# The Reaction of Carbanions with N,S-Diacetylcysteamine. A Model for Enzymatic Carbon–Carbon Condensation[1]

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*Malononitrile, N-acetyl-S-cyanoacetylcysteamine, and ethyl cyanoacetate react with N,S-diacetylcysteamine at 25° in aqueous solution near pH 9 to form N-acetylcysteamine and acetylmalononitrile, N-acetyl-S-2-cyanoacetoacetylcysteamine, or ethyl 2-cyanoacetoacetate, respectively. Between pH 4 and 11 in aqueous solution at 25° mercaptoacetic acid reacts with diethyl acetylmalonate to form acetylmercaptoacetic acid and diethyl malonate. Imidazole, acting as a nucleophilic catalyst, accelerates the transfer of the acetyl group from N,S-diacetylcysteamine to ethyl cyanoacetate. The rate enhancement is approximately $10^3$ with 1 M imidazole. The kinetic measurements show that the carbanions of malononitrile, N-acetyl-S-cyanoacetylcysteamine, and ethyl cyanoacetate are formed in a rapid equilibrium and are the reactive species in the reaction of these compounds with N,S-diacetylcysteamine. The reactive species in the reaction of mercaptoacetic acid with diethyl acetylmalonate are the thiol anion and un-ionized diethyl acetylmalonate.*

A number of enzymic reactions which result in the synthesis of a new carbon–carbon bond may be described, at least formally, as reactions of a carbanion with a thiol ester, usually of coenzyme A (CoA). These reactions include the condensation reaction of thiol esters of acetate and malonate in fatty acid synthesis[2] and the β-ketothiolase reactions,[3] in which the enolate anions of thiol esters are probably the carbanions; the synthesis from glycine and thiol esters of CoA of a number of α-amino ketones, including δ-aminolevulinic acid,[4] a reaction in which the carbanion is presumably formed by the ionization of the α-hydrogen of glycine in a glycine–pyridoxal phosphate Schiff base; and the decarboxylation of oxalyl CoA,[5] in which the carbanion of thiamine pyrophosphate probably attacks the thiol ester. The same type of reaction, in reverse, probably occurs in the oxidative decarboxylation of pyruvate to acetyl CoA and of α-ketoglutarate to succinyl CoA, in which an acylthiamine pyrophosphate intermediate is thought to react with a thiol group of dihydrolipic acid to form a thiol ester and the carbanion of thiamine pyrophosphate.[6]

It has been shown previously that thiol esters undergo a Claisen type of self-condensation to give variable yields of acetoacetate derivatives in the presence of metallic sodium or Grignard reagents under preparative conditions in nonaqueous solutions, and there is evidence that thiol esters are more reactive than oxygen esters under these conditions.[7] More recently, Daigo and Reed have demonstrated a base-catalyzed acetylation of *n*-butylmercaptan by 2-acetyl-3,4-dimethylthiazolium iodide in a solvent of low water content,[8] and Eggerer, *et al.*, have demonstrated the reversible reaction of acetyl cyanide with thiols in aqueous solution.[9]

The present report describes a study of the kinetics, mechanism, and catalysis of the condensation in aqueous solution of N,S-diacetylcysteamine, a model for the thiol ester groups of acetyl CoA and the acyl carrier protein,[2] with a number of potential carbanions, which are somewhat less appropriate models for the malonyl thiol esters and other potential carbanions which are involved in biochemical condensations. The conjugate carbanions of malononitrile (I), N-acetyl-S-cyanoacetylcysteamine (II), and ethyl cyanoacetate (III) yield N-acetylcysteamine and the acetylated carbanion compounds, which are strong acids and are ionized at neutral or alkaline pH (eq. 1). An example

$$R_1R_2\bar{C}H + CH_3\overset{O}{\overset{\|}{C}}SCH_2CH_2NH\overset{O}{\overset{\|}{C}}CH_3 \longrightarrow CH_3\overset{O}{\overset{\|}{C}}{=}\bar{C}R_1R_2 + CH_3\overset{O}{\overset{\|}{C}}NHCH_2CH_2SH \quad (1)$$

I, $R_1$ = CN; $R_2$ = CN
II, $R_1$ = CN; $R_2$ = $COSCH_2CH_2NHCOCH_3$
III, $R_1$ = CN; $R_2$ = $COOC_2H_5$

of the reverse reaction, the reaction of diethyl acetylmalonate with mercaptoacetic acid to yield S-acetylmercaptoacetic acid and diethyl malonate, is also reported.

## Experimental

*Materials.* Diethyl malonate, ethyl cyanoacetate, N-acetylethanolamine, ethyl thioacetate, mercapto-

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(7) (a) R. B. Baker and E. E. Reid, *J. Am. Chem. Soc.*, **51**, 1567 (1929); (b) M. W. Cronyn, M. P. Chang, and R. A. Wall, *ibid.*, 77, 3031 (1955); (c) J. C. Sheehan and C. W. Beck, *ibid.*, 77, 4875 (1955).

(8) K. Daigo and L. J. Reed, *ibid.*, **84**, 659 (1962).

(9) H. Eggerer, E. R. Stadtman, and J. M. Poston, *Arch. Biochem. Biophys.*, **98**, 432 (1962).

acetic acid, S-acetylmercaptoacetic acid, and malononitrile were commercial products and were distilled before use. 1-Acetylimidazole was a gift from Dr. Joseph Gerstein. Diethyl acetylmalonate (acetylmalonic acid diethyl ester), purchased from K & K Laboratories, Inc., was fractionally distilled; b.p. 94–96° (2.1 mm.), $n^{23}D$ 1.4463; reported[10] b.p. 65–70° (0.5 mm.), $n^{25}D$ 1.4435, and[11] b.p. 110–117° (13 mm.), $n^{20}D$ 1.4464–1.4477. Imidazole was recrystallized before use. Acetylmalononitrile, m.p. 138–140°, reported 141°, was prepared by the method of Arndt, *et al.*[12] N,S-Diacetylcysteamine (N-2-mercaptoethylacetamide acetate) was prepared as described by Gerstein and Jencks.[13] N-Acetylcysteamine (N-2-mercaptoethylacetamide) was prepared by the alkaline hydrolysis of N,S-diacetylcysteamine[13] and also by the reaction of thioacetic acid and ethylenimine.[14] Cyanoacetyl chloride was prepared according to the procedure of Patel, *et al.*,[15] and was used within 1 day. Thioacetimidic acid ethyl ester hydrochloride, m.p. 140–142°, reported[16] 143°, was made according to the method of Schmidt.[17] N,O-Diacetylethanolamine (N-(2-hydroxyethyl)acetamide acetate) was prepared by the acetylation of N-acetylethanolamine with acetyl chloride.[18]

Ethyl 2-cyanoacetoacetate was synthesized by a modification of the procedure of Michael and Eckstein.[19] Acetyl chloride (21 ml., 0.3 mole) was added dropwise to a cooled stirred mixture of ethyl cyanoacetate (32 ml., 0.3 mole), pyridine (48 ml., 0.6 mole), and anhydrous ethyl ether (75 ml.). After 15 min. at room temperature the reaction mixture was extracted with 200 ml. of 3 *M* hydrochloric acid and then at once with 100 ml. of cold 3 *M* potassium hydroxide. The basic aqueous extract was acidified to pH 1.5 with concentrated hydrochloric acid, whereupon a red oil separated. The oil was extracted into ether and the solution was dried with sodium sulfate. After removal of the ether with a rotary evaporator, the residue was fractionally distilled to give 12 g. of a colorless oil, b.p. 65° (0.2 mm.); reported[20] b.p. for ethyl 2-cyanoacetoacetate, 88–89° (0.6 mm.). The equivalent weight of the product by titration was 153; molecular weight of ethyl 2-cyanoacetoacetate, 155. The compound was stored at −20° and was redistilled just before use.

