Synthesis and Properties of Dimethyl-β-propiothetin Thiolesters*

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ABSTRACT: The methods usually used for the synthesis of the thiol esters of most organic acids are not applicable to acyl derivatives of "onium" compounds because of the insolubility of these compounds in nonaqueous solvents. Adaptation of the method used for the preparation of the thiolesters of amino acids has permitted the synthesis of the pantetheine and coenzyme A thiolesters of the sulfonium compound, dimethyl-β-propiothetin, and the pantetheine thiolesters of the quaternary ammonium compound, carnitine. The preparation and properties of these compounds have been studied in detail. Dimethyl-β-propiothetin pantetheine is unstable at pH values above pH 5.0 and decomposes spontaneously to acrylylpantetheine. This decomposition follows first-order kinetics and exhibits an activation energy of 24,000 cal.

Previous studies on the mechanism of the fermentation of dimethyl-β-propiothetin (DMPT)[1] by a *Clostridium* (Wagner and Stadtman, 1962) indicated that DMPT was fermented *via* its coenzyme A derivative (C. Wagner and E. R. Stadtman, unpublished results). At the same time a report appeared by Hosein *et al.* (1962) which suggested the presence of the coenzyme A esters of the analogous nitrogen compounds, γ-butyrobetaine, crotonobetaine, and carnitine, in brain. Since the synthesis and chemical properties of thiolesters of sulfonium compounds have not been previously described, we decided to undertake the chemical synthesis of the thiolesters of the "onium" compounds so that they might serve as authentic reference compounds for identification of biological intermediates.

Preliminary work indicated that synthesis of the thiolesters of the "onium" compounds could not be accomplished by simple adaptation of the standard methods used for synthesis of most acyl compounds (Stadtman, 1957; Vagelos, 1963). The present paper describes the preparation and properties of the pantetheine and coenzyme A derivatives of DMPT as well as the pantetheine derivative of carnitine.

Experimental Procedures

Materials and Methods. All chemicals and reagents were obtained from commercial sources. Pantethine was obtained from the Sigma Chemical Co., *dl*-carnitine and DMPT from Calbiochem, and coenzyme A from Pabst Laboratories. Silica gel G was obtained from Research Specialties Co. Absorption spectra were determined with the Perkin-Elmer 202 spectrophotometer.

The reactions were followed by taking advantage of characteristic tests given by certain groups. The presence of an acid anhydride, acid chloride, or thiolester was detected by reaction with neutralized hydroxylamine (Lipmann and Tuttle, 1945). The acid anhydride or acid chloride reacts immediately to form the hydroxamate while thiolesters were treated for about 20 min to ensure complete formation of the hydroxamate before addition of $FeCl_3$. Free sulfhydryl compounds were detected with the nitroprusside reagent of Toennies and Kolb (1951). Thiolesters were specifically detected by first treating the material with alkali to cleave the thiolester and then applying the nitroprusside reagent. Quantitative estimation of the sulfhydryl was carried out by the method of Grunert and Phillips (1951) as described by Stadtman (1957). The presence of the "onium" group was detected by the $KBiI_4$ reagent of Bregoff *et al.* (1953). This reagent was originally developed for the detection of quaternary ammonium compounds, but it serves equally well for the detection of sulfonium compounds. These tests could be used to detect the appropriate compounds on chromatograms.

Reduction of Pantethine. Pantethine was reduced to pantetheine with sodium borohydride. Care was taken to prevent reoxidation by flushing the reaction tube with helium.

Preparation of Acrylylpantetheine. The procedure used by Stadtman (1955) for the preparation of acrylylpantetheine, in our hands, yielded only a very small percentage of the unsaturated thiolester, the major product being a saturated thiolester, presumably the sulfhydryl addition compound of acrylylpantetheine. We were able to obtain a greater percentage of the unsaturated thiolester as compared to the saturated thiolester by carrying out the formation of acrylylpantetheine in a nonaqueous medium. The ethyl formate anhydride of acrylic acid was prepared by the method of Wieland and Rueff (1953). In a typical reaction 40 μmoles of pantetheine dissolved in tetrahydrofuran was added to 33 μmoles of the mixed anhydride in a total volume of 3 ml and allowed to react for 5 min at room temperature. The solution was evaporated to dryness under a

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[1] The abbreviation used is: DMPT, dimethyl-β-propiothetin.

