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THE INTERACTION OF CYCLOSERINE WITH PYRUVATE AND OTHER BIOLOGICALLY RELEVANT α-KETOACIDS

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Abstract—The ability of cycloserine solutions to deplete α-oxoacids has been found to be correlated with the spontaneous transformation of cycloserine into a derivative dimer (2,5-bis-(aminoxymethyl)-3,6-diketopiperazine). Synthetic dimer was found to react rapidly with pyruvate to form the expected oxime. Two lines of evidence indicate that it is the cycloserine dimer and not cycloserine itself that reacts with α-ketoacid. First, the ^{1}H NMR spectrum of the purified oxime is superimposable with that arising when the dimer and pyruvate are mixed and the spectrum taken immediately thereafter. Second, the mass spectrum of the reaction product of cycloserine dimer and methylpyruvate is totally consistent with the formation of a stable oxime derivative. Furthermore, when cycloserine is incubated with pyruvate the oxime derived from the dimer is found. These observations clearly indicate that cycloserine in solution can have chemical activities in addition to its ability to interfere with pyridoxal dependent reactions. On these grounds it is concluded that any biological action of cycloserine should be interpreted cautiously.

L-Cycloserine has been extensively used as a pharmacological probe to inhibit transaminases [1–3]. This use is based on observations that show that L-cycloserine can inhibit these transferase enzymes by one as yet undetermined mechanism [4, 5]. The exact mechanism by which cycloserine acts is of great importance for the purpose of drawing physiological conclusions based on its use.

It was recently demonstrated that L-cycloserine reacted with pyruvate, leading to its disappearance as measured with lactate dehydrogenase [6]. This effect was observed under a wide variety of experimental conditions, including those characteristic of biological systems. Studies carried out by nuclear magnetic resonance spectroscopy indicated that pyruvate disappearance, induced by L-cycloserine, could be stoichiometrically accounted for by the appearance of a new compound with a resonance whose chemical shift was similar to that of acetate. On these grounds, it was suggested that L-cycloserine could remove pyruvate by catalyzing its rapid decarboxylation by an undetermined mechanism [6].

Two considerations prompted to question this possibility. First, the nature of the putative oxidative process leading to pyruvate decarboxylation in the absence of L-cycloserine consumption was not readily understandable mechanistically. Secondly, the extent of pyruvate removal was a function of the L-cycloserine to pyruvate molar ratio. Since pyruvate decarboxylation is a thermodynamically irreversible process, had the L-cycloserine been the catalytic agent the complete removal of pyruvate should be expected at any concentration of cycloserine given sufficient reaction time. These and other considerations strongly suggested that either a contaminant present in the L-cycloserine or a conversion product of it might be responsible for the "cycloserine's" reaction with pyruvate.

The present paper presents conclusive evidence indicating that it is not L-cycloserine but its spontaneously formed dimer, (2,5-bis-aminooxymethyl)-3,6-diketopiperazine, which readily reacts with pyruvate by forming oxime derivatives (Scheme 1). Therefore, any biological experiment with cycloserine needs to be interpreted carefully in view of the potent chemical reactivity of the spontaneously formed dimer.

MATERIAL AND METHODS

Measurement of oxoacids and cycloserine. Pyruvate, oxaloacetate and α-ketoglutarate were measured enzymatically, by following changes in NADH absorbance at 340 nm, in accordance with previously described procedures [7]. Cycloserine was measured photometrically taking advantage of the ability of this compound to react specifically with sodium nitrosopentacyanoferroate [8] to form a blue-coloured complex max 625 nm suitable for quantitative purposes.

Pyruvate decarboxylation. The putative ability of cycloserine to decarboxylate pyruvate was tested by measuring CO_2 formation from 1-[^{14}C]-pyruvate. Appropriate amounts of cycloserine were injected into 50 ml glass bottles, sealed with reversible rubber stoppers, containing 2.5 mM 1-[^{14}C]-pyruvate (S.A.