N-Acetyl-S-cyanoacetylcysteamine was synthesized by the acylation of N-acetylcysteamine. Cyanoacetyl chloride (15.5 g., 0.15 mole) was added dropwise to a cooled, stirred mixture of N-acetylcysteamine (18 g., 0.15 mole) and anhydrous ether (50 ml). During the addition a second phase formed. After removal of the ether with a rotary evaporator, there remained a yellow oil which partially solidified when it was left in an evacuated desiccator over solid sodium hydroxide. A portion (9.3 g.) of the oily solid, presumably the hydrochloride of N-acetyl-S-cyanoacetylcysteamine, was dissolved in 100 ml. of water and was adjusted to pH 3.5 by the addition of 19 ml. of 2 *M* potassium bicarbonate. The solution was extracted four times with 100-ml. portions of ethyl acetate. Upon removal of the ethyl acetate with the rotary evaporator 5.7 g. of yellowish crystals remained. The crude N-acetyl-S-cyanoacetylcysteamine yielded colorless crystals (m.p. 78–79°) after several recrystallizations from benzene. The compound possesses the typical ultraviolet spectrum of a thiol ester (Table I),[21] and its infrared spectrum in chloroform exhibits bands at *ca.* 2260 (w), 1680 (s), and 1510 (m) cm.$^{-1}$, characteristic of the cyano, thiol ester plus amide (amide I band), and amide (amide II band) functions,[22] respectively. *Anal.* Calcd. for $C_7H_{10}N_2O_2S$: C, 45.15; H, 5.38; N, 15.05; S, 17.20. Found: C, 45.38; H, 5.52; N, 14.76; S, 16.97.

N-Acetyl-S-2-cyanoacetoacetylcysteamine was prepared by the acetylation of N-acetyl-S-cyanoacetylcysteamine. Acetyl chloride (1.13 ml., 0.016 mole) was added dropwise to a cooled, stirred mixture of N-acetyl-S-cyanoacetylcysteamine (2.8 g., 0.015 mole), pyridine (2.4 ml., 0.03 mole), and anhydrous ethyl acetate (100 ml.). After the reaction mixture had stood for 30 min. at room temperature it was extracted with 15 ml. of 2 *M* hydrochloric acid and then with 30 ml. of 1 *M* potassium bicarbonate. The aqueous bicarbonate extract was acidified with 2 ml. of 12 *M* hydrochloric acid. The red oil that separated was extracted with ether and ethyl acetate. This extract was dried over sodium sulfate, and the solvent was removed with the rotary evaporator. The residual crude product (1 g.) was dried in an evacuated desiccator over potassium hydroxide and recrystallized several times from carbon tetrachloride to give colorless crystals, m.p. 104.5–106.5°. The equivalent weight determined by titration was 230; molecular weight of N-acetyl-S-2-cyanoacetoacetylcysteamine, 227. *Anal.* Calcd. for $C_9H_{12}N_2O_3S$: C, 47.37; H, 5.26; N, 12.28; S, 14.04. Found: C, 47.29; H, 5.43; N, 12.09; S, 13.97.

Cyanothiolacetic acid ethyl ester was prepared by a modification of the method of Patel, *et al.*[15] Ethanethiol (13.6 g., 0.22 mole) in 20 ml. of anhydrous ether was added dropwise to a cooled, stirred mixture of cyanoacetyl chloride (22.7 g., 0.22 mole) in 25 ml. of anhydrous ether. After the reaction mixture had stood for 1 hr. at room temperature the ether was removed by distillation at room temperature, and the residue was fractionally distilled to yield 19.8 g. of a colorless oil, b.p. 73–75° (0.5 mm.); reported[15] b.p. for cyanothiolacetic acid ethyl ester 126° (10 mm.). The compound possesses the ultraviolet spectrum typical of a thiol ester[21] (Table I), and its infrared spectrum in carbon tetrachloride exhibits bands at *ca.* 2250 (w) and 1690 (s) cm.$^{-1}$, characteristic of the cyano and thiol ester functions,[22] respectively.

*Ultraviolet Spectra and Acid Dissociation Constants.* Ultraviolet spectra were taken with a Cary Model 14 recording spectrophotometer and with a Zeiss PMQ II spectrophotometer. The absorbance was measured against a blank of exactly the same solvent. For those

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(11) D. S. Tarbell and J. A. Price, *J. Org. Chem.*, **22**, 245 (1957).
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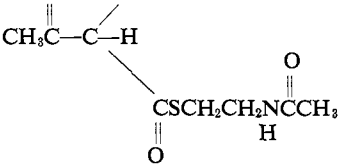
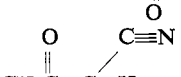
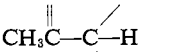
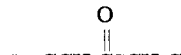
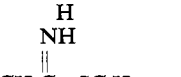
(13) J. Gerstein and W. P. Jencks, *J. Am. Chem. Soc.*, **86**, 4655 (1964).
(14) R. Kuhn and G. Quadbeck, *Ber.*, **84**, 844 (1951).
(15) D. K. Patel, V. Petrow, R. Royer, and I. A. Stuart-Webb, *J. Chem. Soc.*, 161 (1952).
(16) A. Hantzsch, *Ber.*, **64**, 664 (1931).
(17) E. Schmidt, *ibid.*, 47, 2545 (1914).
(18) I. Wadsö, *Acta Chem. Scand.*, **16**, 487 (1962).
(19) A. Michael and O. Eckstein, *Ber.*, **38**, 50 (1905).
(20) J. L. Burdett and M. T. Rogers, *J. Am. Chem. Soc.*, **86**, 2105 (1964).
(21) S. P. Colowick and N. O. Kaplan, *Methods Enzymol.*, **3**, 936 (1957).
(22) L. J. Bellamy, "The Infra-red Spectra of Complex Molecules," 2nd Ed., John Wiley and Sons, Inc., New York, N. Y., 1958.

**Table I.** Acid Dissociation Constants and Ultraviolet Spectra

| Compd. | $pK_a'$[a] | Solvent[b] | $\lambda_{max}$, mμ ($\epsilon$[c]) | $\lambda_{min}$, mμ ($\epsilon$[c]) | Stability of absorbance at $\lambda_{max}$[a] |
|---|---|---|---|---|---|
| $CH_3COCH(CN)_2$ | 1.0[d] | 1 *M* HCl | 250 (14,200) | | 15% decrease in 13 hr. |
| | | *j*, pH 7.6 | 257 (14,700) | | No change in 13 hr. |
| | | 0.1 *M* NaOH | 257 (14,700) | | No change in 13 hr. |
|  | | 1 *M* HCl | 282 (16,000) | | 72% decrease in 14 hr. |
|  | 1.8[d] | *j*, pH 7.6 | 288 (18,500)<br>224 (11,900) | 252 (2500) | No change in 14 hr. |
| | | 0.1 *M* NaOH | 288 (18,500)<br>224 (11,900) | 252 (2500) | No change in 14 hr. |
|  | | 0.02 *M* HCl | 253 (12,000) | | 32% decrease in 24 hr. |
|  | 3.0[d] | *j*, pH 7.6 | 264 (17,700) | 233 (3200) | No change in 24 hr. |
| | | 0.02 *M* NaOH | 264 (17,700) | 233 (3200) | No change in 24 hr. |
| $CH_3COCH(CO_2C_2H_5)_2$ | | 0.10 *M* HCl | 252 (1,700) | | 50% decrease in 10 hr. |
| | 6.68[e] | *j*, pH 7.8 | 270 (15,600) | | 50% decrease in 80 min. |
| | 6.65[f] | 0.01 *M* NaOH | 270 (17,000) | | 50% decrease in 80 min. |
| $N{\equiv}CCH_2C{\equiv}N$ | 11.25[d] | 0.01 *M* HCl | Low end absorption | | |
| | | 0.1 *M* NaOH | *ca.* 225 (*ca.* 20,000) | | 50% decrease in 40 min. |
|  | 8.85[d] | 0.1 *M* HCl | 234 (4,530) | | |
| | | 0.02 *M* NaOH | 270 (15,400) | | 8% decrease in 6 min. |
| $N{\equiv}CCH_2COOC_2H_5$ | 11.7[g] | $H_2O$ | Low end absorption | | |
| | | 0.1 *M* NaOH | 243 (*ca.* 15,000) | | 50% decrease in 3.8 sec. |
| $N{\equiv}CCH_2COSC_2H_5$ | 9.2[d] | 0.01 *M* HCl | 235 (5,100) | | No change in 2 hr. |
| | | 0.1 *M* NaOH | 270 (15,600) | | 14% decrease in 10 min. |
|  | | 0.01 *M* HCl | 228.5 (4,040) | | |
| | | *j*, pH 8.0 | 233.5 (4,375) | | |
|  | | 0.01 *M* HCl | 233 (4,730) | | |
| $HSCH_2COOH$ | 3.44[f] | | | | |
| | 9.95[f] | | | | |
|  | 9.38[h] | | | | |
|  | 6.9[i] | 0.01 *M* HCl | 243 (9,950) | | 11% decrease in 35 min. |
| | | *j*, pH 8.2 | 230 (5,500) | | 18% decrease in 20 min. |

