Biochemical Interactions of Dimethyl Sulfoxide II: Lactate Dehydrogenase-Catalyzed Reactions

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Abstract □ The effect of dimethyl sulfoxide upon the reactions catalyzed by lactate dehydrogenase was studied. Both the forward (lactate to pyruvate) and the reverse (pyruvate to lactate) reactions were investigated using dimethyl sulfoxide concentrations lower than those used by previous investigators. An aldol-type condensation of dimethyl sulfoxide with pyruvate is proposed as an explanation for the observation that the forward reaction is accelerated and the reverse reaction is slowed by the presence of dimethyl sulfoxide in the reaction medium. A product of the aldol-type condensation was identified in the enzyme reaction medium. The aldol condensation product was synthesized and determined to be 2-hydroxy-2-methyl-3-methylsulfinylpropanoic acid.

Keyphrases □ Dimethyl sulfoxide—effect on lactate dehydrogenase-catalyzed reactions, aldol-type condensation with pyruvate proposed, synthesis and identification of condensation product □ Lactate dehydrogenase-catalyzed reactions—effect of dimethyl sulfoxide, 2-hydroxy-2-methyl-3-methylsulfinylpropanoic acid proposed as dimethyl sulfoxide–pyruvate aldol-type condensation product and synthesized □ 2-Hydroxy-2-methyl-3-methylsulfinylpropanoic acid—synthesis, proposed as aldol-type condensation product of dimethyl sulfoxide–pyruvate in lactate dehydrogenase-catalyzed reactions □ Enzyme systems—effect of dimethyl sulfoxide on lactate dehydrogenase-catalyzed reactions (lactate to pyruvate and pyruvate to lactate)

In the previous paper of this series, the inhibition of the respiration of rabbit liver homogenates by dimethyl sulfoxide was reported (1). The effects of dimethyl sulfoxide upon isolated enzyme systems have received little attention in the literature. Ramler (2) studied the effects of high concentrations of dimethyl sulfoxide upon glutamate dehydrogenase- and lactate dehydrogenase-catalyzed reactions. He concluded that the observed inhibition resulted from a change in the secondary and tertiary structures of the enzyme protein.

L-Glutamic acid oxidation, catalyzed by glutamate dehydrogenase, was not affected by dimethyl sulfoxide concentrations of 10–20% (2), but there was a rapid lowering of reaction rate with increasing dimethyl sulfoxide concentrations above 10–20%.

L-Alanine also was reported (3) to be a substrate for glutamate dehydrogenase. L-Alanine oxidation, catalyzed by glutamate dehydrogenase, was observed to increase with increasing dimethyl sulfoxide concentrations (2).

The work described in this paper concerns the effects of dimethyl sulfoxide upon lactate dehydrogenase-catalyzed reactions. Ramler (2) showed an increase in the forward reactions (lactate to pyruvate) with dimethyl sulfoxide concentrations up to 30%. Dimethyl sulfoxide concentrations above 30% produced a marked decrease in the reaction rate.

Ramler did not investigate the effects of dimethyl sulfoxide upon the reverse reaction (pyruvate to lactate), nor did he study the effects of dimethyl sulfoxide at the concentration levels usually employed for enzyme inhibitors. His studies were conducted at pH 9 rather than at pH 7.4.

Therefore, it was decided to study the effects of dimethyl sulfoxide upon the lactate dehydrogenase-catalyzed reactions in less concentrated dimethyl sulfoxide solutions (10^{-3}–10^{-1} *M*) than those employed by Ramler. Both the forward and the reverse reactions were studied at pH 7.4. A product study was conducted on the enzyme reaction mixture to help establish the mechanism of the interaction of dimethyl sulfoxide with the lactate dehydrogenase-catalyzed reaction.

EXPERIMENTAL[1]

Reagents—The following were used: experimental drug grade dimethyl sulfoxide[2], redistilled under reduced pressure (100°/2 torr.); sodium lactate parenteral solution[3]; sodium pyruvate[4]; lactate dehydrogenase[5]; nicotinic adenine dinucleotide[6]; and methyl-*n*-propyl sulfide[7].