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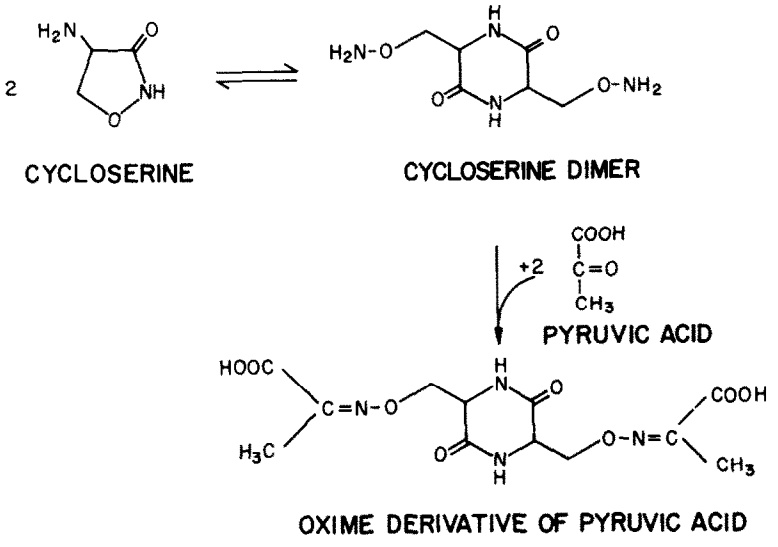
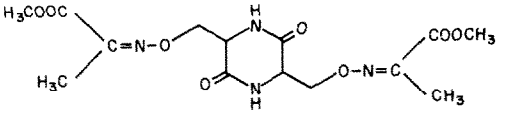


Scheme 1.

10 nCi/μmol) so as to give increasing cycloserine to pyruvate molar ratios. After 30 min incubation at 20° 0.3 ml of diphenylethylamine were injected into small cups suspended from the stoppers and filled with a piece of fluted paper. Immediately thereafter the CO_2 was released by injecting 0.5 ml of 1 N ClH through the rubber stoppers into the reaction mixture. After 60 min incubation at 20° under gentle shaking the diphenylethylamine containing cups were transferred to scintillation vials containing 20 ml of quickscint (Zinsser Analytic, F.R.G.), and the radioactivity determined.

Preparation of cycloserine dimer (2,5-bis-(amino-oxymethyl)-3,6-diketopiperazin). This material was prepared according to a previously described procedure [9]. The product was identified and its purity was ascertained by several means: HPLC chromatography, NMR spectroscopy, mass spectrometry and photometrically (taking advantage of the property [10] of the cycloserine dimer of displaying increasing absorbance at 274 nm when treated with sodium hydroxide).

Preparation of heat stable oxime derivative of cycloserine dimer and methyl-pyruvate. The oxime derivative of cycloserine dimer and pyruvate was prepared by mixing the two compounds in water at neutral pH at a molar ratio of 1:2. The reaction was allowed to proceed to completion at 20° and the product was then freeze-dried. Mass spectroscopic analysis of the compound proved to be impossible due to its lack of volatility coupled with instability. This problem was circumvented by preparing the oxime derivative with methyl-pyruvate. The oxime was obtained by mixing cycloserine dimer and methyl-pyruvate (2:1) in dry methanol and refluxing at 60° under reflux for 1 hr. The precipitate was collected by filtering the product through a fiber-glass membrane, washing it thoroughly with dry methanol and vacuum drying it. The mass spectroscopic determination of this compound was consistent with the alkylidene-aminooxy derivative of methyl pyruvate. It showed a peak corresponding to a 372 molecular weight, that is the same calculated according to the formula:



Scheme 2.

(The stereochemistry about the oxime bond is unspecified because it has not been determined.)

It also showed the peaks corresponding to the molecular weight of the fragments derived from the oxime devoid of the following groups:

254 (M ± 2 ($COOCH_3$))

225 (M ± 2 ($COOCH_3$)—2(CH_3)

256 (M ± ON=C(COOCH$_3$)(CH$_3$))

138 (M ± 2(ON=C(COOCH$_3$)(COOCH$_3$)))

Nuclear magnetic resonance spectroscopy. 360 MH_2 ^1H NMR spectra were obtained in a Bruker WM-360 spectrometer from solutions prepared in deuterium oxide with sodium 3-trimethylsilyl-propionate-d4 as internal reference, under the following conditions: probe temperature of 20°, 8 μsec pulse width (90° flip angle), 4000 Hz spectral width, 16 K data points, 3–5 recycle time and 16–32 scans. The intensity of the water signal was suppressed by a 0.55 presaturation pulse of adequate intensity. When required the pH was adjusted to the desired values with sodium deuteroxide or deuterium chloride.