[a] At 25 ± 3°. [b] All solutions contained about $10^{-4}$ *M* ethylenediaminetetraacetic acid. [c] $M^{-1}$ cm.$^{-1}$. [d] By spectrophotometric titration at an ionic strength of 1 *M* maintained with potassium chloride. The buffers used were: acetylmalononitrile and N-acetyl-S-2-cyanoacetoacetylcysteamine, hydrochloric acid; ethyl 2-cyanoacetoacetate, formate; malononitrile, carbonate and sodium hydroxide; N-acetyl-S-cyanoacetylcysteamine and cyanothiolacetic acid ethyl ester, carbonate. [e] By spectrophotometric titration in 0.01 *M* phosphate buffers, at an ionic strength of 0.05 *M* maintained with potassium chloride. [f] By half-neutralization at an ionic strength of 1 *M* maintained with potassium chloride. [g] From rate constant–pH profile for hydrolysis. [h] From ref. 13. [i] By titration of a freshly prepared 0.01 *M* solution with sodium hydroxide. [j] Tris(hydroxymethyl)aminomethane buffer, 0.01 *M*.

compounds that are unstable at a particular pH, extinction coefficients were obtained by preparing a more concentrated solution of the compound at a pH value at which it is stable, diluting this solution into the buffer in which the compound is unstable, and extrapolating the absorbance to the time of dilution.

Acid dissociation constants were determined by titration with sodium hydroxide, by spectrophotometric titration with buffers of different pH, or by measurement of the pH at half-neutralization. In the spectrophotometric titrations of unstable compounds absorbances were obtained by extrapolation to zero time, as described above. Measurements of pH were carried out with a glass electrode and a Radiometer PHM-4b pH meter.

Ethyl cyanoacetate undergoes rapid hydrolysis in aqueous alkali to form ethanol and cyanoacetate.[23] This rapid hydrolysis made it impossible to measure the p*K* of ethyl cyanoacetate by any of the above methods. It was possible to determine the rate of hydrolysis of ethyl cyanoacetate by following the decrease in the absorbance at 250 mμ of its conjugate carbanion. The rate of hydrolysis was measured in 0.005, 0.010, 0.020, 0.10, and 0.30 *M* sodium hydroxide and in 0.050 and 0.10 *M* carbonate buffer of pH 10.20, at 25° and 1 *M* ionic strength, maintained with potassium chloride. The rate constant for hydrolysis was found to be independent of the hydroxide ion concentration between

(23) W. A. Drushel, *Am. J. Sci.*, **33**, 27 (1912).

0.1 and 0.3 $M$ sodium hydroxide and independent of the carbonate buffer concentration at pH 10.20. A plot of the rate constants for hydrolysis against pH between pH 10 and 13.5 gave a sigmoid curve. These facts show that the rate law for the hydrolysis of ethyl cyanoacetate (CH) and its carbanion ($C^-$) is

$$v = k_2[OH^-][CH] = k_2[OH^-]\frac{(H^+)}{(H^+) + K_{CH}'}[CH + C^-] \quad (2)$$

Values of 1570 $M^{-1}$ min.$^{-1}$ for $k_2$ and of $2.0 \times 10^{-12}$ for $K_{CH}'$, the apparent acid dissociation constant of ethyl cyanoacetate, at 25° and 1 $M$ ionic strength, were obtained.

The acid dissociation constants, ultraviolet spectra, and stabilities of some compounds used in this study are summarized in Table I. The high extinction coefficients for the acidic forms of acetylmalononitrile, N-acetyl-S-2-cyanoacetoacetylcysteamine, and ethyl 2-cyanoacetoacetate indicate that these compounds exist predominantly in the enol form.[24]

*Rate Measurements.* Rates were measured spectrophotometrically with a Zeiss PMQ II spectrophotometer equipped with a thermostated brass block cuvette holder. Reactions were carried out under conditions in which pseudo-first-order kinetics were obtained, with all reactants but one in greater than tenfold excess. Reaction mixtures were prepared in 4-ml., 1-cm. quartz cuvettes and were temperature equilibrated in a water bath. Reactions were initiated by the addition of a small volume containing the reactant of low concentration. In general, a solution which contained all of the components of the reaction mixture except for the reactant of low concentration was used as the blank in the spectrophotometer. In certain cases quartz inserts that reduced the light path to 2.0 or 0.5 mm. were employed to lower the absorbance. All reactions were carried out at 25° and at an ionic strength adjusted to 1.0 $M$ with potassium chloride. Glass-distilled water was used throughout, and all reaction mixtures contained about $10^{-4}$ $M$ ethylenediaminetetraacetic acid to retard any trace-metal-catalyzed reactions. The pH of the reaction mixtures was measured immediately upon the end of the reaction.

With the exception of the reaction of malononitrile with N,S-diacetylcysteamine, rate constants were obtained by plotting the extent of reaction, $x_\infty - x_t$ (for an increase in absorbance) or $x_t - x_\infty$ (for a decrease in absorbance), against time on semilogarithmic graph paper and calculating the pseudo-first-order constants from the slope of the line, using the equation $k = 0.693/t_{1/2}$. All such plots were linear for at least two half-times.

The study of the reaction of malononitrile with N,S-diacetylcysteamine was complicated by several simultaneous reactions. One of these is the rapid reaction of malononitrile with N-acetylcysteamine to yield a stable compound that absorbs strongly in the ultraviolet ($\lambda_{max}$ 274 ($\epsilon_{max}$ 14,300 $M^{-1}$ cm.$^{-1}$)). The nature of this reaction is considered in the Results section. Another is the base-catalyzed dimerization of malononitrile to form 1,1,3-tricyano-2-amino-1-propene,[25] a compound which absorbs strongly in the ultraviolet. Consequently, the rates of reaction of malononitrile with N,S-diacetylcysteamine between pH 8 and 9 were determined by measuring the rate of the initial increase in absorbance at 275 mμ with 0.001–0.01 $M$ thiol ester and 0.1–0.3 $M$ malononitrile against a water blank. Under these conditions the initial product, N-acetylcysteamine, reacts completely and rapidly (about 500 times as fast as N,S-diacetylcysteamine) with the excess malononitrile (see Results section). Thus, the increase in absorbance at 275 mμ is due to the acetylmalononitrile anion, the compound which is formed from the thiol and malononitrile, and malononitrile dimer. The observed increase was corrected for the contribution from the formation of the dimer by measuring separately the increase in absorbance at 275 mμ in the absence of N,S-diacetylcysteamine under otherwise identical conditions. The correction varied from 5 to 45% of the total rate. Measurement of the rate of hydrolysis of N,S-diacetylcysteamine under the conditions of its reaction with malononitrile showed that in no case could hydrolysis and subsequent reaction of malononitrile with the thiol have contributed to more than 5% of the increase in absorbance. Since the extinction coefficients at 275 mμ for the acetylmalononitrile anion and the compound formed from malononitrile and N-acetylcysteamine were known, the rate constants could be calculated. In one instance the method of initial rates was checked by following to completion the increase in absorbance at 250 mμ, with a correction of each reading for the contribution by the malononitrile dimer. An approximately linear first-order plot was obtained, and the rate constant agreed to within 20% with that from the initial rate measurements.

The rates of reaction of N,S-diacetylcysteamine with N-acetyl-S-cyanoacetylcysteamine and ethyl cyanoacetate were followed at 295 and 264 mμ, respectively. The concentrations of the products were measured spectrophotometrically. Above pH 9.5 the pH of some reaction mixtures dropped as much as 0.05 unit in one half-time because of hydrolysis of N,S-diacetylcysteamine, which was the reactant in excess. In these cases the pH was taken as that after 50% reaction, and the rate constants are only accurate to about ±15%.