Enzyme Rate Studies—These studies were carried out using a spectrophotometer[8] equipped with a linear recorder[9] and a constant-temperature chamber set at 37°. The lactate dehydrogenase-catalyzed reactions were studied by assessing the rate of appearance or disappearance of nicotinic adenine dinucleotide by measuring the change in absorbance at 340 nm.

Lactate to Pyruvate Reaction—Studies of the forward reaction were conducted in a 0.06 *M* sodium phosphate buffer at pH 7.4. The initial reaction mixture was 8.0 m*M* in sodium lactate and 2.4 m*M* in nicotinic adenine dinucleotide. The dimethyl sulfoxide concentrations ranged from 0.00 to 10^{-1} *M*. In all studies, a constant lactate dehydrogenase concentration (0.001 mg. protein/ml., representing 0.147 unit of lactate dehydrogenase activity) was maintained. All materials, except the coenzyme, were placed in a cell and allowed to equilibrate to 37°. After 3 min., the reaction was initiated by adding the coenzyme. The coenzyme solution was added to the bottom of the cell and air was introduced to ensure mixing. The volume of each cell was 3 ml. Reaction rates were determined by comparing the absorbance at the end of 1 min. with standards of known concentrations.

Pyruvate to Lactate Reaction—The reverse reaction was run in a similar manner to that described for the forward reaction. The reaction was carried out in a 0.06 *M* sodium phosphate solution buffered at pH 7.4 and was 2 m*M* in sodium pyruvate and 0.6 m*M* in reduced nicotinic adenine dinucleotide; the enzyme concentration was 0.008 mg. protein/ml. (0.840 unit of lactate dehydrogenase activity).

Product Study—*Isolation of Aldol-Type Condensation Product from Enzyme Reaction*—In each of five 50-ml. conical flasks were placed 0.0032 ml. of an 8 m*M* sodium lactate solution, 5.27 ml. (0.1 *M*) of dimethyl sulfoxide, 0.001 ml. (0.003 mg. protein) of lactate dehydrogenase, and 5.63 ml. of 0.06 *M* sodium phosphate

[1] IR spectra were determined with a Beckman IR 20A. NMR spectra were determined using a Varian A60 NMR spectrometer. Carbon–hydrogen analyses were determined using a Hewlett-Packard model 185 C, H, and N analyzer. GC data were obtained using a Beckman GC-2A gas chromatograph. All temperatures are uncorrected.
[2] Crown Zellerbach Corp.
[3] Abbott Laboratories, Lot 825-0808.
[4] Nutritional Biochemicals Corp., Lot 2171.
[5] Worthington Biochemical Corp.
[6] Nutritional Biochemicals Corp.
[7] Aldrich Chemical Co.
[8] Beckman DB.
[9] Bausch & Lomb VOM 7.

Table I—Rates of the Lactate to Pyruvate Reaction

Dimethyl Sulfoxide Concentration, *M*	Mean Rate, mmoles/min.	Standard Error[a] (±)	*p*[b]
0.00	4.8×10^{-2}	7.8×10^{-4}	—
10^{-3}	5.0×10^{-2}	5.5×10^{-4}	0.038
10^{-2}	5.5×10^{-2}	7.5×10^{-4}	<0.0005
10^{-1}	5.9×10^{-2}	1.2×10^{-3}	<0.0005

[a] Degrees of freedom ≥ 40. [b] A one-tailed *t*-test was used.