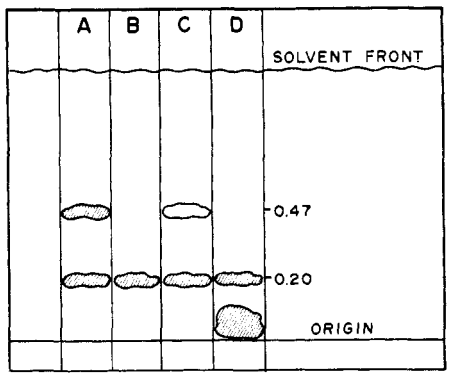


FIGURE 1: Thin layer chromatography of DMPT-pantetheine. The lyophilized reaction mixture was streaked along the origin of the plate as described in the test. The solvent was allowed to ascend for 3 hr. After drying, the plate was inspected under the following conditions: A, ultraviolet light; B, spray with KOH followed by nitroprusside; C, spray with hydroxylamine followed by $FeCl_3$; D, spray with $KBiI_4$ reagent.

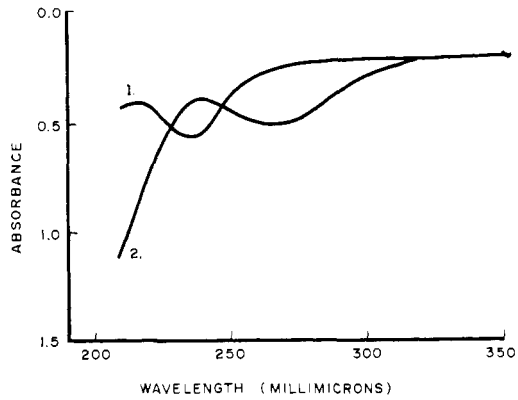


FIGURE 2: Ultraviolet spectrum of DMPT-pantetheine. Curve 1 is the spectrum obtained when the sample of DMPT-pantetheine in 0.0036 M glycine buffer, pH 2.4, was read against water. Curve 2 is the spectrum obtained when the same sample was adjusted to pH 6.5 with phosphate buffer.

stream of helium. The residue was dissolved in water to decompose any unreacted anhydride. This solution contained both an unsaturated as well as a saturated thiolester as shown by the presence of material with absorption maxima at 267 and 235 mμ. From the extinction coefficient for acrylylpantetheine (Stadtman, 1957) it was calculated that 2.0 μmoles of an unsaturated thiolester had been formed. An aliquot of the solution was treated with hydroxylamine and the resulting hydroxamate was chromatographed on paper in water-saturated butanol. The major hydroxamate spot migrated with an R_F of 0.12; a standard of acetyl hydroxamate migrated with an R_F of 0.50. Published R_F values for acrylyl and acetyl hydroxamates are 0.15 and 0.52, respectively (Stadtman and Barker, 1950; Vagelos *et al.*, 1959). The acrylyl hydroxamate was eluted from the paper and quantitated. This indicated that a total of 2.2 μmoles of acrylyl hydroxamate had been formed, which agreed quite well with the value of 2.0 obtained from the spectrum. This corresponded to a yield of 7% based upon the starting materials. The yield of acrylyl hydroxamate was always 20–50% of the total hydroxamate formed.

The acrylylpantetheine was purified further by thin layer chromatography on silica gel G plates using 1-butanol–water (50:9) as solvent. Two thiolester spots were seen at R_F values of 0.46 and 0.52. Both areas were scraped off the plates and the material was eluted from the powder by shaking with tetrahydrofuran. Only the material eluted from the upper spot had an absorption spectrum with a maximum at 267 mμ. Material from the lower spot showed an absorption maximum at 235 mμ and probably is the sulfhydryl addition product of acrylylpantetheine. Approximately 50% of the acrylylpantetheine was recovered from the thin layer plate after elution, as determined by the absorption spectrum. Subsequent rechromatography as described further in the text showed the presence of only a single compound.

