[CONTRIBUTION FROM DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF WASHINGTON]

Studies on Thiols. II. Multiple Forms of Coenzyme A[1]

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Commercial Coenzyme A has been separated by paper and column chromatography, using ethanol/water (70:30) as the solvent system, into four distinct forms (I–IV). I reacts instantaneously with nitroprusside and indophenol sprays and is, therefore, the "free" –SH form. III and IV are disulfides since they react with nitroprusside only in the presence of cyanide, and do not react at all with indophenol. II is an anomalous form, since it reacts slowly with nitroprusside and indophenol, but not with hydroxylamine–$FeCl_3$. A quantitative spectrophotometric assay of the forms with indophenol confirms the assignment of I, III and IV, and reveals that II has a reaction rate similar to a thiazoline. All of the forms are active as coenzymes in the phosphotransacetylase, acetate-activating and succinic decarboxylase systems. The absorption spectra of I–IV show no bands other than the 260 mμ contribution of the adenine moiety. Model compound studies have included the absorption spectra of a typical thiazolidine, thiazoline and thiazole. Based upon a suggestion by Calvin, the cyclization of thiols for forming thiazolines in strong acid has been investigated, using the model compound N-acetyl-β-mercaptoethylamine.

Several investigators[3–5] have observed multiple forms of Coenzyme A[6] and *Lactobacillus bulgaricus* factor (pantathine),[7] when highly purified preparations were examined by paper chromatography. Although two to four components were found, most of the material and CoA activity was present as the "free" –SH form. It was not determined which of the forms pre-existed in the CoA preparation, and which might have been produced artificially during paper chromatography.

In the early part of the present investigation[8] four different components, all with CoA activity, were observed when commercial CoA was subjected to paper chromatography. Accordingly, a detailed study was made of the behavior of the four forms both enzymatically and chemically, making use, in part, of the quantitative indophenol assay described in the preceding paper. Particular attention has been directed toward one of the forms, which does not appear to be the "free –SH," a disulfide or an acyl form of CoA. It is possible that the anomalous form is one in which the sulfur exists in a thiazoline ring. Using a series of model compounds, a study has been made of thiazoline formation from "free" thiols.

Experimental

Chemicals.—Unless otherwise indicated, all chemicals were obtained from commercial sources or as described in Paper I of this series.[9] Coenzyme A and ATP (sodium salt) were obtained from the Pabst Laboratories, and acetyl phosphate from the Mann Research Laboratories. S-Acetyl pantatheine was a gift from Dr. Karl Folkers. Pantatheine was prepared by the reduction of pantathine according to the method of Beinert, *et al.*[4]

Preparation of N-Acetyl-β-mercaptoethylamine.—N-Acetyl-β-mercaptoethylamine was synthesized from thioacetic acid and ethyleneimine by the method of Kuhn and Quadbeck.[10] The crude product was a colorless oil which distilled at 138–140° under 7 mm. pressure. Upon iodometric titration and mercury titration in the cold, the sulfhydryl content was 81 and 87%, respectively, with cysteine used as the standard in each case. Paper chromatography of the material revealed the presence of a hydroxamic-positive impurity, probably the S-acetyl derivative. Attempts to purify the desired compound by conversion to the crystalline disulfide were unsuccessful owing to the extremely slow rate of oxidation in aqueous solution with iodine as a catalyst.

However, the material could be purified by conversion to the crystallline mercury salt according to the following procedure: 2 ml. of the crude oil was dissolved in 50 ml. of water and 0.5 *M* mercuric acetate added until no further precipitation occurred. The semi-crystalline precipitate was removed by centrifugation and recrystallized twice from 95% ethanol and once from hot water. The yield was 1.28 g. of white, lustrous plates which melted with decomposition at 210–220° (melting point block). The free thiol was regenerated by treatment of the mercury salt in ethanol–water (1:2) with hydrogen sulfide, removal of mercuric sulfide by filtration, and evaporation under nitrogen. The final product was a pale yellow oil which showed no hydroxamic-positive material.