Table II—Rates of the Pyruvate to Lactate Reaction

Dimethyl Sulfoxide Concentration, *M*	Mean Rate, mmoles/min.	Standard Error[a] (±)	*p*[b]
0.00	3.72×10^{-4}	1.88×10^{-5}	—
10^{-3}	1.90×10^{-4}	1.25×10^{-5}	<0.0005
10^{-2}	1.01×10^{-4}	4.10×10^{-6}	<0.0005
10^{-1}	0.31×10^{-4}	5.50×10^{-6}	<0.005

[a] Degrees of freedom ≥ 40. [b] A one-tailed *t*-test was used.

buffer, pH 7.4. The flasks were placed in a shaker water bath at 37° and allowed to equilibrate for 5 min. The reaction was started by adding 16 mg. of nicotinic adenine dinucleotide from a 2.4 m*M* solution. The reaction was run for 4 days, after which the flask contents were pooled, cooled in an ice bath, and adjusted to pH 1.4 with cold 15% sulfuric acid. The acidified mixture was extracted with ether in a continuous extractor for 48 hr. The extract was dried over anhydrous magnesium sulfate and the ether was removed *in vacuo*, leaving a small quantity of liquid residue which was subjected to TLC.

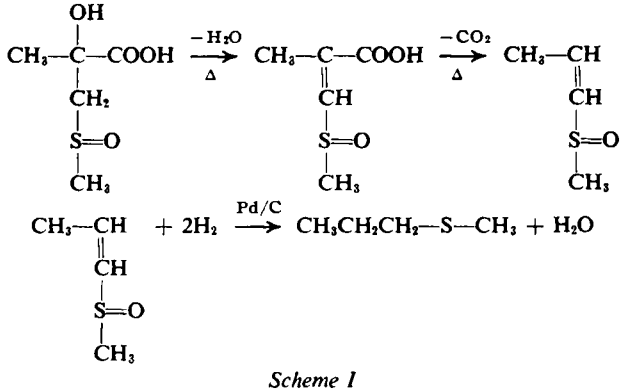
TLC—Ten microliters of the lactate to pyruvate reaction mixture or the residue from the continuous ether extraction was removed and applied to TLC plates[10]. The plates were developed using methanol–chloroform (75:25). The dried plates were treated with iodine vapors for visualization of the spots.

Synthesis of Proposed Intermediate (2-Hydroxy-2-methyl-3-methylsulfinylpropanoic Acid)—To 0.073 mole of potassium pyruvate or sodium pyruvate in 60 ml. of distilled water was added 9.33 ml. (10.30 g., 0.132 mole) of dimethyl sulfoxide. The pH of the mixture was adjusted to 7.5 using a 0.4 *N* sodium hydroxide solution, and the mixture was stirred at room temperature for 24 hr. Following the reaction period, the pH of the mixture fell to 6.5–7.0. The reaction mixture was cooled in an ice water bath to 0°, acidified to pH 2 with cold 15% sulfuric acid, and extracted with ether in a continuous extractor for 48 hr. The extract was dried over anhydrous magnesium sulfate for 48 hr. The ether was removed *in vacuo*, leaving 9.10 g. (75%) of a colorless to light-yellow corrosive liquid possessing a sour odor, b.p. 58°/0.6 torr. IR (neat on sodium chloride cells): 3400 (OH), 2900 [CH(sh)], 1650 (COOH carbonyl), and 1025 [—(O=)S—] cm.$^{-1}$; NMR (60 MHz., dimethyl sulfoxide-d_6): δ 2.6 (s, 3), 4.35 (m, 5), 1.9 (broad, 1), and 7.35 (broad, 1).

Anal.—Calc. for $C_5H_{10}O_4S$: C, 36.14; H, 6.07. Found: C, 36.10; H, 6.04.

The 2-hydroxy-2-methyl-3-methylsulfinylpropanoic acid was subjected to TLC as described, and the chromatogram was compared with that from the enzyme reaction mixture.

Chemical Degradation of 2-Hydroxy-2-methyl-3-methylsulfinylpropanoic Acid—Chemical degradation of the product of the aldol-type condensation was studied according to Scheme I. A solution of 2-hydroxy-2-methyl-3-methylsulfinylpropanoic acid (0.02 mole in toluene) was placed in a flask equipped with a Dean-Starke trap and a condenser. The top of the condenser was fitted with a delivery tube leading to a saturated solution of barium hydroxide. The toluene solution was refluxed overnight. A copious precipitate of barium carbonate formed in the barium hydroxide solution, and 0.5 ml. (0.0278 mole) of water was collected in the Dean-Starke trap.



