LACTIC ACID RACEMIZATION IN CL. BUTYLICUM: ^{18}O EXCHANGE STUDIES[1]

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Evidence recently presented by Shapiro and Dennis (1965) suggests that C. butylicum lactic acid racemase catalyzes the racemization of lactic acid by way of a "direct internal hydride shift". Exchange studies employing L(+) α hydroxy ^{18}O enriched lactic acid support this theory and also eliminate a "hydroxyl ion attack" as an alternative mechanistic proposal for the racemization event. Additional evidence is presented to indicate that free pyruvic acid is not a reaction intermediate.

The data supporting the theory that the racemization proceeds by means of a single enzyme has been reported. (Dennis and Kaplan, 1963; Shapiro and Dennis, 1965). The evidence for a "direct internal hydride shift" is based on the non-exchangeability of the α-H atom during racemization and the observed isotopic rate effect for the stereospecific α-deuterio lactic acids. However, it was still conceivable that the racemization occurred by way of a "hydroxyl ion Sn^2 attack". Such an attack would retain the α-H atom during the Walden inversion, and the isotopic rate effect may have been of a

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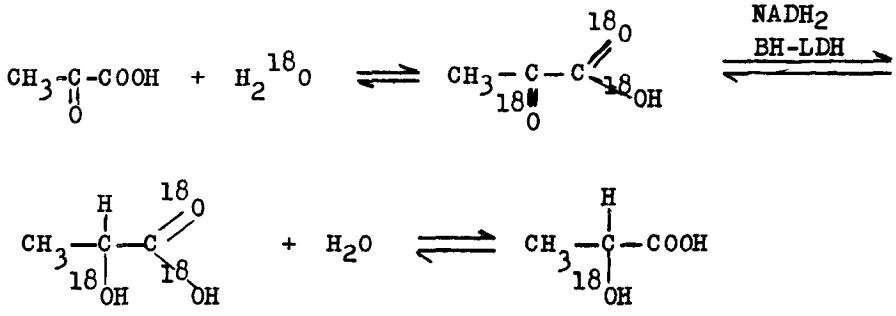
secondary nature. L(+) α-hydroxy ^{18}O enriched lactic acid was synthesized. If the racemization proceeds by means of a hydroxyl ion Sn^2 attack at the α-carbon, we would expect loss of the labeled ^{18}OH from lactic acid during the course of the reaction.

The synthesis of L(+) α-hydroxy ^{18}O lactic acid was conducted according to the following scheme:



EXPERIMENTAL

Preparation of ^{18}O Enriched Li Lactate:

0.2 grams of Na pyruvate was dissolved in 3 ml. of $H_2{}^{18}O$ (Yeda Res. and Dev. Co., Rehovoth, Israel) enriched with 10 atoms % excess and allowed to equilibrate for 1/2 hour at pH 5.0. After 1/2 hour the pH was adjusted to 7.3 with phosphate and 0.2 ml. of 7.2×10^{-4} M BH-LDH (Worthington Biochemical, Freehold, New Jersey) was added. 2.0 grams of $NADH_2$ (Sigma Chem. Co., St. Louis, Mo.), previously dissolved in 7.0 ml. of $H_2{}^{18}O$ enriched with 10 atoms % excess at pH 7.3 were added in increments of about 0.75 ml. Small aliquots were withdrawn and assayed for $NADH_2$ spectrophotometrically at 340 mμ. When the reaction mixture indicated conversion of $NADH_2$ to NAD, more $NADH_2$ was added. 1.0 M H_3PO_4 was added during the course of the reaction to maintain the pH at 7.3. Additional, identical amounts of enzyme were added twice during the reaction.

At completion, the reaction mixture was passed through a Dowex-50 cation exchange resin. The eluate was lyophilized to near dryness, then methanol was added. The precipitated nucleotides and proteins were removed by filtration. The methanol was removed in a Rinko evaporator. The lactic acid with trace amounts of pyruvic acid and degraded nucleotides was then passed through a Dowex-1-formate column and eluted with stepwise increments of formic acid from 0.1 M to 0.6 M. The pooled tubes were taken to dryness in a Rinko evaporator to remove formic acid. The lactic acid was taken up in anhydrous ether and filtered to remove any trace amounts of nucleotides and protein. The ether was removed and the lactic acid was dissolved in H_2O. The lactic acid was assayed enzymatically (Dennis, 1959) and then titrated potentiometrically with standard Li(OH). The number of mmoles of lactic acid determined by enzymatic assay equalled the number of mmoles of standard Li(OH) employed. The solution was taken to dryness and the Li lactate was recrystallized twice from methanol with ethyl ether.

Racemase Reaction:

The reaction mixture contained 20 mg. of L(+) α-hydroxy ^{18}O lactic acid, 0.4 ml. of 0.1 M acetate buffer at pH 5.0, 0.1 ml. of 0.1 M GSH, 2.0 ml. of enzyme solution and 2.50 ml. H_2O. After 2.5 hours at 37°C the reaction was stopped by placing in boiling H_2O for 3 min. The precipitated protein was removed by ultrafiltration. The protein free solution was passed through a Dowex-formate column and eluted with stepwise increments of 30 ml. of formic acid from 0.1 M to 0.6 M. Subsequent isolation of lactic acid as the Li salt was identical to the procedure in the above section.

^{18}O Analyses:

8-9 mg. of Li lactate were placed in a break seal combustion tube with 75 mg. of $HgCl_2$ and sealed at 1 x 10^{-4} mm. of Hg (Rittenberg and Ponticorvo, 1956). The sealed tubes were combusted for 2 hrs. at 400°C. The CO_2 was collected by the method of Williams and Hager (1958). The 44 and 46 mass peak heights were compared employing a consolidated mass spectrophotometer.

Results and Summary:

L(+) $CH_3-\overset{H}{\underset{OH}{\overset{|}{\underset{|}{C}}}}-COOH$ (^{18}O) was enzymatically racemized and the resultant racemic product was analyzed for ^{18}O.

Assay of Racemase Reaction

Lactate (μm)	0 hrs.	2.5 hrs.
L(+)	124	74
D(-)	4	68
Total Lactate Recovered*	128	142
% Racemization	6.4	97

CO_2 Mass Analysis

CO_2 Source	Mass 46/44	Atoms % excess ^{18}O
Li Lactate	0.0038	.196
"0" time, Li Lactate	0.030	1.23
"2.5 hour, Li Lactate	0.028	1.19

From the above data it is evident that the racemization reaction

*The total amount of lactate recovered varied slightly among samples. There is no lactate produced or consumed during the racemization reaction.

proceeds without any appreciable exchange of ^{18}O, thus eliminating the "hydroxyl ion attack" as a possible mechanism. The lack of ^{18}O exchange at pH 5.0 also provides convincing evidence that free pyruvic acid is not a reaction intermediate. One would expect the carbonyl oxygen of pyruvic acid to exchange with the media at this pH. These findings strengthen the validity of involvement of a "direct internal hydride transfer" mechanism for lactic acid racemization in Cl. butylicum.

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References:

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