Enzymes.—Phosphotransacetylase was prepared from *Clostridium kluyveri* (lyophilized cells obtained through the courtesy of Dr. H. A. Barker) by the method of Stadtman and Barker[11] and Stadtman,[12] and the assay of CoA was carried out with this enzyme by the method of Stadtman, *et al.*,[13] and Lipmann and Tuttle.[14] Acetate-activating enzyme was prepared by the method of Beinert, *et al.*,[15] and assayed with CoA by their method. α-Ketoglutaric oxidase was prepared and assayed by the method of Sanadi and Littlefield.[16]

Paper Chromatography.—Whatman No. 1 and 3 filter papers were used with the following solvent systems: $EtOH/H_2O$, (7/3) and isobutyric acid/concd. NH_4OH/H_2O, (66/1/33). Spray reagents for detecting CoA or its functional groups included the nitroprusside spray for –SH and SS groups described previously in Paper I, the molybdate spray of Bandurski and Axelrod[17] for organic phosphate compounds and 1% aqueous indophenol for reducing compounds. CoA

(1) This material is taken from the Dissertation of Robert E. Basford offered in partial fulfillment of the requirements for the degree of Doctor of Philosophy. The work was supported in part by grants from Eli Lilly and Co. and by Initiative 171, State of Washington. A preliminary report of this investigation has been given at the 37th Meeting of the Federated Societies of American Biologists at Chicago in April 1953 (F. M. Huennekens and R. E. Basford, *Federation Proc.*, **12**, 221 (1953)).

(2) Institute for Enzyme Research, University of Wisconsin.

(3) D. M. Buyske, R. E. Handschumacher, H. Higgins, T. E. King, F. M. Strong, V. H. Cheldelin, L. J. Teply and G. C. Mueller, *J. Biol. Chem.*, **193**, 307 (1951).

(4) H. Beinert, R. W. Von Korff, D. E. Green, D. M. Buyske, R. E. Handschumacher, H. Higgins and F. M. Strong, THIS JOURNAL, **74**, 854 (1952).

(5) J. D. Gregory, G. D. Novelli and F. Lipmann, *ibid.*, **74**, 854 (1952).

(6) See reference 5, preceding paper.

(7) G. M. Brown and E. E. Snell, *Proc. Soc. Exptl. Biol. Med.*, **77**, 138 (1951).

(8) F. M. Huennekens and R. E. Basford, *Federation Proc.*, **12**, 221 (1953).

(9) R. E. Basford and F. M. Huennekens, THIS JOURNAL, **77**, 3873 (1955).

(10) R. Kuhn and G. Quadbeck, *Ber.*, **84**, 844 (1951).

(11) E. R. Stadtman and H. A. Barker, *J. Biol. Chem.*, **180**, 1085 (1949).

(12) E. R. Stadtman, *ibid.*, **196**, 627 (1949).

(13) E. R. Stadtman, G. D. Novelli and F. Lipmann, *ibid.*, **191**, 365 (1951).

(14) F. Lipmann and L. C. Tuttle, *ibid.*, **159**, 21 (1945).

(15) H. Beinert, D. E. Green, P. Hele, H. Hift, R. W. Von Korff and C. V. Ramakrishnan, *ibid.*, **203**, 35 (1953).

(16) D. R. Sanadi and J. W. Littlefield, *ibid.*, **201**, 103 (1953).

(17) R. S. Bandurski and B. Axelrod, *ibid.*, **193**, 405 (1951).

was also detected by the "blue quenching" spot of the adenine moiety, which is observable when viewed under a special ultraviolet lamp (Mineralight).[18]

Column Chromatography.—Solka-floc paper pulp[19] was washed with acetic acid and acetone according to the method of Campbell, *et al.*,[20] and was packed in 1.65 × 49 cm. glass tubes by slurrying the powder in acetone, and packing under slight air pressure. The columns were washed with acetone and then with the solvent to be used (EtOH/H_2O, 7/3) until the E_{260} reading had been reduced to *ca.* 0.010. Ten mg. of CoA was dissolved in a small amount of solvent (0.5 cc.), added to the column at incipient dryness, and developed with the solvent. Two and one-half cc. fractions were collected by means of an automatic fraction collector.[21] Aliquots of the individual tubes were examined in the spectrophotometer at 260 mμ and the resulting E_{260} plotted against the volume of effluent solution. The profile thus obtained was used to combine the tubes containing the various fractions of CoA.