The rate of the reaction of ethyl cyanoacetate with ethyl thioacetate was estimated as follows. A solution containing 0.020 $M$ ethyl cyanoacetate, 0.020 $M$ ethyl thioacetate, and 0.50 $M$ potassium carbonate–bicarbonate buffer (1:1) at pH 9.80 was maintained at 25° for 30 min. In this time the ethyl cyanoacetate undergoes complete hydrolysis, whereas less than 10% of the ethyl thioacetate undergoes hydrolysis. The reaction mixture was then extracted with ether to remove the ethyl thioacetate, the absorption of which interferes with the spectrophotometric determination of the ethyl 2-cyanoacetoacetate anion. The spectrum of the ether-extracted reaction mixture, when run against a blank prepared in an identical way with the exception that ethyl cyanoacetate was omitted, was identical with that of ethyl 2-cyanoacetoacetate anion and showed the formation of $7.5 \times 10^{-5}$ $M$ ethyl 2-cyanoacetoacetate anion. The low yield of condensation product (0.37%) shows that hydrolysis of ethyl

(24) A. E. Gillam and E. S. Stern, "Electronic Absorption Spectroscopy," 2nd Ed., Edward Arnold Ltd., London, 1957, p. 257.
(25) F. S. Eberts, Jr., G. Slomp, and J. L. Johnson, *Arch. Biochem. Biophys.*, **95**, 305 (1961).

cyanoacetate is the predominant reaction under these conditions. The first-order rate constant ($k_h$) for the hydrolysis of ethyl cyanoacetate under the same conditions was determined by following the decrease in absorption of the ethyl cyanoacetate anion at 260 mμ and was found to be 0.13 min.$^{-1}$. With these data, the second-order rate constant for the reaction of the ethyl cyanoacetate anion with ethyl thioacetate ($k_2$) was calculated from eq. 3, in which [P] is the final con-

$$k_2 = \frac{[P]}{[CH_3COSR][CH]} \frac{(H^+) + K_{CH}'}{K_{CH}'} k_h \qquad (3)$$

centration of ethyl 2-cyanoacetoacetate anion, [$CH_3$-COSR] and [CH] are the initial concentrations of ethyl thioacetate and ethyl cyanoacetate, respectively, $K_{CH}'$ is the apparent dissociation constant of ethyl cyanoacetate, and ($H^+$) is the hydrogen ion activity. The value of $k_2$ was found to be 1.7 $M^{-1}$ min.$^{-1}$. Similar measurements which were made at pH 9.52 (0.5 $M$ potassium carbonate–bicarbonate buffer, 1:2) and 25° with 0.010 $M$ ethyl cyanoacetate and 0.040 $M$ ethyl thioacetate gave a value of 2.0 $M^{-1}$ min.$^{-1}$ for $k_2$.

The rates of reaction of mercaptoacetate with diethyl acetylmalonate were measured spectrophotometrically at 270 mμ (diethyl acetylmalonate anion disappearance) in the pH range 4.7–10.9 with 0.1 $M$ or less acetate, phosphate, imidazole, and Tris buffers. The mercaptoacetate concentration (0.2–0.4 $M$) was in great excess over that of diethyl acetylmalonate (about $10^{-3}$ $M$). The concentration of S-acetylmercaptoacetate that was formed in the reaction was measured after complete disappearance of the diethyl acetylmalonate by the hydroxylamine–ferric chloride method according to the procedure for the determination of thiol esters of Jencks and Gilchrist.[26] Under these conditions for the determination, diethyl malonate gives no reaction.

*Analysis of Products.* The formation of acetylmalononitrile from malononitrile and N,S-diacetylcysteamine was demonstrated as follows. A solution of malononitrile (10 mmoles) and N,S-diacetylcysteamine (5 mmoles) in 9 ml. of water was maintained at pH 8.5 by the addition of 1 1 $M$ potassium hydroxide with a Radiometer TTTlc titrator, at 22–25°. After 40 min. 4.65 mmoles of base had been consumed. The reaction mixture was then extracted with ether and acidified with 4 ml. of 6 $M$ hydrochloric acid, whereupon an oil separated. The acidic mixture was extracted with ether. The ultraviolet spectrum of a diluted aliquot of the ethereal extract was identical with that of authentic acetylmalononitrile and revealed that the extract contained 4.5 mmoles (90%) of acetylmalononitrile. Evaporation of the dried ethereal extract yielded 0.52 g. (95%) of crude acetylmalononitrile which was recrystallized from toluene (m.p. 138–141°). In another experiment, the spectrum of a reaction solution, when corrected for the absorption of the malononitrile dimer, was found to be that expected for an equimolar mixture of the acetylmalononitrile anion and of the compound formed from malononitrile and N-acetylcysteamine.

The spectra of the reaction solutions after the completion of kinetic runs were obtained in a number of cases for the reaction of N,S-diacetylcysteamine with N-acetyl-S-cyanoacetylcysteamine and with ethyl cyanoacetate in the presence and absence of imidazole. In each case, the difference spectrum between the reaction mixture and the blank was identical with that of the anion of N-acetyl-S-2-cyanoacetoacetylcysteamine or the anion of ethyl 2-cyanoacetoacetate.

S-Acetylmercaptoacetic acid was identified as the product of the reaction between mercaptoacetic acid and diethyl acetylmalonate in the following way. A mixture of 0.50 $M$ diethyl acetylmalonate and 0.05 $M$ mercaptoacetic acid was maintained at pH 7.9 by the addition of sodium hydroxide with the automatic titrator, at 22–25°. After 4 hr. the reaction mixture was adjusted to pH 5 and was extracted with ether. The spectra at pH 2 and 8 of diluted aliquots of the aqueous solution were identical with those of authentic S-acetylmercaptoacetic acid, including the shift in $\lambda_{max}$ and $\epsilon_{max}$ (Table I). Spectrophotometric measurement showed an 81% yield of the thiol ester.

## Results

*Reactions of Carbanions with N,S-Diacetylcysteamine.* The rate constants for the reactions of N,S-diacetylcysteamine with a number of potential carbanions (eq. 1) are summarized in Table II. The reaction with malononitrile was followed spectrophotometrically by measuring the initial rate of appearance of acetylmalononitrile anion and the N-acetylcysteamine–malononitrile adduct, as described in the Experimental section. Pseudo-first-order rate constants for the reactions with N-acetyl-S-cyanoacetylcysteamine and ethyl cyanoacetate were determined by following the appearance of the anions of N-acetyl-S-2-cyanoacetoacetylcysteamine and ethyl 2-cyanoacetoacetate, respectively, in the presence of a large excess of thiol ester. The ester groups of the reactant carbanions undergo concurrent hydrolysis, and the rate constants for carbon–carbon condensation were calculated from the observed rate constants and the fraction of reactant that was acetylated. For example, with 0.25 $M$ carbonate buffer and 0.5 $M$ N,S-diacetylcysteamine, 53% of the N-acetyl-S-cyanoacetylcysteamine at pH 8.96 and 8.8% of the ethyl cyanoacetate at pH 9.1 underwent acetylation. In several cases the rate constants for hydrolysis were measured separately (Table II), and these values are in tolerable agreement with those calculated from $k_{obsd}$ and the fraction of hydrolysis. The rates of reaction of the carbanion compounds with N,S-diacetylcysteamine are proportional to the concentrations of the carbanion compounds and N,S-diacetylcysteamine and increase with increasing pH.

From the variation of the apparent second-order rate constants for the carbon–carbon condensation reactions with the reciprocal of the hydrogen ion activity (Figure 1) or pH (Figure 2), it is evident that in each case the carbanion ($C^-$) is the reactive species and that the rate law is

$$v = k_2[C^-][CH_3\overset{O}{\overset{\|}{C}}SR] = k_2 \frac{K_{CH}'}{K_{CH}' + (H^+)} \times [C^- + CH][CH_3\overset{O}{\overset{\|}{C}}SR] \qquad (4)$$

(26) W. P. Jencks and M. Gilchrist, *J. Am. Chem. Soc.*, 86, 4651 (1964).

