Oxaloacetate Decarboxylase from Cod. Mechanism of Action and Stereoselective Reduction of Pyruvate by Borohydride*

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ABSTRACT: Sodium borohydride has no effect upon the activity of the enzyme, oxaloacetate decarboxylase from cod, regardless of whether substrate is present or absent, or whether pyruvate (the reaction product) is present or absent. However, the reduction of pyruvate by borohydride in the presence of manganous ions and enzyme leads to the formation of an excess of D-lactate, as shown by the dispersion curve for optical activity and by specific enzyme assays with D- and L-lactate dehydrogenases. The enzymic catalysis of borohydride reduction depends upon the presence of metal ions and does not occur in the presence of EDTA. These facts are consistent with the hypothesis that reduction and decarboxylation occur by way of a metal ion–enzyme–substrate complex analogous to that previously suggested for relevant model systems.

The mechanisms of enzymatic decarboxylation of β-keto acids can be distinguished by their dependence upon or independence of metal ions. For example, the oxaloacetate decarboxylases from *Micrococcus lysodeikticus* (Herbert, 1955), from cod (Schmitt *et al.*, 1966), and from other sources (Utter, 1961) require metal ions for activation. By way of contrast, the decarboxylase from *Clostridium acetobutylicum* catalyzes the decarboxylation of acetoacetic acid by way of a Schiff base salt between enzyme and substrate as intermediate (Tagaki and Westheimer, 1968, and references cited therein). The enzyme utilizes a specific lysine residue (Warren *et al.*, 1966; Laursen and Westheimer, 1966) but does not require metal ions. A similar situation obtains with respect to the aldol condensation, where enzymes of class II require metal ions for activity (Rutter, 1964), whereas enzymes of class I react by way of Schiff base salts as intermediates (Grazi *et al.*, 1962).

The oxaloacetate decarboxylase from cod (Schmitt *et al.*, 1966) has now been crystallized (Kosicki, 1968) and shown to depend upon metal ions, preferably Mn^{2+}, for activity. The present investigation was directed toward determining the mechanism of action of this enzyme. The decarboxylation was conducted in the presence of borohydride, in an attempt to trap a Schiff base (see the review by Fischer, 1964); this method has been successful in trapping the Schiff bases from pyridoxal and phosphorylase *a* (Fischer *et al.*, 1958), transaldolase (Horecker *et al.*, 1961), aldolase (Grazi *et al.*, 1962), acetoacetate decarboxylase (Fridovich and Westheimer, 1962), 2-keto-3-deoxy-6-phosphogluconate aldolase, deoxyribose 5-phosphase aldolase (Grazi *et al.*, 1963), and 2-keto-4-hydroxyglutarate aldolase (Kobes and Dekker, 1966). Borohydride reduction, however, does not inactivate the oxaloacetate decarboxylase from cod, either in the presence or absence of oxaloacetate (substrate), or pyruvate (product). On the other hand, borohydride reduction of pyruvate in the presence of enzyme and metal ions leads to the formation of an excess of D-(−)-lactate. This reduction occurs only in the presence of polyvalent cations and is inhibited by oxalate, and so presumably takes place at the active site of the enzyme. These facts are in accordance with mechanisms for decarboxylation and reduction that parallel that for the nonenzymic model system of Steinberger and Westheimer (1951).

Experimental Section

Materials. Oxaloacetate decarboxylase from cod fish was prepared according to the directions of Kosicki (1968). The concentration of enzyme, given in milligrams per milliliter, is based on the 280 mμ to 260 mμ absorbance ratio (Warburg and Christian, 1942) and the units of enzyme activity are given in micromoles of oxaloacetate decarboxylated per minute at 30° (specific activity of crystalline enzyme is 1.8). Some samples of the enzyme were prepared by direct crystallization of the eluates from the calcium phosphate column; these are designated as A. Other samples, here designated as B, were freed of residual calcium ion by the following procedure. The enzyme from the calcium phosphate column was incubated for 15 min at room temperature with 0.1 M EDTA (disodium salt) at pH 5.80 and then precipitated by the addition of $(NH_4)_2SO_4$ (final concentration 313 g/l. of solution). The precipitated enzyme (77.7 mg, specific activity of 1.8) was redissolved in 3.70 ml of 0.02 M potassium phosphate buffer (pH 6.80) and desalted on a 2.5 × 30.0 cm column of Sephadex G-25; the enzyme was eluted from the

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TABLE I: The Effect of $NaBH_4$ on the Activities of Oxaloacetate Decarboxylase and Acetoacetate Decarboxylase.