Scheme I

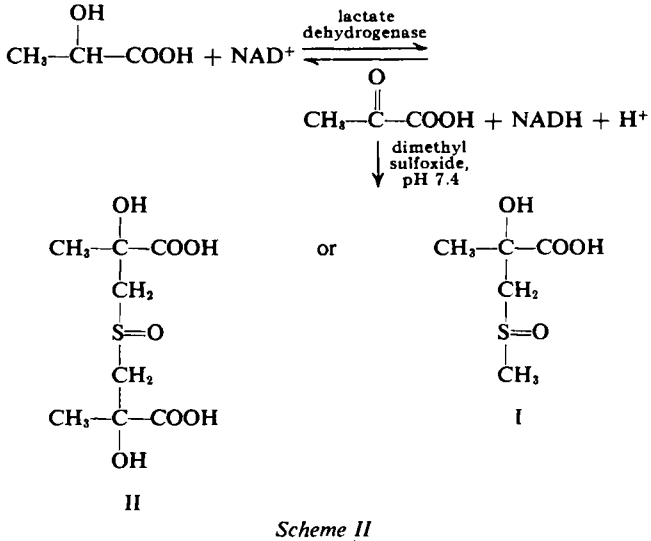
A second degradation was conducted using 0.06 mole of the 2-hydroxy-2-methyl-3-methylsulfinylpropanoic acid in 25 ml. of absolute ethanol. To this solution was added 2 drops of concentrated sulfuric acid. The solution was refluxed for 14 hr. under a nitrogen atmosphere, and the reaction was followed using the barium hydroxide solution described previously.

Following the reaction, the total mixture was hydrogenated on a low-pressure Parr hydrogenator at an initial hydrogen pressure of 65 p.s.i. and using 1 g. of palladium-on-charcoal (100 mg. palladous chloride/1 g. of charcoal) as the catalyst. After 12 hr. of hydrogenation, an additional 1 g. of catalyst was added and the hydrogenation was continued for another 12 hr. The catalyst was removed by filtration and the solution was distilled. The impure methyl-*n*-propyl sulfide fraction was collected at 32°/20 torr.

The methylpropyl sulfide fraction was subjected to vapor phase chromatography using a 1.8-m. (6-ft.) metal column packed with 1.15% silicone rubber SE-30 on Gas Chrom P (100–140 mesh). A thermal conductivity detector was employed. The helium carrier gas was maintained at a flow rate of 20 ml./min., and the column temperature was 70°. The sample volume was 10 μl. The retention time of the peak identified as methyl-*n*-propyl sulfide was 6.5 min. and corresponded to that of a commercial sample determined under identical conditions. An IR spectrum of the hydrogenation product was identical with that of commercial methyl-*n*-propyl sulfide containing 1 ml. of 95% ethanol/100 ml. of sulfide.

RESULTS

The rates of the forward reaction in the presence of varying concentrations of dimethyl sulfoxide are presented in Table I. The rates of the reverse reaction in the presence of varying concentrations of dimethyl sulfoxide are concentration dependent. This concentration dependence is statistically significant (Table II).



Scheme II

[10] Absorbasil-3, Applied Science.

TLC showed the presence of a compound in the enzyme reaction mixture and the ether extract which was neither lactate nor pyruvate nor dimethyl sulfoxide. The R_f value for this compound was identical with that for 2-hydroxy-2-methyl-3-methylsulfinylpropanoic acid. No intermediate spot could be detected in the absence of dimethyl sulfoxide in the reaction medium.

