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Pyruvate Decarboxylating Action of L-Cycloserine

THE SIGNIFICANCE OF THIS IN UNDERSTANDING ITS METABOLIC INHIBITORY ACTION*

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We present evidence which demonstrates that L-cycloserine, structural analog of L-alanine, which is known to be an effective aminotransferase inhibitor, is also a potent inhibitor of cellular pyruvate metabolism. This effect was found to be related to its almost instantaneous action in decreasing pyruvate concentrations in a dose-dependent manner. ^1H nuclear magnetic resonance studies clearly demonstrate that the irreversible removal of pyruvate induced by L-cycloserine is caused by the decarboxylating action of the latter. Pyruvate disappearance induced by L-cycloserine can be stoichiometrically accounted for as acetate. The process does not involve any chemically detected transformation of L-cycloserine. These observations lead to two main considerations regarding the known action of L-cycloserine. First, its inhibitory effect on gluconeogenesis from lactate could be explained only on the basis of its ability to reduce pyruvate availability with no apparent need for transaminase inhibition. Second, its ability as a transaminase inhibitor should be reconsidered in view of its potent decarboxylating action on pyruvate and probably other oxoacids.

4-Amino-3-isoxazolidone (oxamycin) was discovered as a metabolic product of *Streptomyces orchidaceus* by Merck and Co. Inc. Research Laboratories (1), displaying a broad spectrum of antibiotic activity. The comparison of oxamycin with cycloserine, from Commercial Solvents Corp., showed the two compounds to be identical (2). This chemically unique isoxazolidone was thereafter known with the generic name of cycloserine. The usefulness of this drug in metabolic regulation was given by its capacity to inhibit transaminases either *in vivo* (3–6) or *in vitro* (6–8). The differential effects of L-cycloserine in inhibiting only gluconeogenesis from lactate and not from pyruvate (9, 10) were used in support of the hypothesis that intramitochondrially formed oxaloacetate would leave the mitochondria as aspartate when the carbon source was a substrate more reduced than glucose (11, 12).

The present study shows that L-cycloserine is an effective pyruvate decarboxylating agent when the ratio of cycloserine to pyruvate is greater than one. So, at the currently used concentrations of this compound (>1 mM), its inhibitory effect on gluconeogenesis could be primarily associated with its ability to reduce the steady state levels of pyruvate.

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EXPERIMENTAL PROCEDURES

Isolation of Liver Cells—Male Wistar rats, 200 g body weight, starved for 48 h, were used. Liver cells were obtained by perfusing the hepatic vascular system with collagenase according to previously described procedures (13–15). Liver cells were incubated in Krebs-Ringer bicarbonate buffer containing 1.5% gelatin and equilibrated with an O_2/CO_2 (95:5) mixture to adjust the pH to 7.4. Samples of the cell suspension were deproteinized with perchloric acid. The acid-soluble supernatant was brought to pH 6.5, and pyruvate was measured by an enzyme method (16).

Nuclear Magnetic Resonance Spectroscopy—360 MHz spectra were obtained from solutions prepared in deuterium oxide with sodium 3-trimethylsilylpropionate-d_4 as the external reference using a Brucker WM-360 spectrometer. ^1H NMR analysis was carried out using the following conditions: probe temperature of 20 °C, 8-μs pulse width (90° flip angle), 4000 Hz spectral width, 16 kilobyte data points, 3-s recycle time and 16–32 scans. The intensity of the water signal was suppressed by a 0.5-s presaturation pulse. pH was varied when necessary through the addition of sodium deuteroxide or deuterium chloride. Quantitation of specific resonances was achieved in relative terms by comparing the ratio between the intensity of each signal and the intensity of the sodium 3-trimethylsilylpropionate-d_4 reference (17). Integral values were used to determine the intensities of isolated singlets. The sum of heights was found to be more accurate for the quantitation of multiplet structures (18).