Table 1. Effect of cycloserine on CO_2 formation from 1-[^{14}C]pyruvate

	Expected (cpm)	Actual (cpm)
Pyruvate (2.5 mM)	—	108
Cycloserine (25 mM)	—	107
Cycloserine + Pyruvate		
Molar ratios: 1	1100	106
2	3300	133
5	6600	111
10	13200	120

The expected cpm were calculated assuming that the pyruvate disappeared was fully converted into acetate. The results are mean values of two different experiments in duplicate.

RESULTS

Effect of L-cycloserine in producing CO_2 from 1-[^{14}C]pyruvate

A direct approach for testing the putative decarboxylating ability of L-cycloserine was to measure the rates of CO_2 formation from 1-[^{14}C]-pyruvate. Table 1 shows that cycloserine to pyruvate molar ratios ranging from 1 to 10 resulted in no detectable increases of $^{14}CO_2$ production above background. This observation rules out decarboxylation as the chemical basis of the cycloserine interaction with pyruvate.

Effect of different L-cycloserine to pyruvate molar ratios on the time course of pyruvate removal

Figure 1 shows the rates of pyruvate removal resulting when pyruvate was incubated at 20° with increasing amounts of a freshly made solution of L-cycloserine. Pyruvate concentration at the beginning of the incubation was 2 mM and the amounts of L-cycloserine were varied to give molar ratios of cycloserine to pyruvate up to 10. In agreement with our previous report [6], the pyruvate removed is proportional to the concentration of cycloserine at any given time. However, it should be remarked that in this instance where the cycloserine solution was made up immediately before its utilization, a much greater time was needed to decrease the pyruvate concentration similar to that previously observed in 10 min when the cycloserine solution was made up a few hours before utilization.

Figure 2 shows the effect of increasing pyruvate concentration on the time course of cycloserine and pyruvate depletion. Cycloserine alone disappeared as a linear function of time. This finding is related to the ability of cycloserine to dimerize in solution [11–13]. At cycloserine to pyruvate molar ratios above one, pyruvate did not affect the rate of cycloserine disappearance. However, at molar ratios of one or lower, the initial velocity of cycloserine removal appear to be increased. At cycloserine to pyruvate molar ratios above one, or even below one, after the first 2 hr of incubation (Fig. 2), the ratio of cycloserine to pyruvate removed was one, regardless of the absolute concentration of reactants. When a cycloserine solution was made up and its ability to remove pyruvate was tested for 72 hr, it was found that its ability to remove pyruvate correlated with the time that cycloserine had been in solution, independently of whether pyruvate was present along the incubation time or not (Fig. 3). This observation,

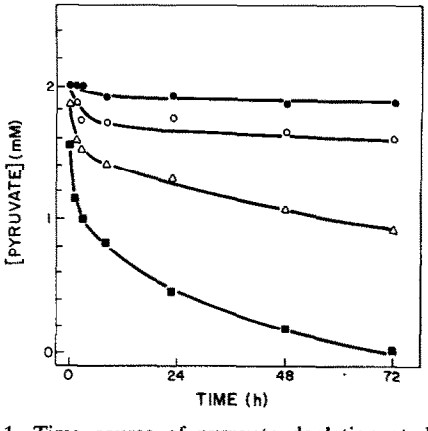


Fig. 1. Time course of pyruvate depletion at different cycloserine to pyruvate molar ratios. Freshly made solutions of cycloserine and pyruvate in water were mixed as to give molar ratios of 1 (●), 2 (○), 5 (△) and 10 (□), and incubated at 20°. At the indicated times pyruvate content was measured enzymatically.