**Table II.** Rates of Reaction of Carbanion Compounds with N,S-Diacetylcysteamine, at 25° and 1 *M* Ionic Strength

| Carbanion compd. | p$K_{CH}'$ | pH | Initial concn. of carbanion compd., *M* | Initial concn. of N,S-diacetyl-cysteamine, *M* | Concn. of acetyl carbanion at $t_\infty$, $M \times 10^3$ | $k_{obsd} \times 10^2$, min.$^{-1}$ | $k_{acylation} \times 10^2$,[a] min.$^{-1}$ | $k_2' \times 10^2$,[b] $M^{-1}$ min.$^{-1}$ | $k_2$,[c] $M^{-1}$ min.$^{-1}$ |
|---|---|---|---|---|---|---|---|---|---|
| $N{\equiv}CCH_2C{\equiv}N$[d] | 11.25 | 8.02[e] | 0.10 | 0.010 | | *d* | *d* | 4.7 | 88 |
| | | | 0.20 | 0.010 | | | | 4.9 | |
| | | | 0.30 | 0.010 | | | | 4.6 | |
| | | | 0.10 | 0.001 | | | | 5.0 | |
| | | | 0.10 | 0.005 | | | | 5.1 | |
| | | 8.52[e] | 0.10 | 0.010 | | | | 16.5 | |
| | | | 0.20 | 0.004 | | | | 15.4 | |
| | | | 0.10 | 0.002 | | | | 16.3 | |
| | | 8.85[e] | 0.10 | 0.004 | | | | 37.8 | |
| $N{\equiv}CCH_2CSCH_2CH_2NCCH_3$ (O, O, H) | 8.85 | 8.33[f] | $5.0 \times 10^{-4}$ | 0.25 | 0.076 | 3.1 | 0.47 | 1.9 | 0.084 |
| | | 8.50[f] | $5.4 \times 10^{-4}$ | 0.25 | 0.094 | 3.4 | 0.59 | 2.4 | |
| | | 8.91[g] | $5.0 \times 10^{-4}$ | 0.25 | 0.198 | 2.9 | 1.15 | 4.6 | |
| | | 8.96 | $5.0 \times 10^{-4}$ | 0.50 | 0.265 | 3.6 | 1.9 | 3.8 | |
| | | 9.00 | $5.0 \times 10^{-4}$ | 0.25 | 0.175 | 3.0 | 1.05 | 4.2 | |
| | | 8.95 | $5.0 \times 10^{-4}$ | 0.125 | 0.112 | 2.8 | 0.63 | 5.0 | |
| | | 9.02 | $5.0 \times 10^{-4}$ | ... | ... | 2.0[i] | | | |
| | | 9.35 | $5.0 \times 10^{-4}$ | 0.50 | 0.285 | 4.5 | 2.6 | 5.2 | |
| | | 9.31 | $5.0 \times 10^{-4}$ | 0.25 | 0.190 | 3.7 | 1.4 | 5.6 | |
| | | 9.29 | $5.0 \times 10^{-4}$ | 0.125 | 0.123 | 3.4 | 0.83 | 6.6 | |
| | | 9.27 | $5.0 \times 10^{-4}$ | ... | ... | 2.6[i] | | | |
| | | 9.43 | $5.0 \times 10^{-4}$ | 0.25 | 0.206 | 4.3 | 1.8 | 7.2 | |
| | | 9.82 | $5.0 \times 10^{-4}$ | 0.25 | 0.225 | 4.5 | 2.0 | 8.0 | |
| | | 10.15 | $5.0 \times 10^{-4}$ | 0.25 | 0.225 | 4.5 | 2.0 | 8.0 | |
| | | 10.35 | $5.0 \times 10^{-4}$ | 0.25 | 0.254 | 4.5 | 2.3 | 9.2 | |
| $N{\equiv}CCH_2COOC_2H_5$ | 11.7 | 9.10[h] | 0.0068 | 0.25 | 0.600 | 2.2 | 0.19 | 0.76 | 3.0 |
| | | 9.10[h] | 0.0034 | 0.50 | 0.385 | 2.9 | 0.33 | 0.66 | |
| | | 9.43[h] | 0.00625 | 0.25 | 0.485 | 5.1 | 0.40 | 1.6 | |
| | | 9.43[h] | 0.00375 | 0.50 | 0.412 | 8.2 | 0.90 | 1.8 | |
| | | 9.77[h] | 0.00625 | 0.25 | 0.496 | 11.1 | 0.88 | 3.5 | |
| | | 9.74[h] | 0.0050 | ... | ... | 9.9[j] | | | |
| $H_2C(CO_2C_2H_5)_2$ | 15.2[k] | | | | | | | | <500[l] |
| $H_2O$ | 15.7 | | 0.010[m] | 0.00017 | | 8.9 | | | 9.1[n] |
| | | | 0.015[m] | 0.00017 | | 13.8 | | | |
| | | | 0.020[m] | 0.00017 | | 18.4 | | | |
| $HC{\equiv}N$[o] | 9.3 | | | | | | | | *ca.* 0.90 |

[a] $k_{acylation} = k_{obsd}$[acetyl carbanion at $t_\infty$]/[carbanion compound initially]. [b] For malononitrile, $k_2'$ = initial rate ($M$ min.$^{-1}$)/[malononitrile]·[N,S-diacetylcysteamine]; for N-acetyl-S-cyanoacetylcysteamine and ethyl cyanoacetate, $k_2' = k_{acylation}$/[N,S-diacetylcysteamine]. [c] For reaction of the carbanion with N,S-diacetylcysteamine, $k_2 = k_2'$ ([$H^+$] + $K_{CH}'$]/$K_{CH}'$). The values given are averages. [d] With malononitrile, each value for $k_2'$ is the average of duplicate determinations of initial rates. [e] In 0.2 *M* tris(hydroxymethyl)aminomethane buffer; a change in buffer concentration from 0.1 to 0.4 *M* at pH 8.02 does not alter the rate. [f] In 0.1 *M* triethanolamine buffer; a change in buffer concentration from 0.05 to 0.10 *M* at pH 8.5 does not alter the rate. [g] In 0.25 *M* carbonate buffer between pH 8.91 and 10.35; a change in buffer concentration from 0.25 to 0.50 *M* at pH 9.3 does not affect the rate. [h] In 0.25 *M* carbonate buffer; a change in buffer concentration from 0.25 to 0.5 *M* at pH 9.4 does not affect the rate. [i] From the decrease in absorbance at 270 mμ. [j] From the decrease in absorbance at 250 mμ. [k] C. Vermesse-Jacquinot, R. Schaal, and P. Rumpf, *Bull. soc. chim. France*, 2030 (1960). [l] An upper limit. [m] Hydroxide concentration. [n] For the reaction of hydroxide ion with N,S-diacetylcysteamine, measured by the decrease in optical density at 234 mμ of acid-quenched aliquots, after the method of P. J. Hawkins and D. S. Tarbell, *J. Am. Chem. Soc.*, **75**, 2982 (1953). [o] From ref. 9, for the reaction of cyanide ion with S-acetyl-N-succinylcysteamine at 25°.

in which $K_{CH}'$ is the apparent acid dissociation constant of the carbanion compound. The validity of this rate equation is most clearly illustrated for the reaction of N-acetyl-S-cyanoacetylcysteamine (Figure 2). The rate constants for this reaction follow eq. 4 in the pH range in which ($H^+$) is equal to or less than $K_{CH}'$. The fact that the rates of reaction of the carbanion compounds with N,S-diacetylcysteamine are not altered by changes in the concentrations of tris(hydroxymethyl)aminomethane, triethanolamine, and carbonate buffers indicates that the reaction is not subject to general base catalysis (Table II, footnotes *e–h*).

The reactions of N-acetyl-S-cyanoacetylcysteamine were carried out at a low concentration ($5 \times 10^{-4}$ *M*) of this compound, because at higher concentrations (above $10^{-3}$ *M*), even in the absence of N,S-diacetylcysteamine, a compound with an absorption maximum at 288 mμ is formed. By analogy with other thiol ester–carbanion reactions, this compound is presumably the anion of N-acetyl-S-2,4-dicyanoacetoacetylcysteamine, the product of the reaction of N-acetyl-S-cyanoacetylcysteamine with its carbanion. If the extinction coefficient for this product is taken to be the same as that for the anion of N-acetyl-S-2-cyanoacetoacetylcysteamine, the second-order rate constant of the reaction of the carbanion of N-acetyl-S-cyanoacetylcysteamine with its conjugate acid is about 4.6 $M^{-1}$ min.$^{-1}$. This rate constant is about 50 times that for the reaction of the same carbanion with N,S-diacetylcysteamine, an increase that would be expected be-

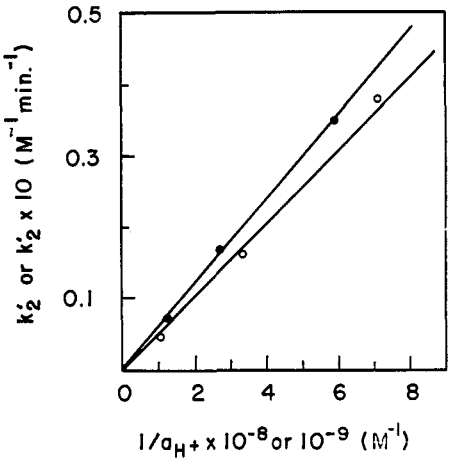



Figure 1. The second-order rate constants for the reaction of N,S-diacetylcysteamine with malononitrile (○, $k_2'$ and $M^{-1} \times 10^{-8}$) and ethyl cyanoacetate (●, $k_2' \times 10$ and $M^{-1} \times 10^{-9}$) as a function of the reciprocal of the hydrogen ion activity.

