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Formation of the Thiazoline Ring in Pantetheine*

David H. Jones and Walter L. Nelson

ABSTRACT: In a study of the possible thiazoline formation in coenzyme A, pantetheine was used as a model compound. The thiazoline ring in pantetheine was formed by heating in strong acid and was measured by absorption at 266 mμ. The rate was proportional to both acid and pantetheine concentration in the time period studied and was temperature dependent. The pK for the conversion of thiazoline into the protonated thiazolinonium form was found to be between pH 2 and 3. The ring was hydrolyzed readily at very acid pH values but was stable in neutral and basic solution. The pantetheine thiazoline was located as a distinct spot on paper chromatography.

The formation of the thiazoline ring in certain biologically important compounds has been proposed several times. Linderstrom-Lang and Jacobson (1941) studied the properties of 2-methylthiazoline and postulated that the thiazoline structure might explain the unreactivity of some sulfhydryl groups in native proteins, and their appearance upon denaturation. Calvin (1954) observed the production of a new absorption peak at 268 mμ when glutathione was heated in strong acid and proposed that thiazoline formation could account for this observation. Further studies on the cyclization of glutathione in acid solution have subsequently been carried out in several laboratories (Préaux and Lontie, 1958; Garfinkel, 1958; Martin and Edsall, 1958; Goodman and Salce, 1965; Jocelyn, 1967).

Basford and Huennekens (1955) made a study of the various forms of CoA in a commercial preparation. They proposed that one of the compounds was the thiazoline form of CoA. In a study of thiazoline formation in compounds related to CoA, they observed the expected peak with β-alanylcysteine but not with pantetheine. In an attempt to study cyclization in CoA analogs we have observed the characteristic absorption peak at 266 mμ with pantetheine and therefore, based on previous studies, thiazoline ring formation was assumed. This report describes our observations concerning the probable thiazoline ring formation in pantetheine.

Experimental Section

Chemicals. D-Pantethine (bis(*N*-pantothenylamidoethyl)-disulfide) was obtained from Sigma Chemical Co. Ellman's reagent, 5,5′-dithiobis(2-nitrobenzoic acid), was obtained from Aldrich Chemical Co.

Reduction of Pantethine. Pantetheine was obtained by the reduction of pantethine with 0.5% sodium amalgam which was prepared by reacting 0.5 g of clean sodium metal with 100 g of mercury under toluene; 2 ml of pantethine of the desired concentration was incubated over 0.2 ml of the sodium amalgam with occasional shaking for 30 min at 37°. The solution was neutralized to pH 7.0 with HCl.

Determination of SH. The SH concentration was determined by the use of Ellman's (1959) reagent, 5,5′-dithiobis-(2-nitrobenzoic acid). To 0.5 ml of the properly diluted solution was added 3.0 ml of 5,5′-dithiobis(2-nitrobenzoic acid), 1.5×10^{-3} M (dissolved in 6×10^{-3} M glycyl-glycine, pH 8.0). The yellow color was allowed to develop for 2 min and then the solutions were read against a blank at 412 mμ in a Beckman D.U. spectrophotometer. The absorbance values were converted into micromoles of SH by use of a standard curve determined with glutathione.

Ultraviolet Spectra. The ultraviolet spectra were obtained on a Bausch & Lomb Spectronic 505 spectrophotometer. The samples were read against a water blank in 1-cm, 1-ml quartz cuvets.

Preparation of Pantetheine Thiazoline. The usual method for preparation of the thiazoline was as follows: 0.5 ml of pantetheine solution (concentration varied depending upon experiment) and 0.5 ml of concentrated HCl were heated at 100° for 8 min. The solution was lyophilized to dryness thus removing most of the HCl. The material was either stored as powder or redissolved in H_2O and the pH was adjusted to the desired value. The material was rather stable as a powder but decomposed rapidly when in weakly acidic solution.