Preparation of DMPT Acid Chloride. To a small round-bottom flask was added 2.0 g (11.8 mmoles) of DMPT (chloride salt) and 10 ml of thionyl chloride. A small magnetic stirring bar was added and the flask was closed with a drying tube containing $CaCl_2$. The mixture was stirred for 20 min at 50° and then at room temperature for 45 min longer. At this time all the DMPT had gone into solution. The excess thionyl chloride was removed under reduced pressure and a thick, yellow oil remained. This was washed several times with petroleum ether (bp 35–70°) to remove traces of thionyl chloride. The oil immediately reacted with neutral hydroxylamine to form an hydroxamate.

Preparation of DMPT-pantetheine. Excess DMPT acid chloride was added dropwise to 1 ml of an aqueous solution containing 720 μmoles of pantetheine kept at 0°, while helium was continuously bubbled through the reaction. Addition of DMPT was continued until the solution no longer gave a positive test for free sulfhydryl. Presence of a thiolester was indicated by first cleaving the thiolester with alkali and then showing presence of sulfhydryl. The solution was lyophilized, dissolved in a small amount of 0.001 N HCl, and purified by thin layer chromatography on silica gel G.

The product of the reaction between pantetheine and DMPT acid chloride was streaked along the bottom of a thin layer glass plate coated with silica gel G. This was chromatographed in 1-butanol–acetic acid–water (4:1:1). The resulting chromatogram was examined under a short-wave ultraviolet light and sprayed with the following reagents: 1, $KBiI_4$ for the presence of a sulfonium group; 2, nitroprusside reagent followed by alcoholic KOH for the presence of a thiolester; and 3, neutralized hydroxylamine followed by $FeCl_3$ after 30 min for the presence of an activated acid.

The results of such a chromatogram are shown in Figure 1. The material at R_F 0.20 was scraped from the

TABLE I: Paper Electrophoresis of DMPT-pantetheine Hydrolysis Products.[a]

Sample	Ultraviolet-Absorbing Spot	Active Acyl	Thiolester	"Onium"
DMPT-pantetheine, pH 4.0	4.7	4.7	4.6, 0.5	4.6, 0.5
DMPT-pantetheine, pH 7.0	0.5	0.5	0.5	0.5
DMPT-pantetheine, pH 9.0	...	...	...	0.5
Acrylylpantetheine	0.5	0.5	0.5	...
DMPT	...	...	...	0.5

[a] Samples of DMPT-pantetheine were treated as described in the text. Paper electrophoresis was carried out in a flat-bed Savant apparatus. The paper used was Whatman No. 3MM. The buffer was 0.01 M ammonium acetate, pH 4.0; 1500 v; 30 ma; 1 hr. The values are centimeters of migration toward the cathode.

plate and the silicic acid was extracted three times by shaking with 0.01 N HCl.

Analysis of DMPT-pantetheine purified in this way showed that of an initial 220 μmoles of DMPT and pantetheine the final product contained 12.6 μmoles of hydroxamate-forming material and 15 μmoles of thiolester, corresponding to an over-all yield of about 6%.

Properties of DMPT-pantetheine

Spectrum of DMPT-pantetheine. When the spectrum of the material eluted from the silica gel plates at R_F 0.20 was run against water it showed a maximum at 235 mμ, which is characteristic of saturated thiolesters (Figure 2). If a difference spectrum were taken, however, in which the blank cuvet contained the same amount of sample which had been adjusted to pH 8 in order to cleave the thiolester bond, the peak at 235 mμ was observed, but, in addition, a new peak was observed in the blank cuvet. This was shown by the appearance of absorbance values below base line with a maximum at 265 mμ. The maximum at 265 mμ slowly disappeared. The same disappearance of the 235 peak in the sample and appearance of the 265 peak in the blank could be shown at lower pH values. Figure 3 shows the development of the difference spectrum at pH 6.5. If the treated sample were itself run against distilled water, a peak was seen at 265 mμ (Figure 2). This indicated that treatment of DMPT pantetheine with alkali changed it from a compound which absorbs maximally at 235 mμ to one which absorbs maximally at 265 mμ. A logical candidate for the latter compound would be acrylylpantetheine. All α,β-unsaturated thiolesters show an absorption maximum at 263–267 mμ (Jaenicke and Lynen, 1963). In addition, treatment of DMPT with cold concentrated alkali causes a decomposition to acrylic acid and dimethyl sulfide (Challenger and Simpson, 1948).