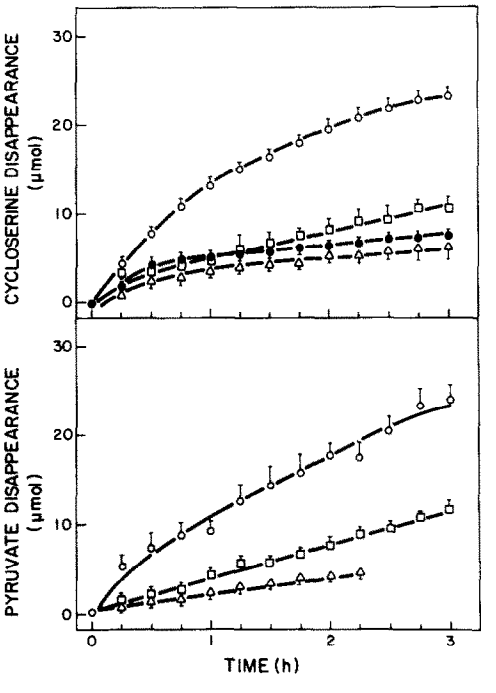


Fig. 2. Effect of increasing pyruvate concentration on the time course of cycloserine and pyruvate depletion. Freshly made solution of cycloserine or cycloserine plus pyruvate at pH 5 were incubated at 65°. At the indicated times their content was determined as described in Methods. Cycloserine concentrations was 30 mM and pyruvate concentration was varied to give molar ratios of 0.25 (○), 1 (□) or 6 (△).

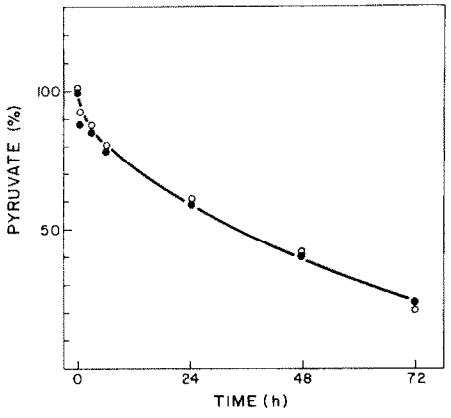


Fig. 3. Time course of the ability of a cycloserine solution to deplete pyruvate. A freshly made solution of cycloserine (10 mM) was allowed to stand at 20° and at the indicated times an aliquot was withdrawn and mixed with a freshly made solution of pyruvate (2 mM) and the concentration of pyruvate remaining was immediately determined (●). Open circles indicate rates of pyruvate depletion when both reagents were mixed at zero time.

taking also into account the stoichiometric relationship show in Fig. 2, clearly indicates that "cycloserine" does not act catalytically, but rather reacts stoichiometrically with pyruvate and implicates the cycloserine dimer in this process.

Interaction of cycloserine dimer with α-oxoacids

Two different observations prompted us to study the action of cycloserine dimer in removing α-oxoacids. The first one, mentioned above, is the reported tendency of cycloserine to dimerize in solution [11–13]. The second one is the reported ability of the cycloserine dimer shared with other aminoxy compounds, to form oxime derivatives [14]. Furthermore, the rate of cycloserine disappearance is proportional to the second power of its concentration (not shown), as expected if the dimerization was a previous step. As it can be seen in Fig. 4, the cycloserine dimer displays an almost instantaneous effect in removing not only pyruvate but other biologically relevant α-oxoacids like α-keto-glutarate or oxaloacetate. The 2:1 stoichiometry, 2 mol of oxoacid removal per mol of dimer, strongly suggested the formation of stable oxime derivatives with the oxoacids according to Scheme 1.

Nuclear magnetic resonance studies

Figure 5 shows ^{1}H NMR spectra of pyruvate, cycloserine, cycloserine dimer and the pyruvate–cycloserine dimer oxime. The cycloserine dimer and the oxime were prepared as described in the experimental section. The spectra indicate the high purity of these compounds, eliminating the possibility of any contaminant being present in significant amounts. The spectral patterns of cycloserine (three pseudo-triplets: 3.89 ppm, 4.00 ppm; and 4.42 ppm) and its dimer (one triplet and two doublets of droplets: 4.36 ppm, 4.10 ppm and 3.96 ppm) as well as the measured coupling constants are entirely compatible with their known structures. The distinct pattern of cycloserine, slightly different than previously observed [6], was obtained by preventing its dimerization. This was achieved by taking the spectra immediately in deuterated water at pH 9. The strong signal (a triplet, 2.01 ppm) present in the oxime (Fig. 5, panel D) arises from the methyl groups of pyruvate.