Paper Chromatography of Pantethine Forms. The paper

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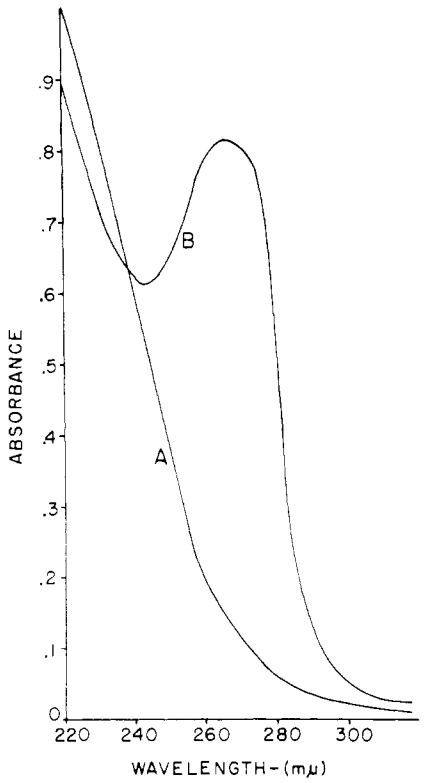


FIGURE 1: Formation of thiazoline ring by heating pantetheine at 100° for 5 min in 6 N HCl. Curve A is a 1.0-ml control solution of 0.42 μmole of pantetheine heated in H_2O. Curve B is a 1.0-ml solution of 0.42 μmole of pantetheine heated in 6 N HCl.

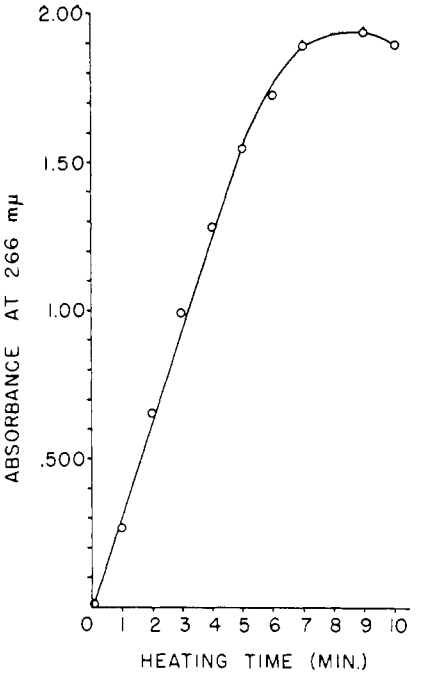


FIGURE 2: Linearity of thiazoline formation with heating time at 100°. Pantetheine (0.87 μmole/1 ml) was heated in 6 N HCl at varying time intervals and the absorption at 266 mμ was determined.

chromatographic method of Basford and Huennekens (1955) was used. Samples were spotted on Whatman No. 1 paper, (18–22.5 in.) and the chromatograms were developed by descending chromatography in an ethanol–H_2O (70:30, v/v) solvent system. The chromatogram was allowed to dry and then the spots were detected with nitroprusside (Joennies and Kolb, 1951). Upon spraying with nitroprusside, the pantetheine and pantetheine thiazoline spots were observed. For the detection of the pantethine spots, the chromatogram was sprayed with an alcoholic NaCN solution subsequent to nitroprusside. The compounds appeared as rapidly fading, pink spots. The indophenol method for reducing compounds was as follows. A 1% solution of sodium, 2,6-dichlorobenzenone–indophenol in water (NaOH added until the compound dissolved) was sprayed onto the chromatogram. The pantetheine and pantetheine thiazoline compounds appeared as white spots on a blue background.