Reagents—Metabolites were obtained from Sigma. Deuterated solvents and sodium 3-trimethylsilylpropionate-d_4 were purchased from Stohler (Waltham, MA), enzymes from Boehringer (Mannheim, Federal Republic of Germany), and collagenase from Worthington. L-Cycloserine was a generous gift from Dr. W. E. Scott of Hoffmann-La Roche Inc.

RESULTS

In order to elucidate some apparent paradoxical effects of L-cycloserine on carbohydrate metabolism *in vivo*, we found it interesting to characterize its effects on transamination. Transamination of alanine and α-ketoglutarate, by purified aminotransferase, was measured by coupling pyruvate production to NADH oxidation with lactate dehydrogenase (19). This process was effectively inhibited by cycloserine at concentrations of 50 μM and below. In contrast, inhibition of the backward reaction required concentrations of L-cycloserine in the millimolar range. It was also observed that although alanine formation was inhibited, pyruvate disappearance was always higher than expected and could not possibly be accounted for by alanine formation. The following observations suggested that some type of interaction between pyruvate and L-cycloserine might occur. First, pyruvate disappearance was detected even in the absence of the aminotransferase in the incubation mixture. Second, the addition of increasing concentrations of L-cycloserine to a solution of pyruvate resulted

in a progressive decline in the amount of pyruvate present as measured with lactate dehydrogenase (Fig. 1).

^1H Nuclear Magnetic Resonance Studies—The fate of pyruvate, not accounted for when combined with L-cycloserine, was studied using ^1H NMR. Fig. 2 (*panel A*) shows the ^1H NMR spectrum of a 5:1 cycloserine to pyruvate mixture. The chemical shift of the pyruvate resonance is 2.37 ppm. An additional resonance with a chemical shift of 1.48 ppm is also detected and assigned to the hydrated form of pyruvate (2,2-dihydroxypropanoate) (20, 21). The pyruvate to hydrated pyruvate ratio of signal intensities was decreased by lowering the pH (*panel B*) and restored to the initial values when the sample was brought back to neutrality (*panel C*). This finding confirms previous observations indicating that the hydration-dehydration exchange of pyruvate is an acid-catalyzed process (21). The study of the eventual influence of pH variations is justified if we consider that extracts of acid-soluble materials are routinely prepared when the biological effects of L-cycloserine are studied. The presence of L-cycloserine characteristically acts by inducing the appearance of a resonance with a chemical shift of 2.01 ppm. This signal is not present in the spectrum of pyruvate or L-cycloserine alone (results not shown) and has been assigned to acetate. The intensity of the acetate signal is accounted for by decreased pyruvate signals. Shifting the pH to lower values results in no change in the absolute amount of acetate. The spectral properties of L-cycloserine (Fig. 2, *left*) are similar to those previously described (22). The spectrum does not show any apparent changes either in chemical shift or in relative resonance intensity when compared with the spectrum taken in the absence of pyruvate (not shown). The influence of increasing L-cycloserine to pyruvate molar ratios on the ^1H NMR spectrum is presented in Fig. 3. It is apparent that a conversion of pyruvate to acetate occurs as a function of the L-cycloserine concentration. A quantitative evaluation of the spectral changes is presented in Fig. 4. Increasing L-cycloserine to pyruvate molar ratios results in an exponential decrease of the pyruvate to acetate ratio. The fact that a similar response is observed when plotting pyruvate plus hydrated pyruvate to acetate ratio, taken together with the constancy of the pyruvate to its hydrated form ratio, indicates that the equilibrium between both pyruvate forms is maintained regardless of the concentration of L-cycloserine. The effect of low pH (<1.0) on the pyruvate decarboxylating potency of L-cycloserine is illustrated in Fig. 5. The only apparent effects are the higher ratio of pyruvate plus hydrated pyruvate to acetate and the lower ratio of pyruvate to hydrated pyruvate which result from the shifting of the equilibrium of pyruvate to its hydrated form. When the signal intensities ratio of L-cycloserine to pyruvate plus hydrated pyruvate plus acetate is plotted against the L-cycloserine to pyruvate ratio (Fig. 6), a straight line with a slope of 1 is obtained. This finding strongly suggests that whatever mechanism mediates the decarboxylating action of L-cycloserine, it does not involve its chemical transformation.