Oxaloacetate Decarboxylase (mg)	Acetoacetate Decarboxylase (mg)	Oxaloacetate (M × 10^3)	Pyruvate (M × 10^3)	Lithium Acetoacetate (M × 10^3)	$NaBH_4$ (M × 10^3)	Rel Enzymic Act.
0.1		7.7				100
0.1		7.6			5.5	76
0.1					5.5	72
0.1			1.0		5.5	62
0.1[a]			1.0		5.5	63
0.1			1.0			56
	0.1			3.3		100
	0.1			3.3	5.5	0
	0.1				5.5	66

[a] Oxaloacetate decarboxylase was added after the reduction by $NaBH_4$ and just prior to the pH 6.6 oxaloacetate decarboxylase assay. Each reaction vessel (1.0 ml, 0°) contained 0.06 M potassium phosphate (pH 6.0), oxaloacetate decarboxylase of specific activity 1.8, or acetoacetate decarboxylase of specific activity 41.

column with the same phosphate buffer and appeared in about 21 ml of eluate. Acetoacetate decarboxylase (specific activity 41) was prepared by a modification of the method of Zerner *et al.* (1966) (*cf.* Westheimer, 1968). The purified, specific D-LDH,[1] prepared from *Leuconostoc mesenteroides*, was a generous gift from Dr. N. O. Kaplan and Mrs. Rebecca Garland of Brandeis University. The D-LDH had been purified by an ammonium sulfate fractionation, and column chromatography on DEAE-Sephadex A-25 and Sephadex G-100. The specific L-LDH was a preparation from rabbit muscle (Boehringer) of specific activity 473 EU/mg of protein. The L-MDH was a preparation from pig heart (Boehringer).

The following materials were commercial preparations: sodium borohydride (98 + % pure, Metal Hydrides, Inc., Beverly, Mass.), oxaloacetic acid, L-(+)- and D-(−)-calcium lactates and sodium pyruvate (Calbiochem), L-(−)- and DL-malic acid (Eastman), D-(+)-malic acid (Nutritional Biochemicals), NAD and acetylpyridine NAD (P-L Biochemicals), dithioerythritol (Cyclo-Chemical). Lithium acetoacetate was prepared by the method of Hall (1963). Other reagents were commercial preparations of analytical grade.

Assay of Oxaloacetate Decarboxylase. The assay (Kosicki, 1968) depends upon following the decrease in absorption of the enol form of the equilibrated tautomeric mixture of the oxaloacetate–Mn^{2+} complex at 260 mμ.

Lactate Assays. Lactate was assayed by measuring the enzymic reduction of acetylpyridine NAD at 365 mμ (log ε 9.1 × 10^3 at pH 9.5) in the presence of the unknown, by a modification of the method of Dennis and Kaplan (1960). Each cuvet contained 0.5 M Tris–Tris-HCl buffer at pH 9.0, 0.015 M acetylpyridine NAD, and either L- or D-lactic dehydrogenase.

Acetoacetate decarboxylase was assayed by following the decrease of the enol absorption of lithium acetoacetate at 270 mμ as described elsewhere (Zerner *et al.*, 1966). L-Malate was assayed using L-malate dehydrogenase (Holzer and Soling, 1963).