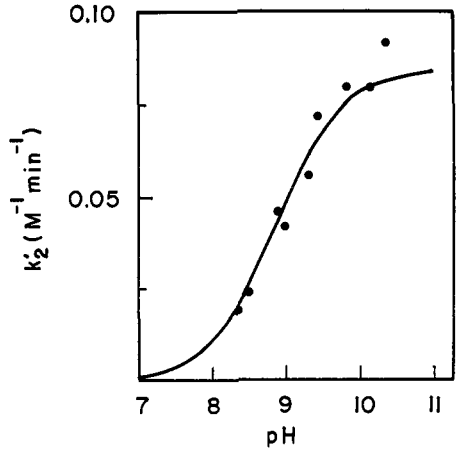



Figure 2. The second-order rate constants for the reaction of N-acetyl-S-cyanoacetylcysteamine with N,S-diacetylcysteamine as a function of pH. The solid line is calculated from the average value of the second-order rate constants for the reaction of the anion with the thiol ester and the acid dissociation constant of $1.41 \times 10^{-9}$ $M$ for N-acetyl-S-cyanoacetylcysteamine.

cause of activation of N-acetyl-S-cyanoacetylcysteamine for nucleophilic attack by the electron-withdrawing cyano group.

The rate constant for the reaction of ethyl cyanoacetate anion with ethyl thioacetate (approximately 1.7–2.0 $M^{-1}$ min.$^{-1}$) is similar to that for the corresponding reaction with N,S-diacetylcysteamine (3.0 $M^{-1}$ min.$^{-1}$). Intramolecular acyl transfer to the amide group[27] of the latter compound, therefore, is not required for acyl transfer to a carbanion to occur.

An attempt was made to measure the reaction of ethyl cyanoacetate with N,O-diacetylethanolamine, the oxygen analog of N,S-diacetylcysteamine. A solution of 0.42 $M$ ethyl cyanoacetate and 0.94 $M$ N,O-diacetylethanolamine was maintained at pH 9.8 with the automatic titrator at 25°. After 40 min., approximately 90% of the ethyl cyanoacetate and 10% of the N,O-diacetylethanolamine had undergone hydrolysis. The ultraviolet spectrum of the reaction mixture showed that less than $10^{-4}$ $M$ ethyl 2-cyanoacetoacetate anion, which is stable under these conditions, had formed. From these data, the rate constant for the hydrolysis of ethyl cyanoacetate at pH 9.8 (*ca.* 0.07 min.$^{-1}$ under these conditions) and the p$K$ of ethyl cyanoacetate, a maximal rate constant of $1.4 \times 10^{-3}$ $M^{-1}$ min.$^{-1}$ for the reaction of N,O-diacetylethanolamine with the ethyl cyanoacetate anion was estimated. Consequently, the sulfur ester is at least 2000 times more reactive than the oxygen ester. If ethyl 2,4-dicyanoacetoacetate anion, the expected product of the reaction of ethyl cyanoacetate anion with ethyl cyanoacetate, is stable under these conditions and if its extinction coefficient is taken to be approximately the same as that of ethyl 2-cyanoacetoacetate anion, then the experiment also shows that the rate constant for the reaction of ethyl cyanoacetate with its anion is less than $6 \times 10^{-3}$ $M^{-1}$ min.$^{-1}$.

An attempt was made to follow the reaction between the diethyl malonate anion and N,S-diacetylcysteamine by following the absorbance at 270 mμ (diethyl acetylmalonate anion formation) of a reaction mixture at 25° that contained 0.5 $M$ thiol ester and 0.05 $M$ diethyl malonate in 0.5 $M$ carbonate buffer, pH 10. Less than $10^{-4}$ $M$ diethyl acetylmalonate anion was formed in 1 hr. A maximal value of 500 $M^{-1}$ min.$^{-1}$ for the rate constant for this reaction can be calculated by assuming that the rate must be less than one-fifth of the rate of diethyl acetylmalonate hydrolysis, the rate constant for which is 0.0081 min.$^{-1}$ at this pH.[28]

Malononitrile was found to react rapidly in the pH range 8–9 with N-acetylcysteamine, a product of its reaction with N,S-diacetylcysteamine, to yield a stable compound with a strong absorption in the ultraviolet ($\lambda_{max}$ 274 mμ ($\epsilon_{max}$ 14,300 $M^{-1}$ cm.$^{-1}$)). The formation of this compound was found to be readily reversible upon dilution to $10^{-3}$ $M$. Mercaptoacetate also reacts with malononitrile to yield a similar compound ($\lambda_{max}$ 273 mμ ($\epsilon_{max}$ about 14,600 $M^{-1}$ cm.$^{-1}$)). Spectrophotometric measurements of the equilibrium constant for the reaction with mercaptoacetate at pH 8.6 (0.10 $M$ Tris buffer) and 25° indicated that the equilibrium involves 1 mole of malononitrile and 1 mole of mercaptoacetate and that the equilibrium constant, $K$ = [adduct]/[malononitrile][mercaptoacetate], is approximately 1000 $M^{-1}$. The rate constants for the reaction of malononitrile with N-acetylcysteamine are summarized in Table III. The second-order rate constants for reaction with the thiol anion are constant to within ±6% over 1 pH unit. These facts suggested that the thiol anion reacts with malononitrile to form an imido thiol ester, $N{\equiv}CCH_2C(SR){=}NH$, as an initial product. The reversible formation of the imido thiolactone structure in 1-cyano-2-mercaptomethylnaphthalene has been described,[29] and hydrogen sulfide adds to malononitrile in basic solution to yield dithiomalonic acid diamide.[30] However, the large difference between the spectrum of the compound and that of thioacetimidic acid ethyl ester (Table I) suggests that the initial product rearranges to a tautomer

(27) J. A. Shafer and H. Morawetz, *J. Org. Chem.*, 28, 1899 (1963); M. T. Behme and E. Cordes, *ibid.*, 29, 1255 (1964), and references therein.

(28) G. E. Lienhard and W. P. Jencks, *J. Am. Chem. Soc.*, 87, 3855 (1965).

(29) G. W. Stacy, A. J. Papa, F. W. Villaescusa, and S. C. Ray, *J. Org. Chem.*, 29, 607 (1964).

(30) H. Lehr, W. Guex, and H. Erlenmeyer, *Helv. Chim. Acta*, 27, 970 (1944).

**Table III.** The Reaction of Malononitrile with N-Acetylcysteamine at 25° and 1 *M* Ionic Strength

| pH[a] | N-Acetylcysteamine,[b] *M* | Adduct concn. at equil.,[c] $M \times 10^5$ | $k_{obsd}$,[d] min.$^{-1}$ | $k_f$,[e] min.$^{-1}$ | $k_2$,[f] $M^{-1}$ min.$^{-1}$ |
|---|---|---|---|---|---|
| 8.02 | 0.010 | 4.4 | 0.28 | 0.25 | 595 |
| 8.55 | 0.005 | 3.8 | 0.49 | 0.38 | 585 |
| | 0.010 | 4.4 | 0.90 | 0.80 | 615 |
| | 0.015 | 4.9 | 1.22 | 1.20 | 615 |
| | 0.020 | 4.6 | 1.54 | 1.42 | 545 |
| 8.93 | 0.010 | 4.3 | 1.67 | 1.44 | 545 |

[a] In 0.2 *M* tris(hydroxymethyl)aminomethane buffer. [b] The malononitrile concentration was $5 \times 10^{-5}$ *M* in all experiments. [c] From absorbance at 275 mμ at equilibrium. [d] From increase in absorbance at 275 mμ. [e] For formation of the adduct; calculated from the equation $k_f = k_{obsd}$ [adduct at equilibrium]/[malononitrile initially], which is derived from the relationships for (pseudo) first-order reactions: $k_{obsd} = k_f + k_r$ and $k_f/k_r$ = [adduct at equilibrium]/[malononitrile at equilibrium]. [f] For reaction with the thiol anion; $k_2 = k_f(1 + (H^+)/K_{RSH}')/[RSH]_{total}$.

mercaptoacetate, divided by the total concentration of mercaptoacetate However, not all of the reaction at the lower pH values results from nucleophilic attack of the thiol, because the hydrolysis of diethyl acetylmalonate is catalyzed by carboxylic acid buffers.[28] The over-all second-order rate constants for the reaction of mercaptoacetate were corrected for such catalysis by assuming that the catalytic constant for hydrolysis by the carboxylate group of mercaptoacetate is the same as that for the carboxylate group of methoxyacetate, which has a $pK_a'$ within 0.15 unit of that of mercaptoacetate.