Results

When a solution of pantetheine was heated at 100° for 5 min in 6 N HCl, an absorption band with a maximum at 266 mμ was observed indicative of a thiazoline ring formation. A typical spectrum is shown in Figure 1. The rate of formation of the 266-mμ peak in 6 N HCl appears to be linear with heating time up to about 6 min, as is shown in Figure 2. The dependency of the reaction upon acid concentration is illustrated in Figure 3. The rate of formation of the thiazoline ring appears to be linear with the (H^+) concentration. The extent of reaction can be estimated to be about 34% complete in this experiment by employing the molar extinction coefficient for 2-methylthiazoline (E_{260} 5300) reported by Martin *et al.* (1959). The extinction of pantetheine thiazoline should be very close to that of 2-methylthiazoline. The observed linearity of ring formation with acid concentration can be explained by the fact that the reaction still approximates initial rate conditions during this time period.

To demonstrate the dependency of the thiazoline formation upon pantetheine and to show the temperature effect, solu-

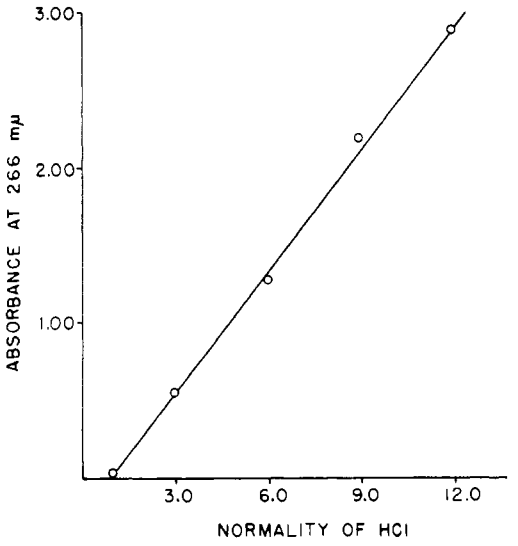


FIGURE 3: Dependence of thiazoline formation upon acid concentration. Pantetheine (0.73 μmole/1 ml) was heated at 100° in 1–12 N HCl for 5 min and the absorption at 266 mμ was determined.

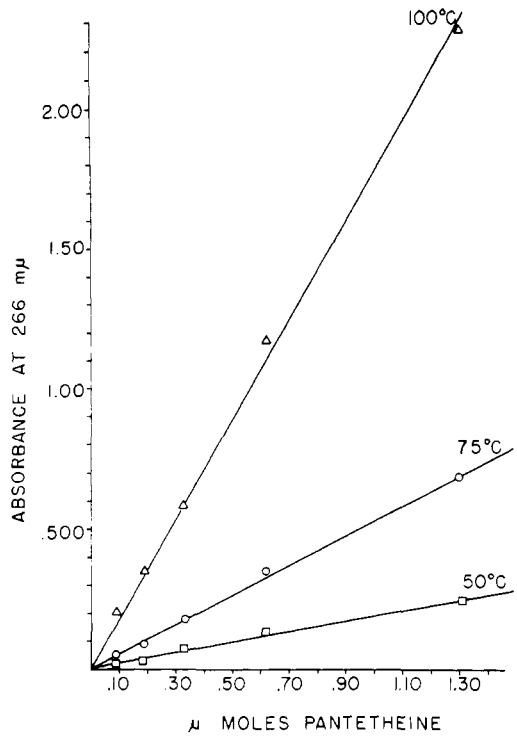


FIGURE 4: Dependence of thiazoline formation upon pantetheine concentration and temperature. Pantetheine solutions (1.0 ml) of varying concentrations were heated for 5 min in 6 N HCl at the temperatures indicated and the absorption was determined at 266 mμ.