Conversion of DMPT-pantetheine to Acrylylpantetheine. Elimination of dimethyl sulfide and resulting α,β-unsaturation of DMPT-pantetheine occurs rapidly at pH values above 6. Proof of this conversion is given by the following experiment. Each of three samples of DMPT-pantetheine, which was routinely stored in 0.01 N HCl, was adjusted to pH 4.0 with dilute KOH, to

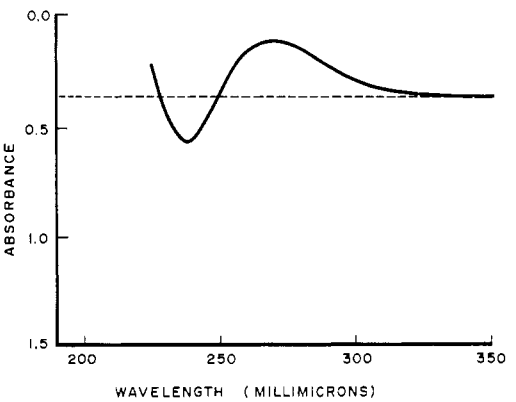


FIGURE 3: Difference spectrum of DMPT-pantetheine. Each cuvet received 0.1 ml of the thiolester and 0.1 ml of 0.05 M glycine buffer, pH 2.4, in a total volume of 1.4 ml. In addition, the blank cuvet received 0.1 ml of 0.1 M phosphate buffer, pH 6.5.

pH 7.0 with potassium phosphate buffer, and to pH 9.0 with Tris buffer. These were allowed to react at room temperature for 90 min. Samples were then spotted on thin layer plates or on paper for chromatography and electrophoresis. Authentic samples of acrylylpantetheine, and free DMPT were also spotted as markers. The results are shown in Tables I–III. These results indicate that DMPT-pantetheine is converted at pH 7.0 to a compound that is chromatographically identical with acrylylpantetheine. This compound is uncharged. It no longer contains a sulfonium group, while the thiolester linkage is still intact. At pH 9.0, however, the acrylylpantetheine is unstable and there is no longer any evidence for a thiolester.

When the chromatographic evidence is considered with the spectral changes it is apparent that the compound formed from DMPT-pantetheine at pH 7.0 is acrylylpantetheine and that at higher pH values this compound is itself cleaved to yield the free acid and the thiol.

Stability of DMPT-pantetheine. We have indicated

TABLE II: Paper Chromatography of DMPT-pantetheine Hydrolysis Products.[a]

Sample	Solvent 1				Solvent 2			
	Ultraviolet-Absorbing Spot	Active Acyl	Thiolester	"Onium"	Ultraviolet-Absorbing Spot	Active Acyl	Thiolester	"Onium"
DMPT-pantetheine, pH 4.0	0.25	0.25	0.25	0.28, 0.19	0.68	0.67	0.67	0.65, 0.57
DMPT-pantetheine, pH 7.0	0.88	0.88	0.89	0.20	0.87	0.87	0.87	0.56
DMPT-pantetheine, pH 9.0	...	...	...	0.22	...	...	...	0.57
Acrylylpantetheine	0.82	0.82	0.82	...	0.87	0.87	0.87	...
DMPT	...	...	...	0.19	...	...	...	0.67

[a] Samples of DMPT-pantetheine were treated as described in the text. Paper chromatography was carried out in an ascending system on Whatman No. 40 paper in two solvents. Solvent 1 was 1-butanol–acetic acid–water (4:1:1). Solvent 2 was methanol–acetic acid (10:1). Values are for the R_F values.