Method of Reduction. A solution of about 6 mg of crystalline oxaloacetate decarboxylase was prepared in 3–4 ml of 0.30 M potassium phosphate buffer at pH 6.00. To this solution, solutions of sodium borohydride (9.0 ml of 0.028 M solution in 10^{-3} M NaOH) and sodium pyruvate (9.00 ml of 0.10 M solution adjusted to pH 6.0) were added over a period of 60–80 min with stirring at about 25°; the slow rate of addition was maintained with a Buchler peristaltic pump. Each reaction was stopped by acidification to pH < 1 with concentrated HCl, and the mixture evaporated with a rotary evaporator to a moist white solid. The residue was extracted with four 20-ml portions of ether. The ether solution was in turn evaporated to an oily residue which was then dissolved in 2.0 ml of 2 N NaOH (final pH about 12). A 400-μl sample of the alkaline concentrate was purified by thin-layer chromatography as described below. Control experiments with D-lactic acid showed that this method of concentrating and purifying recovered 35–45% of the lactate present.

Thin-Layer Chromatography. Lactate (and malate) formed during $NaBH_4$ reductions were purified from borates and other products by chromatography on silica gel plates (20 × 20 cm, Silica-GF 254, Merck). Each sample, applied in a band, was developed in a mixture of ethanol (95%), NH_4OH (concentrated), and water in a ratio of 80:4:16 (Prey *et al.*, 1962) for 2 hr at room temperature. Under these conditions the lactate migrates with an R_F of 0.51 and is separated from pyruvate (R_F of 0.41), malate (R_F 0.15), and acetate (R_F of 0.66). The respective areas were located by

[1] Abbreviations used are: D-LDH, lactate dehydrogenase specific for D-(−)-lactic acid; L-LDH, lactate dehydrogenase specific for L-(+)-lactic acid; L-MDH, malate dehydrogenase specific for L-(−)-malic acid.

TABLE II: The Effect of Pyruvate, Lactate, and Borate on the Activity of Oxaloacetate Decarboxylase.[a]

Reaction	Preincubation of Oxaloacetate Decarboxylase 1:1 ratio	Rel Rate of Decrease in 260-mμ Absorption			
		+ Mn^{2+}	+ Mn^{2+} + Borate	+ Borate	+ H_2O
1	Oxaloacetate decarboxylase + D-(−)-lactate	32	54		
2	Oxaloacetate decarboxylase + L-(+)-lactate	74	110		
3	Oxaloacetate decarboxylase + pyruvate	70	101		
4	Oxaloacetate decarboxylase + water	100	130	28	11
5	Water	3	18	13	3

[a] Each reaction cuvet (1.0 ml, 30°, pH 6.6 oxaloacetate decarboxylase assay) contained 3×10^{-2} M potassium phosphate buffer (pH 6.60), 10^{-3} mg/ml of oxaloacetate decarboxylase, 7.7×10^{-3} M oxaloacetate as well as 10^{-3} N D-(−)- or L-(+)-calcium lactate, or 10^{-3} N sodium pyruvate, or 10^{-4} M $MnSO_4$, or 5×10^{-3} M sodium borate (pH 6.2) as indicated.

slight ultraviolet fluorescence quenching or by bromocresol green pH indicator spray. The silica gel was scraped off and extracted with 5.0 ml of 0.15 M NH_4OH, followed by two further 3.0-ml washes. The washes and eluate were combined and concentrated on a rotary evaporator, and the final solution was adjusted to a weight of 2.00 g. The samples were acidified with concentrated HCl before optical rotatory dispersion measurements (pH < 1).

Instrumentation. The ultraviolet absorbance changes were measured on a Cary 15 spectrophotometer with a thermostatted cell chamber at 30°. The optical rotatory dispersion measurements were made on a Cary 60 instrument with cells thermostatted at 25°.

Results

Borohydride Reduction. Sodium borohydride does not appreciably inhibit the activity of oxaloacetate decarboxylase whether or not it is in the presence of its substrate, oxaloacetate (Table I). The same reduction conditions, however, do inhibit acetoacetate decarboxylase activity (Warren *et al.*, 1966). Sodium borohydride dissolved in 0.001 N NaOH was added in two 0.1-ml portions 2 min apart. The oxaloacetate decarboxylase and acetoacetate decarboxylase were assayed by taking aliquots of the reaction mixtures as the source of the enzyme for the assays described in the Experimental Section.