Results and Discussion

Paper Chromatography of CoA.—Although it was observed that CoA could be separated by paper chromatography into four forms using either isobutyric acid/ammonia/water, or alcohol/water as solvent systems, the latter was used almost exclusively, since it is a more innocuous solvent, and can be removed easily by lyophilization after the samples have been eluted from the paper. The R_f value for each form is roughly the same in either solvent system.

In Table I the R_f values for the four forms of CoA (I–IV) are listed along with certain of their chemical characteristics. None of the forms is produced artificially during the paper chromatography of the CoA, since the separated forms move with the same R_f values upon re-chromatography. Under the ultraviolet light all four forms can be seen as "blue quenching" spots. I (R_f 0.42) and II (R_f 0.35) are considerably darker, *i.e.*, they contain much more material than III (R_f 0.15) and IV (R_f 0.00). From the instantaneous reactivity of I with both the nitroprusside and indophenol sprays, it can be readily identified as the "free" –SH form. II, III and IV react with nitroprusside after the paper has been sprayed with cyanide, indicating that they are disulfide forms, probably the S–S form of CoA and the mixed disulfide of CoA and glutathione. The mixed disulfide might be expected as a contaminant since large quantities of glutathione are admixed during the isolation of CoA.[4] However, II, but not III and IV reacts slowly with indophenol, and it was this observation which first prompted the investigation of this form, since all disulfides or thiol esters previously examined[9] do not reduce indophenol, even slowly. None of the forms react with the hydroxylamine–ferric chloride spray for thiol esters.[22]

TABLE I

MULTIPLE FORMS OF CoA SEPARATED BY PAPER CHROMATOGRAPHY

+ and − indicate a positive reaction and no reaction, respectively, with the spray reagent. The nitroprusside reaction is seen as a magenta spot on a light yellow background, the indophenol reaction as a white spot on a blue background, and the phosphate color is blue on a white background.

Designation	R_f[a]	Nitroprusside Without CN	Nitroprusside With CN	Indophenol	Phosphate
I	0.42	+	+	+	+
II	.35	−	+	+(S)[b]	+
III	.15	−	+	−	+
IV	.00	−	+	−	+

[a] Solvent system EtOH/H_2O, (7/3). [b] (S) indicates indophenol bleached very slowly.

Column Separation of Multiple Forms of CoA.—In addition to paper chromatography as a means of separation of the forms of CoA, the use of Solka-floc paper columns was explored (see Experimental section). Table II shows the distribution of 260 mμ absorbing material, and indophenol and nitroprusside-positive material in the fractions collected. Ten mg. of CoA (*ca.* 10.5 μmoles on the basis of 80% purity) were placed on the column with a subsequent recovery of 73% based on adenine content, or 67% based on nitroprusside-sulfur. Separation of the same batch of CoA in bulk by preparative paper chromatography (see following section) gave the results shown in Table III where the forms have been assayed both chemically and enzymatically.

TABLE II

DISTRIBUTION OF CHEMICAL ACTIVITIES IN COLUMN FRACTIONS

Fractions[a]	260 mμ Absorption μmoles	260 mμ Absorption % Total	Indophenol μmoles	Indophenol % Total	SH, μmoles	SH + SS, μmoles	% Total[b]
8–26	7.06	92	4.11	80	5.25	5.25	75
68–90	0.45	6	0.90	18	0.30	1.28	18
146–150	0.09	1	0.04	1	0.05	0.10	1
153–155	0.05	1	0.05	1	0.00	0.35	5
Total	7.65		5.10		5.60	6.98	

[a] Fraction numbers represent tube numbers, each tube containing 2.5 ml. of effluent. [b] Based upon total SH plus SS.