Effect of L-Cycloserine on Gluconeogenesis from Pyruvate—

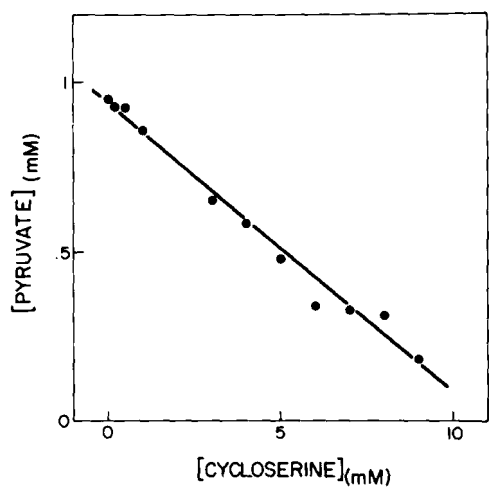

FIG. 1. **Effect of increasing L-cycloserine concentrations on pyruvate availability as measured with lactate dehydrogenase.** Increasing concentrations of L-cycloserine were added to an imidazole-buffered solution (pH 7), containing 1 mM pyruvate. The mixture was allowed to stand at room temperature and 5 min thereafter the pyruvate concentration measured enzymically.

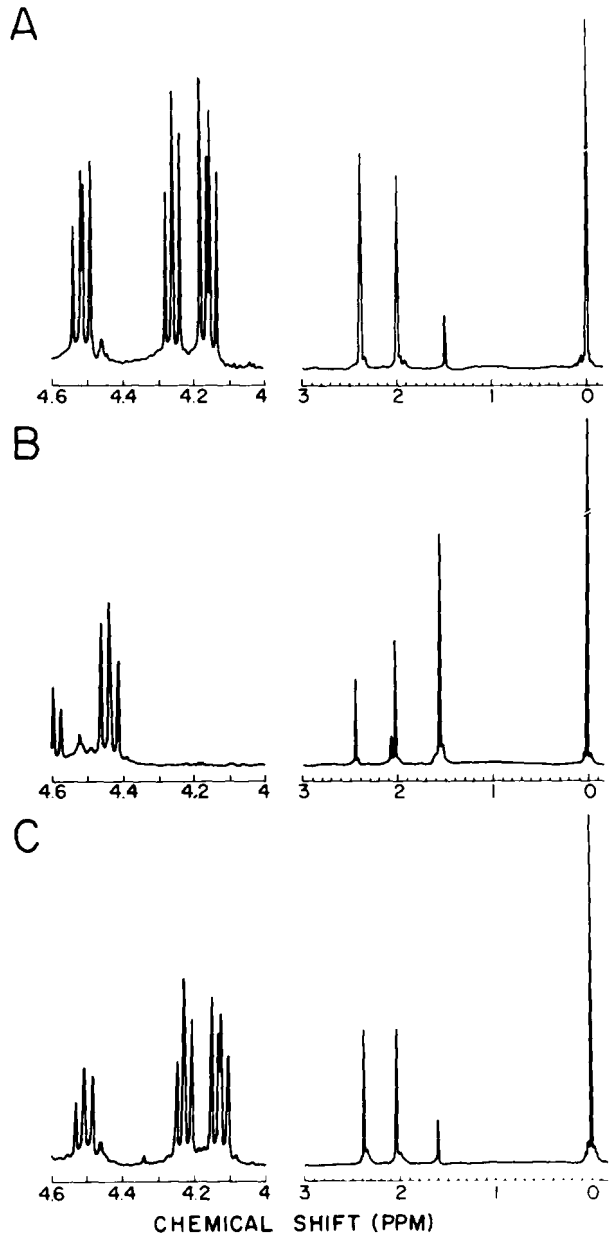

FIG. 2. **Effect of pH on ^1H NMR spectrum of a mixture of pyruvate and L-cycloserine.** A mixture of L-cycloserine and pyruvate, molar ratio 5:1, was made up in deuterium oxide and the spectrum obtained in the next few minutes (*panel A*). After lowering the pH to 1.0 with DCl, a second spectrum was taken (*panel B*). The pH was brought back to neutrality and the spectrum acquired once more (*panel C*). Assignments of signals at neutral pH are from left to right: L-cycloserine (δ = 4.52, 4.24, 4.16 ppm), pyruvate (δ = 2.37 ppm), acetate (δ = 2.01 ppm), and hydrated pyruvate (δ = 1.48 ppm).

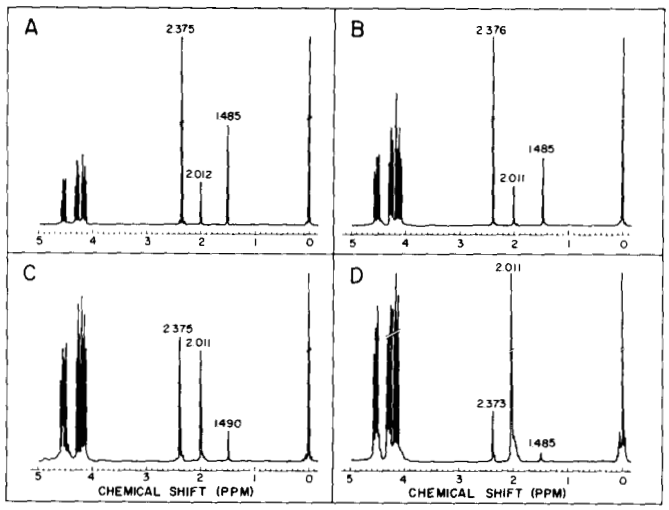

FIG. 3. **^{1}H NMR spectra of a mixture of different L-cycloserine to pyruvate molar ratios.** Mixtures of L-cycloserine and pyruvate were prepared in deuterium oxide (pH 6.9) yielding molar ratios of 1, 2, 5, and 10 (*panels A–D*) and the ^{1}H NMR spectrum obtained within the next 5 min. Resonance assignments are those presented in Fig. 2.

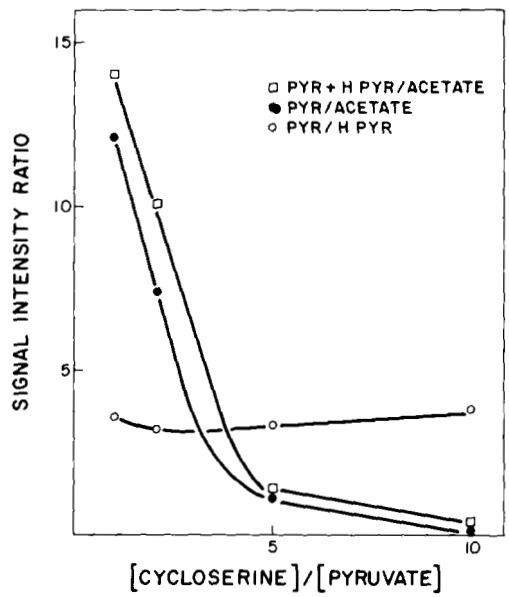

FIG. 4. **Effect of increasing the L-cycloserine to pyruvate molar ratio on acetate formation at neutral pH.** Signal intensity ratios were calculated from spectra taken under similar conditions to those described in Fig. 3. *H-PYR* stands for the hydrated form of pyruvate.