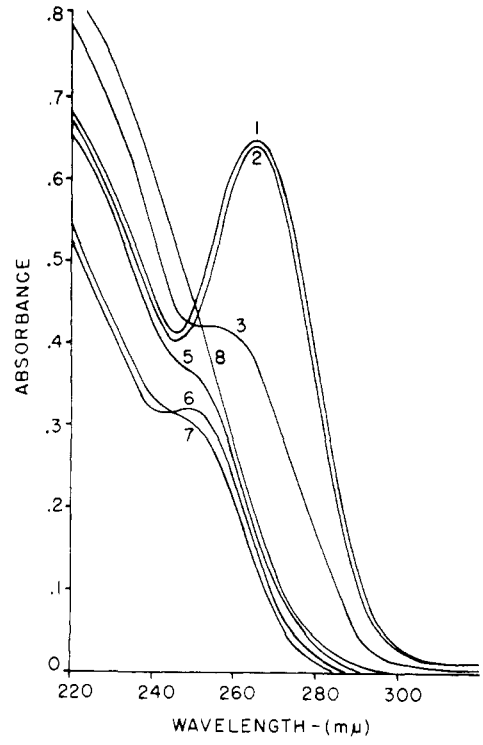


FIGURE 5: Effect of varying pH on the ultraviolet spectra of pantetheine thiazoline (0.3 μmole) adjusted to the pH values indicated and the spectra were determined.

tions of increasing pantetheine concentration were heated for 5 min in 6 N HCl at three different temperatures. The concentration of pantetheine present in each sample before acid treatment was determined by the use of Ellman's (1959) reagent. The ring formation is apparently linear with pantetheine concentration during this time period and is temperature dependent (Figure 4). The maximum extent of reaction completion in this case is, as in the previous experiment, 34% for the 100° incubation. The linearity of the concentration curve is therefore again explained by approximation of initial rate conditions.

Once the thiazoline ring has been formed by the acid-dependent cyclization of pantetheine, the ultraviolet spectrum is dependent upon the pH of the solution. Figure 5 shows the spectral changes observed in the pantetheine thiazoline at different pH values.

It can be observed that after the initial cyclization of pantetheine in strong acid, the characteristic peak can be readily lost by adjusting the pH to values above 3. From the strong 266-mμ peak at pH values 1 and 2 the spectrum shows a shift to a lesser 250-mμ peak at pH values above 3. At values above pH 8 the end absorption becomes very strong and thus no peaks or shoulders can be observed. This shift of the spectrum is similar to the behavior of 2-methylthiazoline at various pH values described by Calvin (1954). In this case a pK at 5.3 was indicated which corresponds to the addition of a proton to the ring nitrogen forming the thiazolinonium ion. The protonated thiazolinonium ion is the species which gives the strong 265-mμ absorption. At pH values near neutrality and above, the ring exists as the unprotonated thiazoline form having a lesser absorption at 250 mμ.

Apparently the pK for the conversion of thiazolinonium into thiazoline found in our studies of pantetheine thiazoline is lower than the value determined by Calvin for 2-methylthiazoline. From the spectral data it appears to be between pH 2 and 3. Titration of the pantetheine thiazoline with HCl indicated a pK of about 2.5 for this conversion. These titration data give only an approximate value since considerable decomposition of the ring at acid pH values interfered with the determination. Nevertheless, it can be concluded that the pK for the pantetheine thiazoline is considerably lower than the 2-methylthiazoline value given by Calvin.

Garfinkel (1958) in his study of thiazoline formation into glutathione, concluded that the loss of the 265-mμ peak due to exposure to nonacidic conditions was irreversible. We have found, however, that the observed spectral changes in pantetheine are readily reversible. The thiazoline ring of pantetheine was formed in 6 N HCl as described and the excess HCl was removed by lyophilization. The residue was dissolved in water and acidified by addition of 1 drop of 1 N HCl and the spectrum was taken. The pH was then adjusted to 6.0 by addition of 1 drop of 1 N KOH and the spectrum was taken again. After reacidification by addition of 1 drop of 1 N HCl, the spectrum was obtained a third time. The results are shown in Figure 6.