TABLE III

DISTRIBUTION OF CHEMICAL AND ENZYMATIC ACTIVITIES IN PAPER CHROMATOGRAPHY FRACTIONS

CoA form	I	II	III	IV
Adenine,[a] % total	76	19	4	1
CoA,[b] % total	79	17	3	1

[a] Assayed by light absorption at 260 mμ. [b] Assayed by Acetate-activating enzyme.[15]

It can be seen that both methods separate the mixture into the same forms and in almost the same proportions. Although the column method is more suitable for large-scale separations, chromatography on paper is a much simpler procedure, gives nearly the same separation and recovery, and was adopted, therefore, for routine use in this investigation.

Chemical Reactivity of CoA Forms.—Ten or twenty mg. of CoA can be chromatographed conveniently on heavy Whatman No. 3 paper (a single sheet, 48 × 57 cm.), with the separated forms being subsequently eluted and concentrated by lyophilization. The chromatographically separated forms, obtained in this manner, were assayed

(18) Mineralight Model V-41 obtained from Ultra Violet Products, Inc., 145 Pasadena Ave., South Pasadena, California.

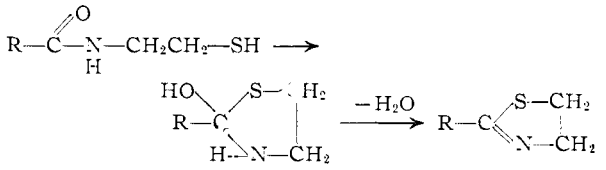
(19) Solka-floc paper pulp, BW 200, obtained from the Brown Co., 150 Causeway St., Boston 14, Mass.

(20) P. N. Campbell, T. S. Work and E. Mellanby, *Biochem. J.*, **48**, 106 (1951).

(21) Technicon Time-Flow Fraction Collector No. 2, Technicon Chromatography Corp., 215 East 149th St., New York 51, N. Y.

(22) E. R. Stadtman, *J. Biol. Chem.*, **196**, 535 (1952).

by the standard indophenol method described previously.[9] In each case 0.12 μmole of the form was assayed, assuming that all forms had the same molar extinction coefficient (15.9×10^3) at 260 mμ due to the adenine moiety. The results of this assay, expressed in terms of the initial velocity in each case, are illustrated in Table IV. Similar data obtained with cyclic thiols and glutathione (*cf.* Table III of the preceding[9]) are included for comparison. In confirmation of the evidence obtained previously with the spray reagents, the above studies revealed that I and glutathione have very similar rates in the indophenol assay systems and that I may be assigned unequivocally the "free –SH" structure. III and IV have no reactivity with indophenol, which is consistent with the assumption that they are disulfides. It is of considerable interest that II reacts with indophenol at a slow rate comparable to the "potential" thiols, wherein a cyclic structure must open hydrolytically to yield the "free" thiol. By an intramolecular reaction between the carbonyl group of the terminal amide linkage and the thiol group of CoA, a similar cyclic structure can be envisaged



where R represents the remainder of the CoA molecule. 2-Methyl-Δ^2-thiazoline may be considered, therefore, as a model compound for a possible cyclic structure of CoA.

TABLE IV

REACTIVITY OF CoA FORMS WITH INDOPHENOL

Assay method described in the preceding paper.[1]

Compound	V_0, μmole/ml./min.
Glutathione	2.4×10^{-2}
Thiazolidinecarboxylic acid	2.1×10^{-3}
2-Methyl-Δ^2-thiazoline	2.8×10^{-5}
2-Aminothiazole	2.4×10^{-5}
CoA form I	2.6×10^{-2}
CoA form II	3.5×10^{-5}
CoA form III	0[a]
CoA form IV	0[a]

[a] Less than 1×10^{-6} μmole/ml./min.

Coenzymatic Activity of Multiple Forms of CoA.—After the demonstration that forms I–IV can be separated physically, and that they differ chemically, it was of interest to ascertain quantitatively whether all four forms were active as coenzymes in various CoA-requiring enzyme systems.