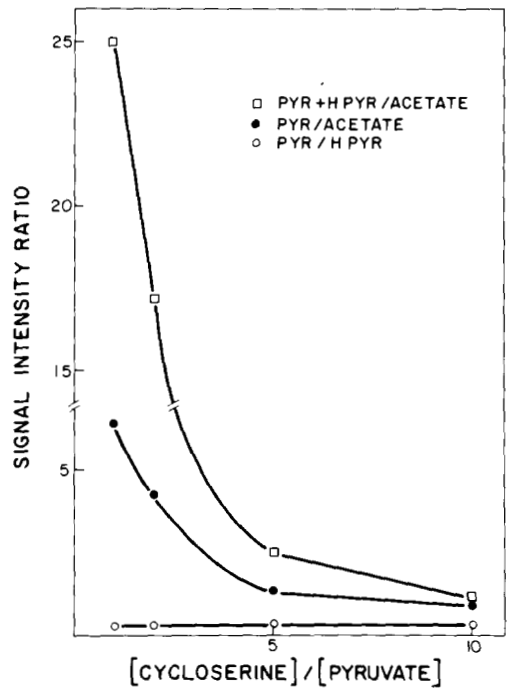

FIG. 5. **Effect of increasing the L-cycloserine to pyruvate molar ratios on acetate formation at acidic pH.** Signal intensity ratios were calculated from spectra obtained under similar conditions to those described in Fig. 4 but at low pH (<1.0). *H-PYR* stands for the hydrated form of pyruvate.

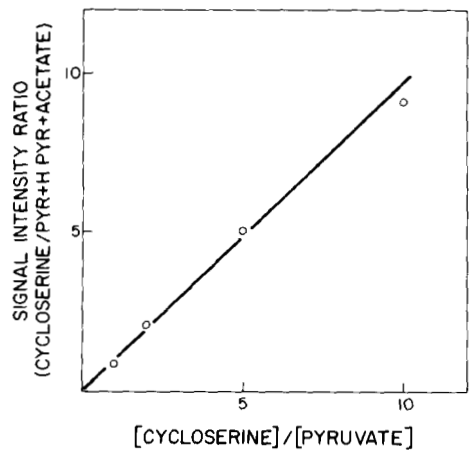

FIG. 6. **Ratio of L-cycloserine to pyruvate plus hydrated pyruvate (*HPYR*) plus acetate signal intensities as a function of L-cycloserine to pyruvate ratio.** Calculations were made from spectra similar to those in Figs. 2 and 3.

The biological relevance of the above observations was evaluated in intact cells by studying the ability of L-cycloserine to perturb gluconeogenesis from low, probably close to physiological, pyruvate concentrations. Fig. 7 is a plot of rates of glucose production from 1 mM pyruvate *versus* increasing L-cycloserine to pyruvate molar ratios. The analysis of this plot allows us to conclude that L-cycloserine acts effectively in preventing pyruvate conversion into glucose under the extra- and intracellular microenvironmental conditions prevailing in current experimental procedures. The inhibitory effect of L-cycloserine is an almost linear function of its extracellular concentration. The similarity of this response to the data shown in Fig. 1, in which pyruvate disappearance was determined *in vitro*, clearly indicates that the major L-cycloserine effect is to reduce substrate availability.

DISCUSSION

L-Cycloserine has been widely used as a metabolic inhibitor (4, 5, 10, 23, 24), on the basis of its apparent ability to inhibit aminotransferases (3–8). The results presented unveil a novel and relatively potent action of this structural analog of alanine. Its ability to induce pyruvate decarboxylation in a dose-dependent manner strongly suggests that the currently available studies of its inhibitory action on alanine aminotransferase by measuring initial velocities of pyruvate formation are probably inaccurate. Preliminary evidence indicates that L-cycloserine decarboxylating activity is not limited only to pyruvate but to other biologically important oxoacids. Thus, the kinetics of the L-cycloserine inhibitory action on other aminotransferases should be reconsidered when more information on its relative effects on the catalyzed processes and