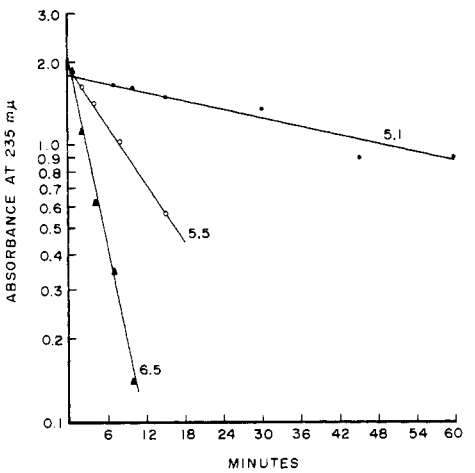


FIGURE 4: Rate of DMPT-pantetheine decomposition. The rates were followed by measuring absorbance changes at 235 mμ. Each sample contained 0.1 ml of the purified thiolester and 0.1 ml of 0.1 M buffer in a total volume of 1.4 ml. This was read against a blank cuvet containing buffer but no thiolester. Citrate buffer was used for pH 5.0 and 5.5; phosphate buffer was used for pH 6.5; the temperature was 34°.

TABLE III: Thin Layer Chromatography of DMPT-pantetheine Hydrolysis Products.[a]

Sample	Active Acyl	Thiolester	"Onium"
DMPT-pantetheine, pH 4.0	0.16	0.16	0.16, 0.03
DMPT-pantetheine, pH 7.0	0.69	0.69	0.03
DMPT-pantetheine, pH 9.0	...	...	0.03
Acrylylpantetheine	0.69	0.69	...
DMPT	...	...	0.03

[a] Samples of DMPT-pantetheine were treated as described in the text. Thin layer chromatography was carried out on 8 × 8 in. glass plates coated with silica gel G. The solvent used was 1-butanol–acetic acid–water (4:1:1) and allowed to ascend for 3 hr. Values are R_F values.

above that DMPT-pantetheine is more stable under acid conditions. The rate of conversion of DMPT-pantetheine to acrylylpantetheine under various conditions was followed by measuring the decrease in absorbance at 235 mμ. The rates obtained at three different pH values at 34° are shown in Figure 4. It can be seen that the rates follow first-order kinetics. The rate of acrylylpantetheine formation can be followed by measuring the increase in absorbance at 265 mμ in like manner. This also follows first-order kinetics. From the molar extinction coefficients (Stadtman, 1957) for the saturated and unsaturated thiolesters the amount of DMPT-pantetheine decomposed and the amount of acrylylpantetheine formed can be calculated. At pH 6.5 and 5.5, 75 and 80%, respectively, of the decomposed DMPT-pantetheine appears as the α,β-unsaturated thiolester. The pH dependence of the reaction is shown by a plot of the first-order rate constant *vs.* pH (Figure 5). From these data it may be calculated that the half-life of DMPT-pantetheine at pH 5.9 and 34° is only 2.6 min.

Activation Energy for the Decomposition of DMPT-pantetheine. The elimination of dimethyl sulfide and subsequent formation of the acrylyl derivative of pantetheine occurs much more readily than the corresponding reaction of the free DMPT (Challenger and Simpson, 1948). It would therefore be expected that the activation energy for the elimination reaction is decreased by the presence of the thiolester group. We have determined

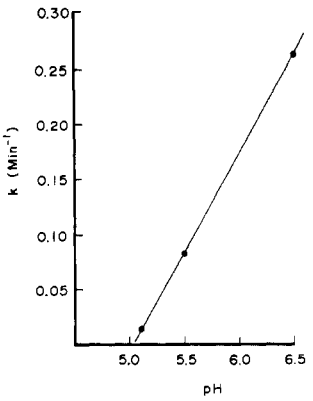


FIGURE 5: Decomposition of DMPT-pantetheine as a function of pH. The first-order rate constants for the decomposition of DMPT-pantetheine are plotted against pH. The temperature was 34°.