Rate constants for the hydrolysis of diethyl acetylmalonate in the presence of methoxyacetate were determined at the same temperature, ionic strength, pH values, and concentrations of carboxylate ion as were used in the experiments with mercaptoacetate. Catalysis by methoxyacetate becomes negligible at pH values at which diethyl acetylmalonate is converted to

**Table IV.** Rates of Reaction of Mercaptoacetate with Diethyl Acetylmalonate at 25° and 1 *M* Ionic Strength[a]

| Mercaptoacetate, *M* | Initial concn. of diethyl acetylmalonate, $M \times 10^3$ | pH | $k_{obsd}$,[b] min.$^{-1}$ | $k_{acylation}$,[c] $M^{-1}$ min.$^{-1}$ | Fractional yield of S-acetylmercaptoacetate | |
|---|---|---|---|---|---|---|
| | | | | | Calcd.[d] | Obsd.[e] |
| 0 | 0.50 | 8.32 | 0.0175 | | | |
| 0.20 | 2.0 | 8.32 | 0.030 | 0.063 | 0.42 | |
| 0.20 | 8.2 | 8.42 | | | | 0.45 |
| 0.20 | 9.1 | 8.42 | | | | 0.39 |
| 0.40 | 2.0 | 8.34 | 0.0446 | 0.068 | 0.60 | |
| 0.40 | 17.7 | 8.52 | | | | 0.59 |
| 0.40 | 18.5 | 8.52 | | | | 0.56 |

[a] Each reaction was carried out in 0.05 *M* tris(hydroxymethyl)aminomethane buffer. [b] From the disappearance of diethyl acetylmalonate. [c] $k_{acylation}$, the rate constant for reaction with mercaptoacetate, $= (k_{obsd} - k_{hydrol})$/[mercaptoacetate]. [d] Fractional yield $= (k_{obsd} - k_{hydrol})/k_{obsd}$. [e] Measured with hydroxylamine–ferric chloride test, fractional yield = [S-acetylmercaptoacetate, final]/[diethyl acetylmalonate, initial].

of the imido thiol ester, N≡C—CH=C(SR)$NH_2$ (or possibly HN=C=CH—C(SR)=NH). These tautomers, because of conjugation with the cyano group, might be expected to have the observed spectrum of the adduct and to be more stable than the imido thiol ester itself.

*Thiol Ester Formation from Diethyl Acetylmalonate and Mercaptoacetate.* The rates of disappearance of diethyl acetylmalonate in the presence and absence of mercaptoacetate and the fractional yields of S-acetylmercaptoacetate were measured at pH 8.3–8.5 (Table IV). The yields of S-acetylmercaptoacetate expected from the enhancement of the rate of diethyl acetylmalonate disappearance in the presence of thiol agree closely with the measured yields, indicating that the rate enhancement at this pH occurs by thiol ester formation. The increase in the observed first-order rate constant for diethyl acetylmalonate disappearance is proportional to the concentration of mercaptoacetate (Table IV); *i.e.*, the reaction is first-order with respect to each reactant.

The second-order rate constants for the reaction of diethyl acetylmalonate with mercaptoacetate are plotted as a function of pH in Figure 3. The values indicated by the solid circles were obtained from the difference in the observed rate constants for diethyl acetylmalonate disappearance in the presence and absence of the conjugate carbanion, which suggests that the carboxylate group catalyzes the hydrolysis of the uncharged form of this compound. The corrected second-order rate constants, calculated by subtracting the rate constant for the disappearance of diethyl acetylmalonate in the presence of methoxyacetate from that in the presence of mercaptoacetate and dividing this value by the concentration of mercaptoacetate, are shown as the open circles in Figure 3.

The pH–rate profile of the corrected second-order rate constants for the reaction of mercaptoacetate with diethyl acetylmalonate is a bell-shaped curve with points of inflection at pH 6.7 and 10.0, which correspond to the $pK_a'$ values of diethyl acetylmalonate and mercaptoacetate, respectively. This profile for the rate of thiol ester formation shows that the reaction occurs between the thiol anion ($RS^-$) and undissociated diethyl acetylmalonate (CH), according to the rate law

$$v = k_2[RS^-][CH] = k_2 \frac{K_{RSH}'}{K_{RSH}' + (H^+)} \times \frac{(H^+)}{K_{CH}' + (H^+)}[RSH]_{total}[CH]_{total} \quad (5)$$

The value of $k_2$ at 25° and 1 *M* ionic strength is 130 $M^{-1}$ min.$^{-1}$.

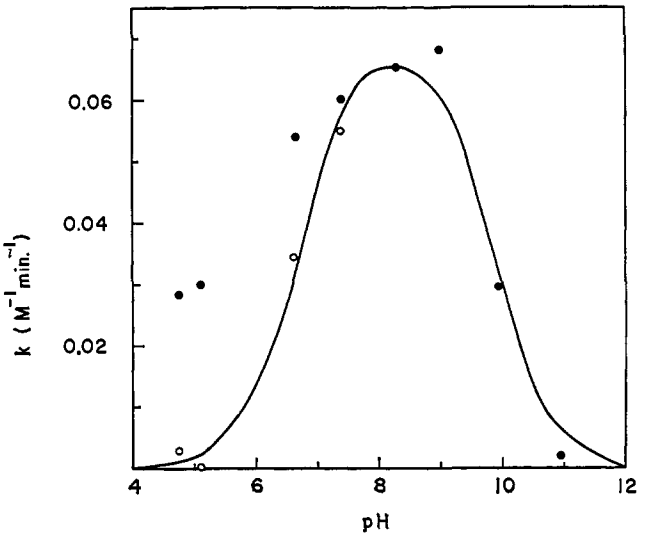



Figure 3. pH–rate profile for the reaction of mercaptoacetate with diethyl acetylmalonate. The closed circles are uncorrected second-order rate constants at 25° and 1 *M* ionic strength. The open circles are second-order rate constants corrected for carboxylate catalysis (see text). The line is the theoretical curve calculated from the $pK_a'$ values of diethyl acetylmalonate (6.65) and the sulfhydryl group of mercaptoacetate (9.95) and the measured second-order rate constant at pH 8.3.

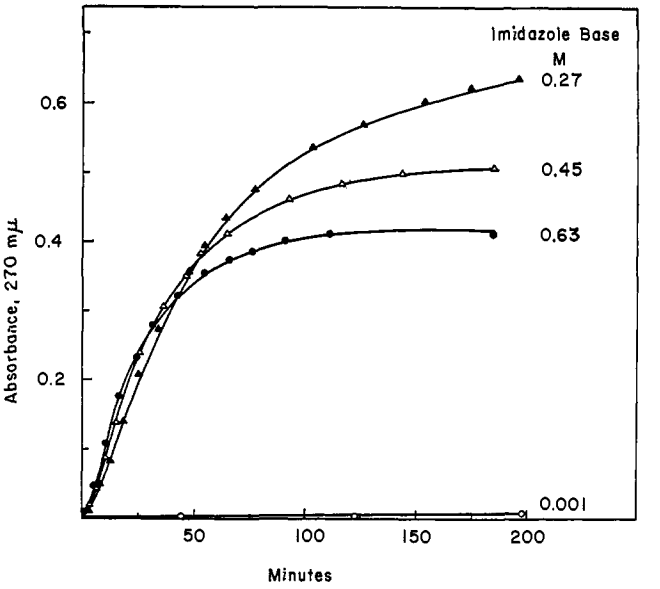



Figure 4. Imidazole catalysis of acetyl transfer from N,S-diacetylcysteamine to ethyl cyanoacetate at 25° and 1 *M* ionic strength, measured spectrophotometrically by the formation of ethyl 2-cyanoacetoacetate anion. The reaction mixture contained initially $10^{-4}$ *M* N,S-diacetylcysteamine, 0.25 *M* ethyl cyanoacetate, and 1.4, 1.0, 0.6, or 0.002 *M* imidazole buffer, 45% as the free base. The mixture with 0.002 *M* imidazole buffer was maintained at pH 7 with a pH-Stat. The fractional yields of ethyl 2-cyanoacetoacetate from N,S-diacetylcysteamine at completion are given in Table V.