DISCUSSION

Baker *et al.* (4) showed that the lactate dehydrogenase reaction sites are the same. Therefore, inhibition at the reaction site should decrease the rates of both the forward and the reverse reactions. The increase in the forward reaction rate and the decrease in the rate of the reverse reaction could be explained by the presence of a concentration gradient on the reversible reaction in a direction favoring pyruvate formation. Such a concentration gradient could result from a complexation or condensation of dimethyl sulfoxide with pyruvic acid, thus removing that species from the reaction medium. A possible reaction that could remove effectively the keto acid salt (pyruvate) is an aldol-type condensation between the methyl groups of dimethyl sulfoxide and the carbonyl function of the pyruvate. Such a condensation may involve either a single methyl group or both methyl groups of the dimethyl sulfoxide (Scheme II). The effect on the enzyme-catalyzed reaction would be concentration dependent (Tables I and II).

The presence of 2-hydroxy-2-methyl-3-methylsulfinylpropanoic acid in the enzyme mixture leads to the conclusion that dimethyl sulfoxide interferes with the lactate dehydrogenase-catalyzed reaction by condensing with pyruvate in an aldol-type condensation.

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Cinchona Alkaloids on Ion-Exchange Resins V: Ammonium Form of Resins

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Abstract □ Equilibrium exchange of cinchona alkaloid sulfates and column runs of cinchona bark were carried out with the ammonium form of sulfonic acid cation-exchange resins. The results indicate that, for 100 meq. of the resin capacity, the amounts of the alkaloid-containing material extracted from cinchona bark are 15 and 8 g. for the ammonium forms of two commercially available ion-exchange resins. Dilute aqueous sulfuric acid was used as the extraction solvent, and ammonical ethanol was used as the eluant.

Keyphrases □ Cinchona alkaloids—separation from cinchona bark using ion-exchange resins in ammonium form, comparison of two commercially available resins, separation capacity □ Alkaloids, cinchona—separation from cinchona bark using ion-exchange resins in ammonium form, comparison of two commercially available resins, separation capacity □ Resins, ion exchange, in ammonium forms—comparison of separation capacities of two commercially available resins, cinchona alkaloids □ Ion-exchange chromatography—comparison of separation capacities of two commercially available resins, cinchona alkaloids □ Chromatography, ion exchange—comparison of separation capacities of two commercially available resins, cinchona alkaloids

A previous paper (1) reported the effect of the particle size of styrene divinylbenzene copolymer-based sulfonic acid cation-exchange resins on equilibrium exchange and column studies for cinchona alkaloid sulfates. The work suggested that a fine mesh resin with a relatively low degree of cross-linking (Resin I[1]) or with an expanded structure (Resin II[2]) in ammonium form could be suitable for recovery of cinchona alkaloids, with aqueous sulfuric acid as the solvent for the exchange run and ammoniacal ethanol as the solvent for the elution run. The present work includes a study of equilibrium exchange of cinchona alkaloid sulfates and column runs of cinchona bark with the ammonium form of sulfonic acid cation-exchange resins.

EXPERIMENTAL

Resins—The resins were conditioned and converted into hydrogen form. The experimentally estimated values of percent moisture content and capacity in milliequivalents per gram of air-dried resin were: Resin I (50–100), 24.0, 3.89; Resin I (100–200), 22.0, 3.72; and Resin II (20–50), 26.0, 3.53.

The ammonium form of the resin was prepared by treating the hydrogen form with excess aqueous ammonia. The moisture content of the air-dried resin in the ammonium form was estimated, and its capacity was calculated by taking the oven-dried capacity of the resin in hydrogen and ammonium forms to be the same.

Chemicals and Materials—Chemicals were from earlier stocks. Cinchona bark[3] was powdered below 60 mesh, and samples were estimated for alkaloid content (2). The value was 6.5%. Samples of extracted alkaloid material were analyzed (3), and the mixture of quinine and cinchonidine obtained was estimated for each by UV absorption (4). The percent analysis was: quinine, 18.0; quini-

[1] Dowex 50W-X4 (50–100, 100–200).

[2] Amberlite-200 (20–50).

[3] Obtained from the Government Quinine Factory, West Bengal, India.