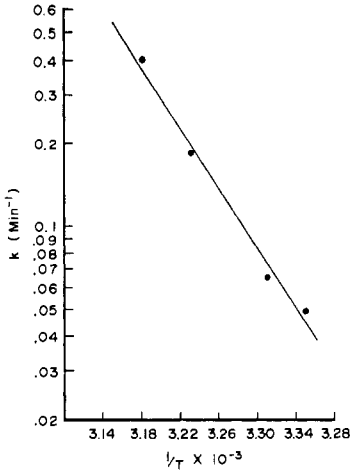


FIGURE 6: Decomposition of DMPT pantetheine as a function of temperature. The first-order rate constants are plotted against the reciprocal of the absolute temperature. The procedure was the same as that described in Figure 4 except that phosphate buffer was used to maintain the pH at 5.9.

the activation energy at pH 5.9 by measuring the first-order rate constants for the reaction at different temperatures. Figure 6 presents a plot of the reciprocal of the absolute temperature *vs.* the log of the first-order rate constant. The activation energy at pH 5.9 is calculated to be approximately 24,000 cal. While the corresponding value for DMPT is not known, the activation energy for the total unimolecular reaction involving the decomposition of *t*-butylsulfonium iodide by way of elimination and substitution reactions at pH 7.0 is 33,000 cal (Cooper *et al.*, 1948). At pH 5.9 the activation energy would be expected to be even higher. It is thus apparent that the presence of the thiolester has markedly reduced the activation energy for decomposition of the sulfonium compound.

Preparation of Other Thiolesters of Onium Compounds. DMPT-coenzyme A was prepared in a manner similar to that described for preparation of DMPT-pantetheine. The acid chloride of DMPT was added to 2.0 ml of an aqueous solution containing 13 μmoles of coenzyme A. Helium was bubbled through the reaction mixture to prevent oxidation of the thiol. The pH was kept between 2 and 3 by the addition of dilute NaOH to prevent loss of the phosphate group of coenzyme A under conditions of high acidity. The reaction was continued until all the free sulfhydryl had reacted. The reaction mixture was purified by thin layer chromatography in methanol–acetic acid (5:1) (R_F 0.40). The yield was about 20% based upon coenzyme A.

Carnitylpantetheine was synthesized in a similar manner. One gram of *dl*-carnitine was converted to the acid chloride by reaction with thionyl chloride as described for DMPT. The acid chloride was converted to the thiolester by dropwise addition to an aqueous solution containing 200 mg of pantetheine in a manner similar to that described for DMPT. The resulting thiolester was purified by chromatography on thin layer plates in methanol–acetic acid solvent (5:1). A spot was observed with an R_F of 0.33 which was positive for the quaternary ammonium group, thiolester, and activated acid. Upon elution it gave an ultraviolet spectrum with a maximum at 233 mμ.

Both the carnitylpantetheine and the DMPT-coenzyme A were analyzed for content of active acyl groups and thiolester groups by means of the quantitative applications of the hydroxamic acid and nitroprusside reactions. The results are presented in Table IV. Equivalent amounts of thiolester and active acyl groups were present.

TABLE IV: Analysis of Thiolesters.[a]

Compound	Active Acyl	Thiolester
DMPT-coenzyme A	1.30	1.34
Carnitylpantetheine	0.40	0.55

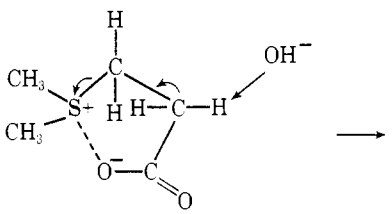
[a] Samples of DMPT-coenzyme A and carnitylpantetheine which had been prepared and purified as described in the text were analyzed for active acyl content and thiolester content. Values are expressed as μmoles per milliliter.

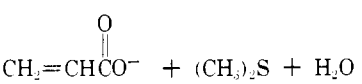
Discussion

Preparation of the thiolester derivatives of "onium" compounds presents certain difficulties due to their ionic character. The insolubility of these compounds in nonaqueous solvents precludes activation of the carboxyl group in a manner similar to that used for synthe-

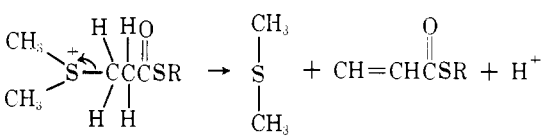
sis of the thiolesters of organic acids (Stadtman, 1957; Vagelos, 1963). We have, therefore, adapted the method of Wieland and Schafer (1952) for the preparation of the aminoacyl thiolesters. The intermediate conversion of the acyl chloride to the thiophenyl ester has been eliminated because of the instability of the thiolester derivatives of DMPT. Direct formation of the thiolester from the acyl chloride in aqueous solution has been carried out. Most of the acyl chloride hydrolyzed to the free acid, but by using a large excess of the acyl chloride, the thiolester was also formed.