The products of oxaloacetate decarboxylase decarboxylation and of $NaBH_4$ reduction, that is, pyruvate and both D- and L-lactates, inhibit the activity of oxaloacetate decarboxylase. The inhibitions are relieved in the presence of borate (Table II). In experiments designed to show this effect, the oxaloacetate decarboxylase (specific activity 1.8) was preincubated with pyruvate or lactate at room temperature for 1 min before adding the mixture as the final component (0.2 ml) to the cuvet. A decrease in the rate of absorbance change (260 mμ) was observed with borate replacing the $MnSO_4$ (Table II, reaction 4). The enzyme requires a metal ion, preferably Mn^{2+}, for activity, a shown in Table III.

Stereoselectivity. The reduction of pyruvate by borohydride in the presence of oxaloacetate decarboxylase yields lactate. The product is optically active, where the D-(−)-acid is formed in excess over the L-(+). The identification of lactate and the quantitative analysis of the products has been carried out by two different means: (1) analysis for each enantiomer has been conducted with the corresponding specific dehydrogenase, and (2) the lactic acid has been purified by extraction from the reaction mixture, purified by thin-layer chromatography on silica gel, and its optical rotatory dispersion has been determined; these methods are described in the Experimental Section. The stereoselective reduction, like the decarboxylation, is dependent upon

TABLE III: The Stimulation of Oxaloacetate Decarboxylase Activity by $MnSO_4$.[a]

Additions	Enzymic Act. (units/ml)
Buffer + oxaloacetate	0.0025
Buffer + oxaloacetate + oxaloacetate decarboxylase	0.0025
Buffer + oxaloacetate + oxaloacetate decarboxylase + $MnSO_4$	0.0113
Buffer + oxaloacetate + oxaloacetate decarboxylase + $MnSO_4$ + EDTA	0.0024
Buffer + oxaloacetate + $MnSO_4$	0.0025

[a] Each cuvet contained the components of the pH 6.6 assay described in the Experimental Section except for the additions where indicated of $MnSO_4$ and/or the disodium salt of EDTA, to final concentrations of 10^{-4} and 2×10^{-3} M, respectively.

TABLE IV: Stereoselective Reduction of Pyruvate by BH_4^- and Oxaloacetate Decarboxylase.

Reaction	Oxaloacetate Decarboxylase,[b] mg (units)	Type	Pyruvate (M × 10^3)	Mn^{2+} (M × 10^3)	EDTA (M × 10^2)	$NaBH_4$[c] (M × 10^3)	Lactic Acid Added: M × 10^2	Lactic Acid Added: Type	μmoles of Lactic Acid Produced: L-LDH[d]	μmoles of Lactic Acid Produced: D-LDH	μmoles of Lactic Acid Produced: % D	Optical Rotation Obsd 225 mμ[e]	Total Excess D: μmoles[f]	Total Excess D: % D[g]
1	22 (10)	A	3.92	4.35		1.10			153	238	61	−0.055	92	61
2	22	A[a]	3.92	4.35		1.10			186	196	51	0.000	0	50
3	22	A	3.92			1.10			164			−0.025	42	56
4	22	A	3.92		9.00	1.10			150			0.000	0	50
5	6.3 (11.5)	B	3.92	4.35		1.10			148			−0.176	294	75
6	6.3	B	3.92			1.10			121			−0.030	50	59
7	6.3	B	3.92	4.35		1.10			143			−0.176	294	76
8	6.3	B	3.92		0.46	1.10			170			0.000	0	50
							2.0	D-(−)				−0.120		100
							2.0	L-(+)				+0.120		0
							2.0	65-D:35-L				−0.036		65

[a] Oxaloacetate decarboxylase added *after* the reduction was completed. [b] The concentrations of the reaction mixtures were calculated for a final volume of 23.0 ml containing 3.92 × 10^{-2} M potassium phosphate buffer (pH 6.0). The pyruvate and $NaBH_4$ were added as described in the Experimental Section. [c] Lactic acid solutions of known concentration acidified with HCl to pH < 1 used for standards of optical activity. [d] The total amount of lactic acid recoverable used to calculate the amount of racemic mixture of D- plus L-lactic acids needed for (*f*) below. [e] The optical activity measured from 300 to 200 mμ in a cell of 0.5-cm light path using an acidified aliquot (purified by thin-layer chromatography) consisting of one-fifth of the total sample dissolved in 2.0 ml. [f] The excess amount of D-(−)-lactic acid recoverable as calculated from *c* and *e*. [g] Calculated from *d* and *f*.