Table V shows the activity of the four forms in the phosphotransacetylase system[11] which, in the presence of *catalytic* amounts of CoA (5.0×10^{-4} to 4.0×10^{-3} μmole), converts acetyl phosphate to acetyl CoA, which is decomposed by arsenate to regenerate CoA. The rate of disappearance of acetyl phosphate in the system is directly proportional to the amount of CoA present. Each of the multiple forms of CoA was adjusted spectrophotometrically to equivalent adenine concentration and assayed in the above system with unchromatographed CoA as the standard. It should be noted that good agreement was obtained between the calculated amount of CoA in I (3.78×10^{-3} μmole) based upon light absorption at 260 mμ and the amount assayed enzymatically as CoA (3.90×10^{-3} μmole). Although the same amount of CoA, based on 260 mμ absorption, was taken for the assay of each form, the ratio of activities of forms I:II:III:IV is roughly 4:2:1:2. However, the amount of CoA activity ascribed to each form is based upon a standard curve prepared from unchromatographed CoA. Thus, the differences observed in the activity of the different forms should not be considered as quantitative, since the action of the parts of a mixture is being evaluated by comparing them with the action of the entire mixture. This difficulty cannot be obviated at present, since, on a dry weight basis, 100% pure CoA of known structure is not available.

TABLE V

ACTIVITY OF CoA FORMS IN VARIOUS ENZYME SYSTEMS

CoA form	Phosphotransacetylase system[a] CoA assayed, μmole × 10^3	Acetate-activating system[b] CoA assayed, μmole × 10^3	Succinic decarboxylase system,[c] μl CO_2
I	3.90	7.0	305
II	2.22	6.0	307
III	0.99	4.9	284
IV	1.85	5.6	..[d]

[a] Each form was assayed using 3.78×10^{-3}, μmole of CoA (on the basis of adenine content) using the protocol of Stadtman, *et al.*,[13] and Lipmann and Tuttle.[14] [b] Each form was assayed using 5.5×10^{-2} μmole of CoA (on the basis of adenine content) by the method of Beinert, *et al.*[15] [c] Each form was assayed using 2.5×10^{-2} μmole of CoA (on the basis of adenine content) by the method of Whiteley.[23] [d] In this experiment the particular lot of CoA used did not contain any appreciable amount of IV.

In another experiment, equal quantities of the four forms of CoA were assayed with acetate-activating system of Beinert, *et al.*[15] In this system there is a *stoichiometric* relationship between the CoA present and the product formed, acetyl CoA. The results of this experiment are shown also in Table V, where it can be seen that all of the forms show nearly the same reactivity, the ratios being I:II:III:IV about 1.4:1.2:1.0:1.1. On the basis of adenine content 5.5×10^{-2} μmole of CoA was added in each case, and it is seen that for each form the CoA assay is in good agreement. If appreciable amounts of non-specific 260 mμ absorbing material had been eluted from the filter paper along with the CoA forms, the adenine would have been higher than the CoA values.

Table V shows also the activity of I, II and III in the succinic decarboxylase system from *M. lactilyticus*[23] which converts succinate to succinyl-CoA in the presence of ATP and CoA, and further converts succinyl-CoA to CO_2 and propionyl-CoA.[24] In this case the results are expressed in an arbitrary unit, μ liters of CO_2 evolved, corrected for the blank wherein CoA is absent. As in the preceding case, all the forms exhibited approximately equal activity.

(23) H. R. Whiteley, *Proc. Natl. Acad. Sci.*, **39**, 772, 779 (1953).

(24) The authors are indebted to Dr. Helen Whiteley for suggesting this experiment and performing the assays.

In all three assay systems, an excess of cysteine or glutathione (*ca.* 100 μmoles) was used to convert any disulfide forms of CoA to the "free –SH" form. In the experiments utilizing phosphotransacetylase and acetate-activating enzyme, attempts were made to assay the forms in the absence of a reducing agent. In these cases, III and IV were completely inactive, hence confirming that they are disulfides, but, in addition, forms I and II showed sub-optimal activity. The role of the added thiol is therefore, at least twofold, *i.e.*, the maintenance of the coenzyme in the free –SH form and, perhaps, maintenance of thiol groups on the enzymes in their reduced forms.