From these results, it is obvious that the shift from the thiazoline to the thiazolinonium form is readily reversible. Thus, once the pantetheine has been cyclized to the ring form by the heat- and acid-dependent dehydration the equilibrium may

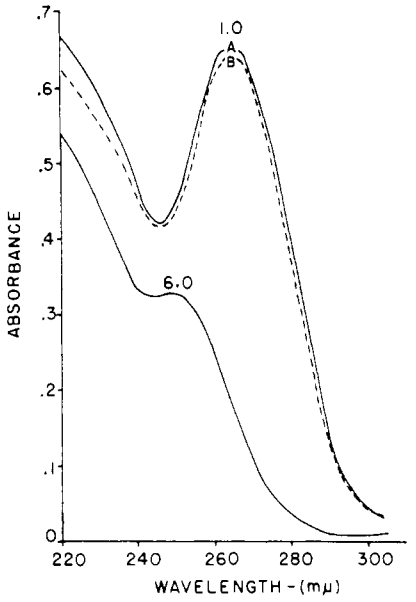


FIGURE 6: Stability of thiazoline ring to pH changes. Curve A is the original spectrum of pantetheine thiazoline (0.3 μmole). Curve B is the spectrum after adjusting to pH 6.0 and then reacidifying to 1.0.

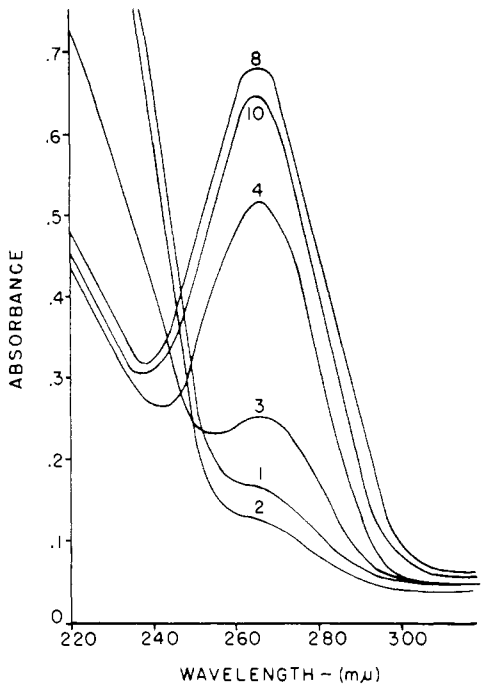


FIGURE 7: Stability of the thiazoline ring at various pH values. Aliquots of the thiazoline (0.3 μmole) were incubated at the pH values indicated and the spectra were determined.

be shifted back and forth from the thiazolinonium to the thiazoline without significant ring cleavage.

Since the isolation of the pantetheine thiazoline was contemplated, it was of interest to determine the stability of the ring at different pH values. Samples of pantetheine were cyclized and the acid was removed as previously described. The residues were dissolved in H_2O and the pH was adjusted to values of 1–10. The samples were allowed to stand for 1 hr at room temperature at the various pH values, each was reacidified, and the spectrum was obtained. In this manner the degree of ring cleavage was observed at the various pH values.

It can be seen from Figure 7 that the ring is almost completely hydrolyzed at acid pH values. From these results the point of minimum ring stability is around pH 2. The ring appears quite stable at neutral and slightly basic conditions but is hydrolyzed somewhat above pH 10. Therefore, the thiazolinonium ion appears to be more readily cleaved than the thiazoline form. Not until the pH reaches 10 does ring cleavage begin to take place.

Attempts in the past to isolate a single thiazoline structure from glutathione have not been successful. Goodman and Salce (1965) have stated that at least four distinct products are observed on paper chromatography after treatment of glutathione with 12 N HCl. Pantetheine thiazoline formed under our conditions was located as a distinct spot on paper chromatography using an ethanol–water (70:30, v/v) solvent system. The various spots were located by the nitroprusside and indophenol spray techniques. Upon thiazoline formation with pantetheine a single new spot was observed. The R_F values determined for pantethine, pantetheine, and pantetheine thiazoline are given in Table I.