Figure 6 shows the time course of the reaction which occurred when cycloserine dimer and pyruvate were mixed up at neutral pH. In less than 2 min, the time needed to load the probe with the sample and seal it (Fig. 6, panel A), the appearance of two distinct signals was observed. One of them (2.01 ppm) is similar to that observed in the oxime (Fig. 5, panel D). The second one, located at higher field (1.33 ppm) is likely to be associated with the CH_2–O of the tetrahedral intermediate adduct formed between the hydroxyl amino moiety and the carbonyl group. This resonance, as well as those corresponding to pyruvate (2.37 ppm) and its hydrated form (1.48 ppm) disappeared as a function of time (Fig. 6, panels B and C) in the same proportion as the resonance with a chemical shift of 2.01 ppm increased. The similarity of the ^{1}H NMR

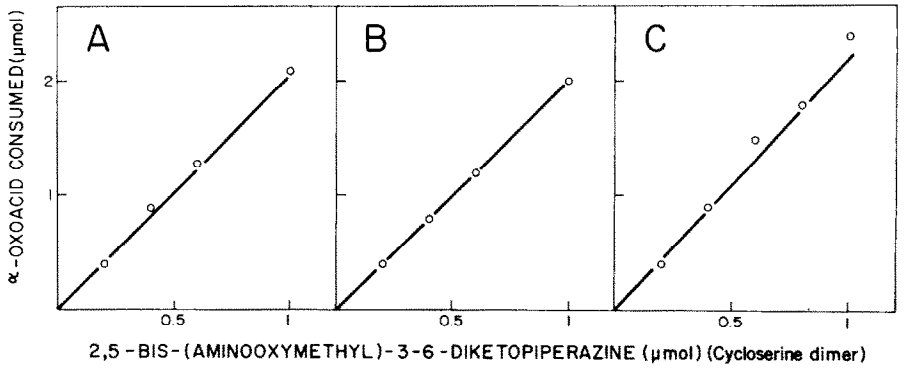


Fig. 4. Ability of cycloserine dimer (2,4-bis-(aminooxymethyl)3,b-diketopiperazine) to remove α-oxoacids. Cycloserine dimer and pyruvate (A), α-oxoglutarate (B) or oxaloacetate (C) were mixed up in water at neutral pH, allowed to stand at 20° for 15 min and then the oxoacid content was determined enzymically.

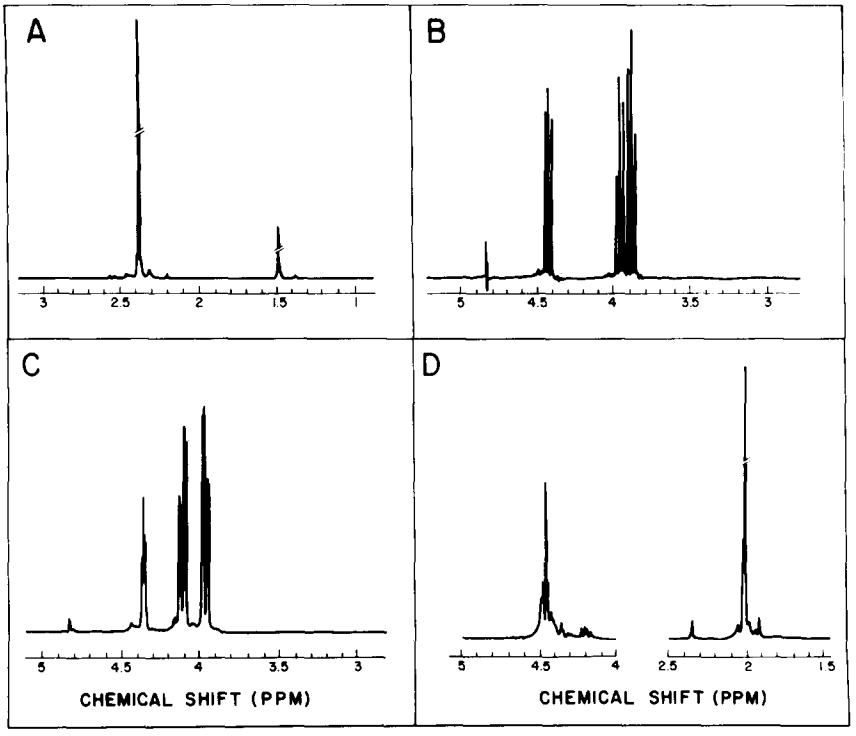


Fig. 5. ^{1}H NMR spectra of pyruvate (A) cycloserine (B), cycloserine dimer (C) and the pyruvate–cycloserine dimer oxime (D). The solutions were made up in deuterated water and the ^{1}N NMR spectrum obtained within the next 5 min.

spectra taken when all the pyruvate was exhausted (Fig. 6, panel C) and that of the oxime previously made up and characterized by different criteria (Fig. 5, panel D), strongly suggests that the interaction of cycloserine dimer and pyruvate leads to the formation of a stable oxime derivative.