In connection with the experiment utilizing phosphotransacetylase, where the greatest variation was observed among the forms, Sanadi and Littlefield[16] have reported that only about 65% of the added CoA is converted to succinyl-CoA by α-ketoglutaric oxidase, although the remainder can be estimated as CoA by phosphotransacetylase. One possible explanation of this finding would be that one or more of the possible CoA forms is not "active" in the α-ketoglutaric oxidase system and that this form is fully active in the transacetylase system.

Spectral Examination of CoA Forms and Model Compounds.—If II is a cyclic thiol compound, similar to the model compounds, thiazolidinecarboxylic acid (A), 2-methylthiazoline (B), or 2-aminothiazole (C), evidence for its structure might be obtained in the ultraviolet absorption spectrum. Figure 1 shows the spectra of (A), (B) and (C) in phosphate buffer, *p*H 7. No maximum is observed in the spectrum of (A), merely a small "shoulder" at 240 mμ. (B) however, shows maxima at 230 and 246 mμ with about the same molar extinction coefficient (ε). (C) exhibits a strong maximum at 253 mμ and has a much larger ε value (*ca.* 6×10^3) than (A) or (B) (*ca.* 10^2). It is evident that the low ε values of the model compounds, especially A and B, and the fact that the absorption maxima are in the region 240–260 mμ, makes it difficult to demonstrate ring formation in a CoA form owing to the masking effect of the adenine contribution ($\epsilon = 1.59 \times 10^4$ at 260 mμ). It is not surprising, therefore, that CoA forms I, II and III have nearly identical spectra and show only the large peak at 260 mμ.

Cyclization of Thiols.—The assumption that CoA form II possesses a cyclic, thiazoline-like, structure rests principally on the evidence that II is oxidized by indophenol at a rate comparable to the cyclic rather than the open thiols. In addition, certain observations on the restriction or cyclization of thiols may be cited in support of this view.

Recently, Benesch, *et al.*,[25] and Pasinski and Tcherniak[26] have made the interesting observation that urea and guanidinium ion increase the amount of nitroprusside-positive material in complex thiols,

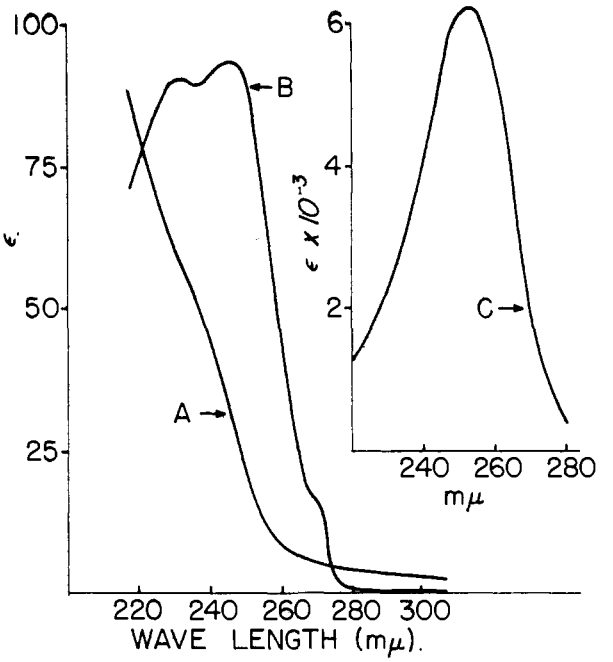


Fig. 1.—Spectra of model compounds. Spectra taken of aqueous solutions in 1-cm. cuvettes, with a Beckman spectrophotometer, Model DU.

including CoA and certain thiol-containing peptides. They consider this effect to be analogous to the appearance of thiol groups in proteins after treatment with these denaturating reagents. Urea and guanidinium are believed to break the hydrogen bonds between the thiol and other groups in the molecules, such as >C=O or $-NH_2$.