TABLE V: Inhibition of Stereoselective Reduction[a] of Pyruvate by Oxalate.

mg	Oxaloacetate Decarboxylase (units)	Type	Pyruvate (M × 10^2)	Mn^{2+} (M × 10^5)	EDTA (M × 10^4)	Lithium Oxalate (M × 10^3)	$NaBH_4$ (M × 10^2)	μmoles of L-Lactic Acid, L-LDH Assay	μmoles of D-Lactic Acid Optical Act.[b]	% D-(−)-Lactic Acid
3.4	3.4	A	2.5	6.3			1.5	7.1	8.4	68
3.4	3.4	A	2.5		6.3		1.5	6.7	8.4	69
3.4	3.4	A	2.5				1.5	4.7	6.2	69
3.4	3.4	A	2.5			6.3	1.5	7.5	0.0	50

[a] The reactions and purifications were carried out as described in the Experimental Section except that the $NaBH_4$ (9.0 ml, 2.75 × 10^{-2} M) was added over a period of 20 min to the mixture already containing pyruvate. The reaction mixture, in a final volume of 16 ml of 5.6 × 10^{-2} M potassium phosphate buffer (pH 6.0) was maintained at 0°. [b] Calculated as in Table IV.

TABLE VI: Inhibition of Oxaloacetate Decarboxylation by Oxalate.[a]

Additions	Enzymic Act. (units/ml)
Buffer + oxaloacetate + Mn^{2+} + oxaloacetate decarboxylase	0.0169
Buffer + oxaloacetate + Mn^{2+} + oxaloacetate decarboxylase + oxalate	0.0010
Buffer + oxaloacetate + Mn^{2+}	0.0030
Buffer + oxaloacetate + oxaloacetate decarboxylase	0.0022

[a] Each reaction cuvet (1.0 ml, 30°) contained the indicated components of the pH 6.6 assay described in the Experimental Section. To reaction cuvet 1, lithium oxalate (final concentration of 5×10^{-3} M) was added after 1-min reaction. The inhibited rate was reached after a lag of 1 min (reaction 2).

metal ion, although of course borohydride reduces pyruvate to racemic lactate whether enzyme is present or not. Presumably the part of the reduction that occurs on the enzyme is stereospecific and the mixtures of D- and racemic lactate in our experiments arise from competition between reduction on the enzyme and reduction in the bulk of the solution.

The data showing the reduction of pyruvate by borohydride in the presence of oxaloacetate decarboxylase are given in Table IV, and a graph of the critical experiments with optical rotatory dispersion is shown as Figure 1. It should be noted that enzyme A, which presumably contains considerable calcium ion, promotes stereoselective reduction to a small extent which could be eliminated only in the presence of 0.09 M EDTA (disodium salt). Enzyme B, which has been purified so as to reduce its content of metal ions, causes some stereoselective reduction of pyruvate even when no extra metal ion is added, but the stereoselectivity is much greater in the presence of Mn^{2+} and is totally eliminated in the presence of 0.0046 M EDTA (disodium salt).

TABLE VII: Reduction of Oxaloacetate with $NaBH_4$ in the Presence of Oxaloacetate Decarboxylase.[a]

Product	R_F	μmoles of L Form	Assay	Optical Act. 200–300 mμ
DL-Malate	0.15	200	L-MDH	0.00
DL-Lactate	0.51	16	L-LDH	

[a] The reaction mixture (25°) of 16.0 ml contained 3.4 mg (3.4 units, type A) of oxalacetate decarboxylase, 5.6×10^{-2} M potassium oxaloacetate, 6.3×10^{-5} M $MnSO_4$, and 1.5×10^{-2} M $NaBH_4$. The $NaBH_4$ was added over a 5-min period. The reaction was stopped and the mixture was concentrated and purified as described in the Experimental Section except that two additional acetone washes were used to extract the acidified and evaporated reaction mixture to elute the malic acid. Using D-malate as a control showed that the method of concentration and purification recovered 50% of the malate present.