The spectra shown in Fig. 7 add further evidence in support of the conclusion that it is not cycloserine but rather its dimer which reacts with α-oxoacids. Heating a mixture of cycloserine and pyruvate at 65° at an acidic pH led to displacement as well as to qualitative changes in the pattern of resonances, indicating the formation of dimer (Fig. 7, panel B). This was accompanied by a depletion of pyruvate and appearance of a signal at 2.01 ppm. In contrast, at pH 10 (a pH which is known to slow down cycloserine dimerization [5]), although the sample was again heated, the cycloserine spectral pattern did not change significantly, most of the pyruvate remained, and the signal arising at 2.01 ppm was very small.

DISCUSSION

The ability of cycloserine to remove pyruvate is a function of the absolute cycloserine concentration and of the time it has been in solution (Figs 1 and 3). This finding, also taking into consideration the stoichiometric relationship between cycloserine disappearance and the removal of pyruvate, eliminates the possibility that cycloserine acted catalytically in depleting pyruvate. In addition, the fact that CO_2 is not released from pyruvate when incubated with cycloserine eliminates decarboxylation as the cause for pyruvate removal. The evidence presented here shows that pyruvate is irreversibly removed together with stoichiometric amounts of cycloserine. Pyruvate removal is a linear function of cycloserine concentration at cycloserine to pyruvate molar ratios above one (Fig. 1). In addition, there is a temporal correlation between cycloserine disappearance and its ability to remove pyruvate (Fig. 2). Taken together these two observations strongly suggested that it was not cycloserine but some derivative of it which reacted with pyruvate.

On the basis of the previously reported ability of cycloserine to dimerize in solution [11–13], the possibility was considered that the active agent in removing pyruvate was the cycloserine dimer (2,4 - bis - (aminoxymethyl) - 3,6 - diketopiperazine) (Scheme 1). The 1:2 stoichiometric reaction of purified cycloserine dimer with pyruvate and other oxoacids (Fig. 4) strongly supports the notion that the dimer was the mediator of this process. The ^{1}H NMR studies provided definitive evidence concerning the nature of the reaction between "cycloserine" and oxoacids. The compound prepared by incubating cycloserine dimer with pyruvate shows the same ^{1}H NMR spectrum (Fig. 6) as the reaction product obtained by incubating cycloserine with pyruvate (Fig. 7, panel B). That this compound is the oxime derivative of the dimer was confirmed by mass spectrometry on the dimethyl ester. The formation of stable oxime derivatives between cyclo-

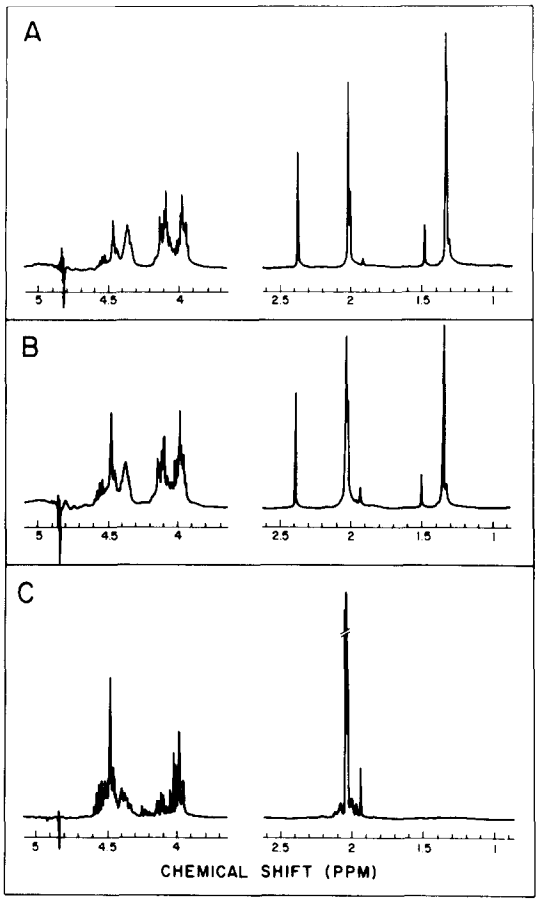


Fig. 6. Time course of the reaction of cycloserine dimer and pyruvate. Solutions of cycloserine dimer and pyruvate in deuterated water at neutral pH were mixed and the ^1H NMR spectrum taken within the next 5 min (A), at 10 min (B) and 5 hr (C) respectively.