In addition to this mechanism wherein the thiol group of CoA might be restricted by hydrogen bonding, the possibility of thiazoline ring formation must be considered.

As shown in a previous diagram, such a cyclization is envisioned as occurring through an interaction of the thiol group and the carbonyl of the terminal peptide linkage to yield first a hydroxythiazolidine and finally a stable thiazoline. Fisher–Hirschfelder models show that this process can be carried out without any resulting strain in either product. Linderstrøm-Lang and Jacobsen[27] have postulated a similar mechanism to explain the "shielding" of sulfhydryl groups in proteins and have demonstrated, moreover, that guanidinium ion can open methylthiazoline, while Newton and Abraham[28] and Craig, *et al.*,[29] have also considered the possibility of thiazoline ring formation in the peptide, Bacitracin A.

Finally, in support of belief that glutathione is capable of thiazoline ring formation, Calvin[30] has made the important observation that both thiazoline and reduced glutathione in 12 *N* HCl show a new absorption band ($\epsilon = 10^3$) in the region 260–270

(25) R. Benesch, R. E. Benesch and W. I. Rogers, in "Glutathione, A Symposium," Academic Press, Inc., New York, N. Y., 1954, p. 31. We are indebted to Dr. Benesch for making available to us his manuscript prior to publication.

(26) A. G. Pasinski and R. C. Tcherniak, *Biochimia (Russian)*, **17**, 198 (1952).

(27) K. Linderstrøm-Lang and C. F. Jacobsen, *J. Biol. Chem.*, **137**, 443 (1941).

(28) G. G. F. Newton and E. P. Abraham, *Biochem. J.*, **53**, 604 (1953).

(29) L. C. Craig, W. Hausmann and J. R. Weisiger, THIS JOURNAL, **76**, 2839 (1954).

(30) M. Calvin presented at the Symposium on Glutathione, Ridgefield, Conn., Nov. 20–21, 1953.

mμ.[31] Oxidized glutathione did not exhibit this effect. We have confirmed and extended these observations using other model compounds related to CoA in anticipation of studies to be carried out with CoA form II. S-Acetylglutathione and thiazolidinecarboxylic acid do not give rise to this characteristic band in acid. The latter result is not surprising since this substance would have to undergo *oxidation* to the thiazoline, whereas in the cyclization process between a thiol and carbonyl group, the resulting hydroxythiazolidine could be *dehydrated* readily to the thiazoline.

A very curious finding is the fact that alatheine (β-alanylcysteine) shows the expected band in acid, whereas the closely related compound, pantatheine (pantoyl-β-alanylcysteine) does not.

The presumed addition of a proton to thiazoline, giving rise to the characteristic absorption band, is a reversible process, since, if the acidified solution is readjusted to *p*H 7, the 260–270 mμ band is replaced by the original spectrum. This finding has been used in the following experiment to provide further evidence that model thiol compounds can be converted to thiazolines. In Fig. 2 the spectra of N-acetyl-β-mercaptoethylamine are shown at *p*H 7, in 12 *N* acid, and returned to *p*H 7 after exposure to acid. It can be seen that the expected 260–270 mμ band appears in acid, and that after neutralization the material now retains a thiazoline-type spectrum indicating an acid catalysis of the ring closure or dehydration step.[32] In this connection it is of interest to recall that our sample of commercial CoA was prepared essentially by the process of Beinert, *et al.*,[4] in which the CoA is exposed to acid during its isolation, and it is possible that a thiazoline form of CoA may arise in this manner. Also, the effects noted by Benesch, *et al.*,[25] may be referable unequivocally to the breaking of hydrogen bonds in certain cases (*e.g.*, β-mercaptoethanol or β-mercaptoethylamine) where ring formation is impossible, and in others involving carbonyl groups the cleavage of the actual thiazoline ring may be promoted by the basic conditions (*p*H 11) of the nitroprusside assay.