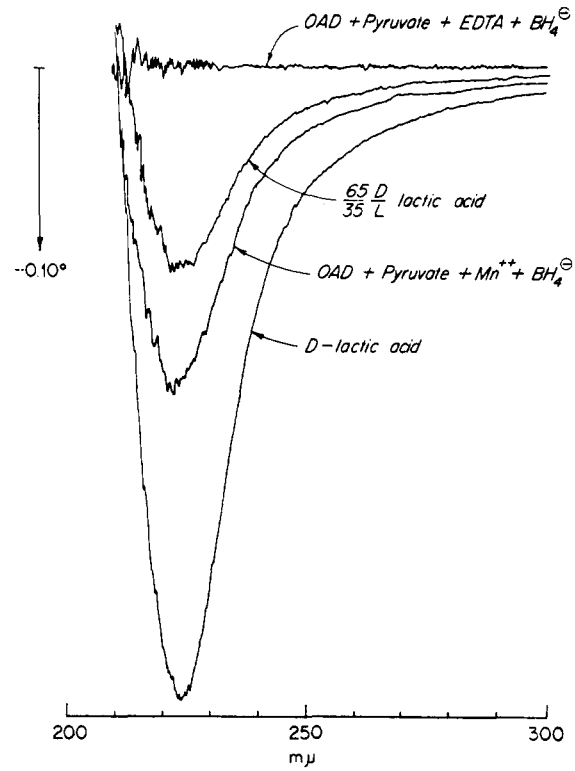
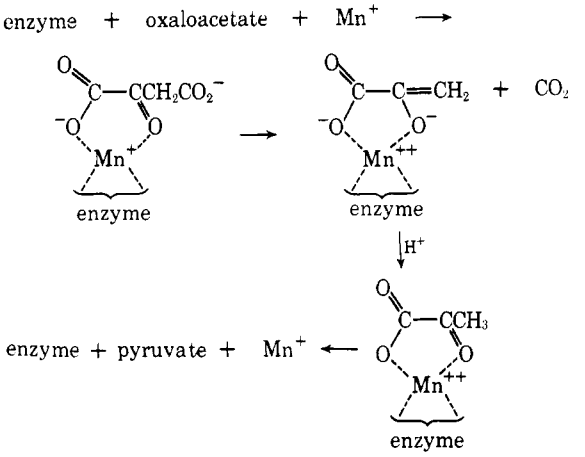


FIGURE 1: The optical activity of stereoselectively reduced pyruvate by BH_4^- and oxaloacetate decarboxylase. The compositions of the mixtures are presented in Table IV (reactions 7 and 8). The known mixture of D-(−)-lactic acid and D/L (Table IV, reactions 9 and 11) was diluted to 5.8×10^{-2} M in total lactate to correspond in concentration to the complete reaction mixture (Table IV, reaction 7).

Rates. In one experiment with 11.5 units of oxaloacetate decarboxylase and 4.6×10^{-5} M Mn^{2+}, reduction of pyruvate with borohydride yielded 294 μmoles of excess D-lactate in 67 min. This corresponds to 4.4 μmoles of lactate produced per min, as compared with a decarboxylation rate for the same quantity of enzyme of 11.5 μmoles/min. No attempt was made to study the conditions for saturation of the enzyme with pyruvate for reduction; such a study would be complicated by the rapid acid-catalyzed destruction of borohydride. But in any case the rate for the reduction is at least one-third of that for the decarboxylation.

Oxalate is a strong competitive inhibitor for the decarboxylase, with a K_I of about 10^{-5} M (Schmitt *et al.*, 1966). It is also a strong inhibitor for the borohydride reduction of pyruvate (Tables V and VI).

SCHEME I



Malate. Although the enzyme catalyzes the reduction of pyruvate with the production of an excess of the D enantiomer, it does not produce optically active malate by the reduction of oxaloacetate in the presence of enzyme. The reduction to malate can be proved with malic dehydrogenase, but the purified malic acid shows no rotation in measurements of optical rotatory dispersion down to 210 mμ (Table VII).