The spot at R_F 0.68 produced after acid treatment of pantetheine is a distinct spot and apparently corresponds to the ring form of pantetheine. The reaction of the thiazoline spot with the spray reagents is identical with that of pantetheine. It therefore appears that the thiazoline form of pantetheine can be isolated on paper chromatography as a distinct form.

Discussion

The formation and degradation of the thiazoline ring most likely proceeds by the mechanism proposed by Martin *et al.* (1959) (Scheme I).

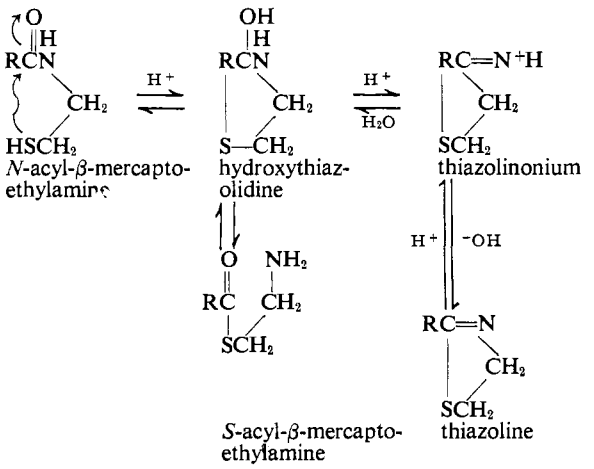
From our results it appears that the initial ring formation is an acid-catalyzed step which is probably first order in both acid and pantetheine concentration. The linearity of pantetheine thiazoline formation with acid concentration is in contrast to that observed with glutathione by Garfinkel (1958). In his study the optical density at 265 mμ did not increase

TABLE I: R_F Values of Pantetheine Forms.[a]

Compound	R_F	Nitroprusside: Without CN^-	Nitroprusside: With CN^-	Indophenol
Pantethine (SS)	0.81	−	+	−
Pantetheine (SH)	0.77	+	+	+
Pantetheine acid treatment	0.68	+	+	+

[a] + and − indicate a positive reaction or no reaction, respectively.

SCHEME I



linearly with acid concentration indicating that more than one acid-catalyzed pathway was involved in cyclization.

Once cyclization of pantetheine has occurred, the ring may exist predominantly as the thiazolinonium ion in very acid pH or as the thiazoline above the pK value. This shift of thiazolinonium to thiazoline can be observed by the spectral changes.

Our results which indicate that the ring is most readily cleaved around pH 2 and stable at neutral and slightly basic conditions are in agreement with the work of Martin *et al.* (1959). In this report, the hydrolysis rate of 2-methylthiazoline was studied as a function of pH. It was found that the maximum rate of thiazoline hydrolysis is around pH 3. Thus the thiazolinonium ion is subject to hydrolysis more readily than is the thiazoline form. We have also observed that the ring is more stable in very strong acid (6–12 N HCl) than in more dilute acid. This indicates that the ring cleavage is indeed a hydrolysis reaction since dehydration conditions inhibit it. This observation agrees with the results of Préaux and Lontie (1957) with glutathione.

It is of interest to note that the thiazoline spot on paper chromatography reacts with nitroprusside and indophenol reagents immediately as does pantetheine. The proposal by Basford and Huennekens (1955) that the thiazoline form of of CoA existed in a commercial preparation was based on evidence that one of the spots (R_F 0.35) observed on paper chromatography showed the slow reduction of indophenol. This difference in indophenol reduction may indicate that if the thiazoline form was present in their preparation the concentration must have been low. In work reported elsewhere (Jones and Nelson, 1968) we have repeated the procedure of Basford and Huennekens for the separation of CoA forms and have shown evidence that the R_F 0.35 corresponds to oxidized CoA (SS). However, since we did not observe a slow reduction of indophenol in our preparation, the presence of CoA thiazoline cannot be ruled out.

Since the thiazoline ring can be formed with pantetheine it must also be possible with CoA. However, it seems unlikely that the pyrophosphate linkages could survive the acidic conditions required for the cyclization of pantetheine.

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