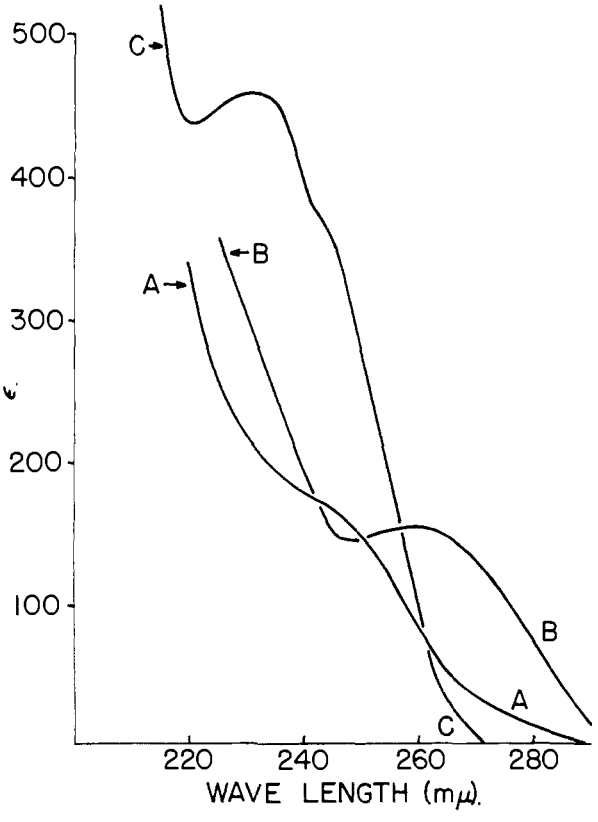
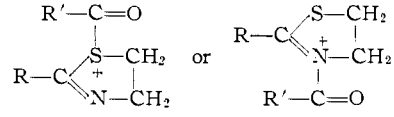


Fig. 2.—Spectra of N-acetyl β-mercaptoethylamine: (A) *p*H 7; (B) in 12 *N* HCl; (C) *p*H 7 after exposure to 12 *N* HCl. All spectra obtained in 1-cm. cuvettes with a Cary recording spectrophotometer.

(31) In neutral solution glutathione has no absorption in this region, whereas thiazoline has only weak absorption between 240 and 260 mμ (*cf.* Fig. 1).

If form II is a thiazoline structure, then the enzymatic assays, wherein this form is active, are of further interest. It can be shown from spectral evidence that cysteine at neutral *p*H is *unable* to open the thiazoline ring. Thus, it must be concluded either that the thiazoline ring is opened enzymatically, or that during the enzymatic reaction an acylthiazoline structure is formed, *viz.*



or perhaps the corresponding hydroxythiazolidine derivatives. In view of the fact that S-acetyl-β-mercaptoethylamine rearranges spontaneously to the N-acetyl compound,[10] the second structure would appear more probable.

Either of the above structures would possess the requisite "high-energy" acyl–CoA bond, since they are examples of "onium" type compounds, whose importance in enzymatic reactions has been emphasized recently by Woolley's review[33] and by Cantoni's important discovery that S-adenosylmethionine is the "active methyl" donor for creatine and N-methylnicotinamide synthesis.[34] The recent finding of Stadtman and White[35] that N-acetylimidazole can serve as an acetyl donor in the phosphotransacetylase system lends weight to the plausibility of the second structure.

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(32) Lynen and Ochoa, *Biochem. Biophys. Acta*, **12**, 299 (1953), have been able to substitute S-acyl-N-acetyl-β-mercaptoethylamine for S-acyl-CoA in at least two enzymatic processes of the fatty acid oxidizing system: (a) the reduction of β-keto CoA derivatives to β-hydroxy CoA derivatives by "β-keto-reductase" and (b) the reduction of α-β unsaturated fatty-acyl-CoA derivatives by "ethylene reductase." S-Acetoacetyl-glutathione could not replace S-acetoacetyl-CoA in the β-keto reductase system.

(33) D. W. Woolley, *Science*, **171**, 323 (1953).

(34) G. L. Cantoni, *J. Biol. Chem.*, **204**, 403 (1953).

(35) E. R. Stadtman and F. H. White, THIS JOURNAL, **75**, 2022 (1953).