At first, all attempts to carry out the esterification were carried out at pH 7.5 in order to neutralize the acid formed and promote formation of products. No thiolester of a sulfonium compound was detected under these conditions. When the reaction was carried out at pH 2–3 in aqueous solution, thiolester formation did occur. It is apparent from our studies on the stability of the DMPT thiolesters that failure to observe thiolester formation at pH 7.5 was due to the spontaneous decomposition to dimethyl sulfide and the acrylyl thiolester under these conditions. Decomposition of the free DMPT by alkali to yield acrylic acid and dimethyl sulfide probably occurs by the bimolecular mechanism developed by Hughes *et al.* (1948).



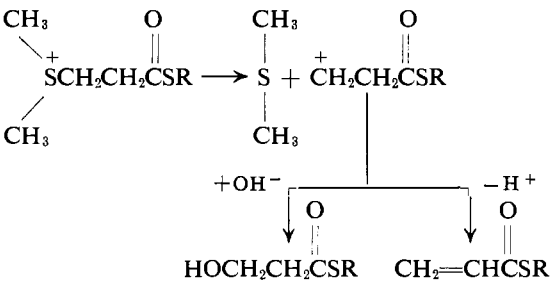


The electrophilic nature of the sulfonium group is probably diminished by its tendency to form the salt-like thetin with the carboxyl group. It is evident that formation of the thiolester labilizes the sulfonium group and permits elimination to occur under milder conditions. This may be due partly to a decreased tendency of DMPT for ring formation when present as the ester with consequent increased inductive effect of the sulfonium group. A possible mechanism is β elimination of a proton followed by elimination of dimethyl sulfide. Loss of the proton would be favored by the presence of both the thiolester and the sulfonium groups. This



mechanism is favored by OH^- and it is difficult to visualize it occurring at such low pH; however, the pH dependence of the decomposition suggests that it may operate.

Simple solvolysis followed by stabilization of the carbonium ion either through loss of a proton or addition of OH^- to form the β-hydroxypropionyl thiolester is also a possibility. Our data indicate that almost all of



the degraded DMPT thiolester can be accounted for as acrylyl thiolester. A choice between the alternative mechanisms is difficult with the available data.

Acknowledgments

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References

Bregoff, H. M., Roberts, E., and Delwiche, C. C. (1953), *J. Biol. Chem. 205*, 565.

Challenger, G., and Simpson, M. I. (1948), *J. Chem. Soc.*, 1591.

Cooper, K. A., Hughes, E. D., Ingold, C. K., Maw, G. A., and MacNulty, B. J. (1948), *J. Chem. Soc.*, 2049.

Grunert, R. R., and Phillips, P. H. (1951), *Arch. Biochem. 30*, 217.

Hosein, E. A., Proulx, P., and Roushan, A. (1962), *Biochem. J. 83*, 341.

Hughes, E. D., Ingold, C. K., and Maw, G. A. (1948), *J. Chem. Soc.*, 2072.

Jaenicke, L., and Lynen, F. (1963), *Enzymes 3*, 27.

Lipmann, F., and Tuttle, L. C. (1945), *J. Biol. Chem. 159*, 21.

Stadtman, E. R. (1955), *J. Am. Chem. Soc., 77*, 5765.

Stadtman, E. R. (1957), *Methods Enzymol. 3*, 931.

Stadtman, E. R., and Barker, H. A., (1950), *J. Biol. Chem. 184*, 769.

Toennies, G., and Kolb, J. J. (1951), *Anal. Chem. 23*, 823.

Vagelos, P. R., (1963), *Methods Enzymol. 6*, 544.

Vagelos, P. R., Earl, J. M., and Stadtman, E. R. (1959), *J. Biol. Chem. 234*, 765.

Wagner, C., and Stadtman, E. R. (1962), *Arch. Biochem. Biophys. 98*, 331.

Wieland, T., and Rueff, L. (1953), *Angew. Chem. 65*, 186.

Wieland, T., and Schafer, W. (1952), *Ann. Chem. 576*, 104.