serine dimer and pyridoxal phosphate has been previously communicated [5, 14, 15]. The results in Fig. 2 show that the rates of cycloserine and pyruvate disappearance when cycloserine is in excess over pyruvate, are virtually the same. In addition, the rate of cycloserine consumption was the same in the presence or absence of pyruvate, suggesting a rate limiting formation of dimer. However, it should be mentioned that when the pyruvate to cycloserine ratio was one or greater the initial, but not the final rate, of cycloserine consumption was greater than that of pyruvate. A mechanism that would explain this result has not been determined. Nevertheless, this observation does not alter a main conclusion of this study, that is, that under all pyruvate to cycloserine ratios studied, the oxime is the major end-product.

The capacity of cycloserine dimer to interact with some unspecified keto compounds [16] and to interfere with the assay of pyruvate [17] has also been previously communicated briefly. The cycloserine dimer has been reported to be at least as effective as cycloserine in inhibiting aspartate and other aminotransferases [5, 14], and also in inhibiting microbial growth [12]. All these processes depend on pyridoxal phosphate as a cofactor. However, the findings reported here confirm the idea [6] that the biological action(s) of cycloserine cannot merely be ascribed to its ability to inhibit pyridoxal phosphate linked enzymes. The more than one order of magnitude difference between the cycloserine concentration needed to inhibit alanine aminotransferase [17] and that needed to inhibit gluconeogenesis from non-amino substrates [18] strongly indicates that the latter process may be affected by decreasing the availability of pyruvate or other key oxoacids whose concentration may be limiting [19, 20]. It is evident that cycloserine dimer is potentially a powerful biologically active agent. An understanding of the effect of cycloserine on any biological process may be inaccurate if it does not take this into account.

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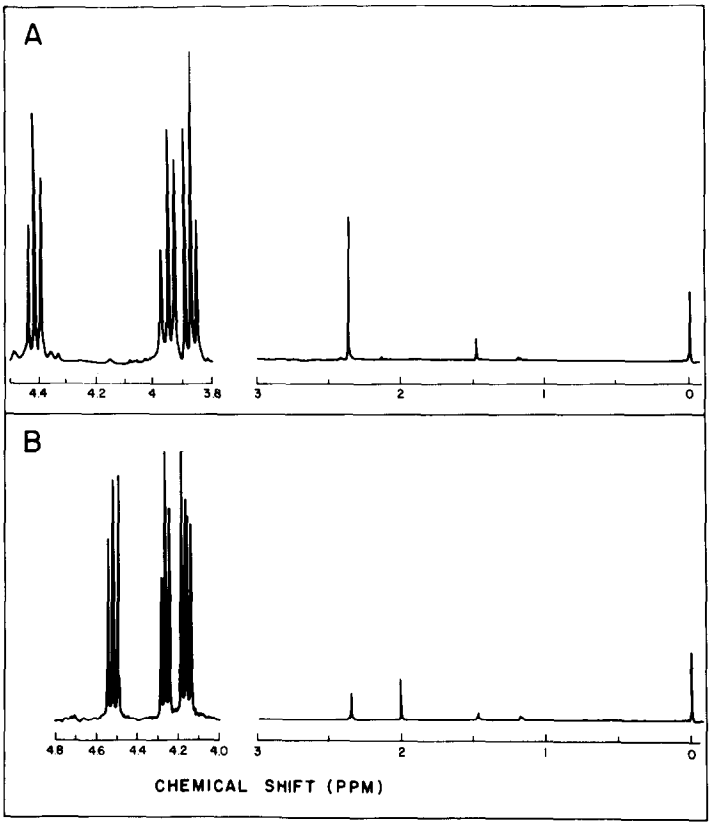

Fig. 7. Effect of pH and temperature on the ^{1}H NMR spectra of a mixture of cycloserine and pyruvate. Mixtures of cycloserine and pyruvate (10:1) in deuterated water were incubated at pH 9 for 15 min at 20° (A) or at pH 7 and 70° for 60 min (B) and then the ^{1}H NMR spectrum was obtained.

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