.

7. After inhibition by chelating agents, and subsequent dialysis, the enzyme activity is partially restored by various metal ions; cyanide enhances this reactivation in some cases.

8. The possible function of the metal component in the mechanism of action of the enzyme is considered.

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# Hydrolysis of some Casein Fractions with Plasmin

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Derechin (1961) showed that, when the proteolysis of whole casein is followed by estimating the ultraviolet-absorbing material remaining in solution after treatment of the reaction mixture with perchloric acid, a curve was obtained showing a lag period before a linear portion. The slope of this linear region is proportional to the amount of enzyme present and on this basis an assay for human plasmin was devised. However, when different batches of casein were used for the assay of a single plasmin preparation, apparent differences in activity up to 15% were obtained. These results necessitated a further study of casein to establish firstly the causes for these differences from batch to batch, secondly the reasons for the curve with a lag period and finally to seek for a more suitable protein substrate for plasmin assay.

Casein consists of several electrophoretically distinguishable components (Mellander, 1939), and a procedure for separating these in the presence of urea was described by Hipp, Groves, Custer & McMeekin (1952). From electrophoretic observations made on the protein fractions obtained by this procedure it was suspected that the α-casein precipitable in 4·7M-urea differed from that remaining with the β- and γ-casein in the supernatant solution, and further experiments confirmed this. Both the components of α-casein were purified and the action of plasmin on each of them was examined.

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## EXPERIMENTAL

*Buffers.* Phosphate buffers, pH 8 (65 mM-$Na_2HPO_4$–4 mM-$KH_2PO_4$) and pH 7·6 (63 mM-$Na_2HPO_4$–9 mM-$KH_2PO_4$), were used to dissolve the casein for electrophoresis and for hydrolysis with plasmin respectively. Phosphate–urea: 6·6M-urea in phosphate buffer, pH 8. Phosphate–NaCl soln.: 10% (v/v) phosphate buffer, pH 7·6, in 0·2M-NaCl.

pH *determination.* Values of pH were determined with a MacInnes & Belcher (1933)-type glass electrode. Measurements were made at room temperature with a valve potentiometer and standardized with HCl–KCl mixture, pH 2·10 (0·01M-HCl–0·09M-KCl).

*Electrophoresis.* Analyses were carried out in the Tiselius apparatus at 0°, the diagonal schlieren optical system, with monochromatic light λ 546 mμ, being used. Protein concentrations were between 1·0 and 1·75 g./100 ml. The proportion of each protein in a mixture was calculated from its relative area in the electrophoresis pattern.

*Ultracentrifugal analysis.* Protein solutions in phosphate buffer, pH 8, containing in addition 0·15M-NaCl, were subjected to 250 000g in the Svedberg oil-turbine ultracentrifuge (Svedberg & Pedersen, 1940), with a 12 mm. cell. Optical observations by the diagonal schlieren method (Philpot, 1938) were photographically recorded on Ilford rapid-process panchromatic plates, a high-pressure Hg arc being used as a light source, from which a monochromatic light, λ 546 mμ, was isolated with a suitable filter.