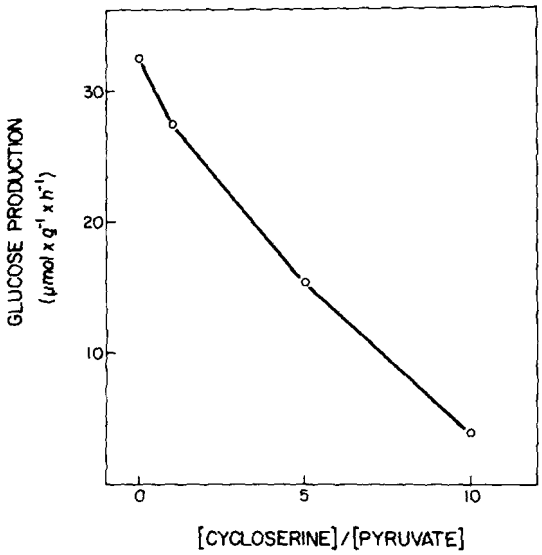

FIG. 7. **Effect of increasing L-cycloserine concentrations on rates of glucose production from 1 mM pyruvate in isolated rat liver cells.** Duplicate samples of rat liver cells (≈15 mg wet weight/ml) were incubated in Krebs-Ringer bicarbonate buffer, equilibrated with an O_2/CO_2 mixture (95:5) at 36.5 °C, in the presence of 1 mM pyruvate as substrate and the indicated L-cycloserine to pyruvate ratios. Samples were processed as described under "Experimental Procedures." Rates were calculated for the incubation interval between 0 and 30 min.

on products or substrates removal becomes available.

The usefulness of L-cycloserine as a metabolic inhibitor lies entirely on its ability to inhibit aminotransferases. L-Cycloserine has been utilized in two main ways: first, to study amino acid production and/or removal under several metabolic or nutritional conditions (4–5), or to study the biological effects of certain amino acids when their metabolic conversion was prevented (25, 26); second, it has been used as a selective inhibitor of hepatic gluconeogenesis from substrates more reduced than glucose (10). From stoichiometric evaluations of carbon and hydrogen balances, it was postulated (11–12) that during gluconeogenesis from substrates yielding pyruvate, intramitochondrially formed oxaloacetate, would leave the mitochondria predominantly as aspartate or as malate when the substrate was more reduced (lactate) or more oxidized (pyruvate) than glucose, respectively. If oxaloacetate leaves the mitochondria as aspartate, then transamination is essential to form aspartate in the mitochondrial matrix and to yield oxaloacetate in the cytosol. The observation that L-cycloserine inhibited only gluconeogenesis from lactate with no detectable effects when pyruvate was the carbon source was utilized in support of this hypothesis (10). The results presented seriously question the validity of these arguments. The inhibitory effect of L-cycloserine on gluconeogenesis from 1 mM pyruvate (Fig. 7) suggests that its inhibitory effect on lactate to glucose flux could probably be explained solely on the basis of its ability to remove pyruvate. When 10 mM lactate is utilized as a substrate, the expected intracellular concentration of pyruvate should be approximately 0.3 mM. According to the data presented in Figs. 1 and 7, L-cycloserine concentrations above 1 mM (10) would cause an inhibition of gluconeogenesis whether or not the transamination steps were inhibited. Failure to previously observe an inhibition of gluconeogenesis from pyruvate can be explained taking into account that large supraphysiological concentrations of pyruvate (>10 mM) were utilized (10, 24).

The well-known pharmacological actions of L-cycloserine on the central nervous system (27), characterized by confusion, disorientation, and convulsions, can now be better understood considering its remarkable effect in reducing pyruvate availability, which is the main, almost exclusive, source of energy for the neurons (28, 29).

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