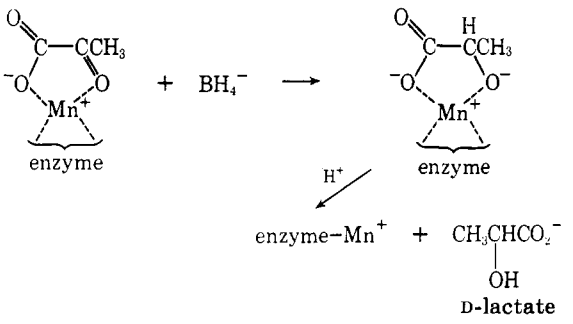
Discussion

The present research shows that oxaloacetate decarboxylase from cod requires a metal ion for activity, but since the enzyme is not inactivated by borohydride and substrate, it presumably does not operate by way of the Schiff base mechanism that obtains for acetoacetate decarboxylase. The mechanism for the decarboxylation then is probably that suggested, from studies of model systems, by Steinberger and Westheimer (1951) (Scheme I).

The activity of the metal ion is presumably enhanced by appropriate chelation or by a change in local dielectric constant, or both, in accordance with the researches of Rund and Plane (1964) and of Rund and Claus (1967). (The diagrams in this section are intended to show chelation of metal ion to enzyme, but are not intended to imply the number of enzymic ligands.)

The enzyme-promoted reduction of pyruvate by borohydride fits exactly into the pattern for the model system. Metal ion is required for the enzyme-catalyzed borohydride reduction, so that the pyruvate is presumably bound for the reduction just as it is bound as the product of the decarboxylation. The formation of optically active lactic acid as a result of reduction in the presence of the decarboxylase is positive proof that the reaction is an enzymic one. Further evidence that the reaction occurs at the active site of the decarboxylase comes from the strong and specific inhibition of reduction, as of decarboxylation, by oxalate. In all probability, then, the reduction of pyruvate takes place according to Scheme II. The internally consistent view of the reduction and decarboxylation strengthens the interpretation of the mechanism of each. The formation of lactate of the D configuration will eventually prove

SCHEME II



useful in indicating the stereochemistry of the binding of pyruvate to the enzyme.

Two problems remain that require further discussion. First, it is necessary to comment on the enzymic catalysis of the borohydride reduction. Borohydride rapidly reduces pyruvate in solution; in order for the enzymic reaction to compete effectively at low absolute concentrations of enzyme, the rate on the enzyme surface must be very much more rapid than that in solution. The rate is in fact large, and comparable with that for the enzymic decarboxylation. Presumably one of the factors that promotes the reaction is that the Mn^{2+} strongly polarizes the carbonyl group of pyruvate, so as to increase the positive charge on the carbonyl carbon atom, and so make it more susceptible to attack by the incipient H^- of borohydride. This is the same electronic effect that promotes the decarboxylation, and any influence of the enzyme on the metal ion that promotes one reaction should likewise promote the other. But in addition, there is at least the possibility that the enzyme requires a positive binding site that, in the decarboxylation, holds the second carboxyl group of the oxaloacetate; if so, such a binding site could attract the borohydride ion. This highly speculative hypothesis, if correct, could explain the second mechanistic problem: Why doesn't the enzyme promote the borohydride reduction of oxaloacetate itself to malate? Presumably it does not, since the malate produced with BH_4^- and enzyme is racemic. Putting aside as unlikely the possibility that the enzyme has promoted a nonstereoselective reduction, one must conclude that the rate of enzyme-promoted reduction is low compared with that for simple borohydride reduction in solution. This could be accounted for if the second carboxyl group of the oxaloacetate normally occupies the postulated positive binding site, so that borohydride cannot be accomodated. This explanation is similar to that that has been advanced to explain why, in the borohydride reduction of acetoacetate and acetoacetate decarboxylase, the Schiff base of the product (acetone) undergoes reduction, rather than the Schiff base of acetoacetate itself.

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