In an attempt to determine whether there is a detectable accumulation of a hemimercaptal intermediate in the early stages of the reaction of mercaptoacetate with diethyl acetylmalonate, the initial absorbances at 270 mμ of identical concentrations of diethyl acetylmalonate in solutions of pH 5.5, in the presence and absence of 0.2 *M* mercaptoacetate, were measured. Since hemimercaptals probably have a very low absorption in the 270 mμ region,[31] the finding that there was no difference in the initial absorbances indicates that a rapid, pre-equilibrium formation of a measurable amount of hemimercaptal does not occur.

No reaction of 0.1 *M* N-acetylcysteamine with acetylmalononitrile, N-acetyl-S-2-cyanoacetoacetylcysteamine, and ethyl 2-cyanoacetoacetate at pH 7, 9, and 9, respectively, could be detected spectrophotometrically in 2 hr. at room temperature.

*Imidazole Catalysis of Acyl Transfer.* Transfer of the acetyl group from N,S-diacetylcysteamine to ethyl cyanoacetate is catalyzed by imidazole (Figure 4). The larger the imidazole concentration the faster is the rate of transfer, but the smaller is the yield of ethyl 2-cyanoacetoacetate. With 0.63 *M* imidazole a 24% formation of ethyl 2-cyanoacetoacetate occurs with a half-time of 18.5 min. and with 0.27 *M* imidazole, there is a 39% yield with a half-time of 38 min. These observations may be explained by the following scheme.

$$CH_3\overset{O}{\overset{\|}{C}}SR + Im \rightleftharpoons CH_3\overset{O}{\overset{\|}{C}}Im \xrightarrow[Im]{>C^-} CH_3\overset{O}{\overset{\|}{C}}{-}\overset{O}{\overset{\|}{C}}H \quad (6)$$
$$+ \quad RSH \qquad + \quad CH_3\overset{O}{\overset{\|}{C}}OH$$

Imidazole reacts with N,S-diacetylcysteamine to yield 1-acetylimidazole,[32] which may (a) react with N-acetylcysteamine to regenerate thiol ester,[33] (b) react with ethyl cyanoacetate anion ($C^-$) to form ethyl 2-cyanoacetoacetate, or (c) undergo hydrolysis, which has been shown to be catalyzed by imidazole.[33] A high imidazole concentration will increase the rate of acetyl transfer by increasing the rate of acetylimidazole formation and breakdown, but will decrease the yield of acetylated carbanion by increasing the rate of hydrolysis of the acetylimidazole.

For verification of the above scheme, the reaction of 1-acetylimidazole with ethyl cyanoacetate in imidazole buffers was studied. It was found that acetylimidazole reacts with 0.25 *M* ethyl cyanoacetate near pH 7 to yield the ethyl 2-cyanoacetoacetate anion at a rate which is similar to the rate of hydrolysis of acetylimidazole (Table V). The fractional yields of ethyl 2-cyanoacetoacetate from acetylimidazole were measured at concentrations of imidazole and ethyl cyanoacetate identical with those used in the investigation of acetyl transfer from N,S-diacetylcysteamine. The fact that the fractional yields with the two acetyl donors agree closely (Table V) is substantial evidence that the imidazole-catalyzed reaction proceeds through an acetylimidazole intermediate.

The kinetics of the imidazole-catalyzed reaction were not studied in detail. On the basis of published rate constants for the reaction of mercaptoethanol with acetylimidazole[33] and the free energies of hydrolysis of acetylimidazole and N,S-diacetylcysteamine,[13] the formation of acetylimidazole and its reaction with the thiol

(31) E. A. Fehnel and M. Carmack, *J. Am. Chem. Soc.*, **71**, 84 (1949).

(32) M. L. Bender and B. W. Turnquest, *ibid.*, **79**, 1656 (1957); T. C. Bruice, *ibid.*, **81**, 5444 (1959); E. R. Stadtman in "Mechanism of Enzyme Action," W. D. McElroy and B. Glass, Ed., The Johns Hopkins Press, Baltimore, Md., 1954, p. 581.

(33) W. P. Jencks and J. Carriuolo, *J. Biol. Chem.*, **234**, 1272, 1280 (1959).

**Table V.** Fraction of Acetyl Transfer from Acetylimidazole and N,S-Diacetylcysteamine to Ethyl Cyanoacetate, in Imidazole Buffers

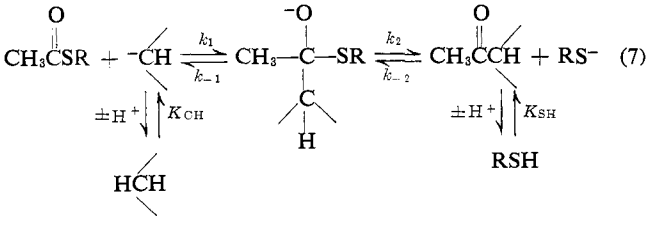
| Imidazole buffer, 45% free base, $M$ | Ethyl cyanoacetate, $M$ | Initial concn. of N,S-diacetylcysteamine, $M \times 10^4$ | Initial concn. of acetylimidazole,[a] $M \times 10^4$ | Final concn. ethyl 2-cyanoacetoacetate,[b] $M \times 10^4$ | $k_{obsd}$,[c] min.$^{-1}$ | $k_2$,[d] $M^{-1}$ min.$^{-1}$ | Fractional yield of ethyl 2-cyanoacetoacetate[e] |
|---|---|---|---|---|---|---|---|
| 0.60 | 0.25 | 1.0 | | 0.39 | | | 0.39[f] |
| 0.60 | 0.25 | | 1.34 | 0.56 | 0.068 | 0.114 | 0.42 |
| 0.60 | 0.25 | | 1.08 | 0.45 | 0.068 | 0.114 | 0.42 |
| 1.0 | 0.25 | 1.0 | | 0.30 | | | 0.30[f] |
| 1.0 | 0.25 | | 1.18 | 0.38 | 0.088 | 0.112 | 0.32 |
| 1.0 | | | *ca.* 1.25 | | 0.065[g] | | |
| 1.4 | 0.25 | 1.0 | | 0.24 | | | 0.24[f] |
| 1.4 | 0.25 | | 1.31 | 0.34 | 0.102 | 0.106 | 0.26 |

[a] Measured spectrophotometrically at 245 mμ, where $\epsilon_{max}$ is 3000 $M^{-1}$ cm.$^{-1}$ (see ref. 13). [b] Measured spectrophotometrically at 264 mμ. [c] From the increase in absorbance at 270 mμ, at 25° and 1 $M$ ionic strength. [d] For reaction of ethyl cyanoacetate with acetylimidazole, from $k_2 = k_{obsd}$[fractional yield of ethyl 2-cyanoacetoacetate]/[ethyl cyanoacetate]. [e] Equal to [ethyl 2-cyanoacetoacetate, final]/[N,S-diacetylcysteamine or acetylimidazole, initial]. [f] These data are from the experiments shown in Figure 4. [g] For hydrolysis of acetylimidazole, measured spectrophotometrically at 245 mμ.

would be expected to occur under the conditions of the experiments shown in Figure 4 at rates of the same magnitude as its hydrolysis and reaction with ethyl cyanoacetate. Thus, the kinetics are complex. When the data of Figure 4 are plotted according to the first-order rate law, linear plots are obtained after the first 20% of reaction. An approximately constant value of 0.060 $M^{-1}$ min.$^{-1}$ is obtained as the second-order rate constant upon division of the pseudo-first-order rate constants by the imidazole free base concentration. If one takes this value as a crude measure of the rate of the catalyzed reaction, it can be calculated from the rate constant for the direct reaction of ethyl cyanoacetate anion with N,S-diacetylcysteamine that catalysis by 1 $M$ imidazole increases the rate by a factor of about $10^3$ at pH 7.

## Discussion

The detailed mechanism proposed for the reactions described here is shown in eq. 7. The rate of the forward reaction is first order with respect to the thiol ester and the source of the carbanion and shows a dependence on basicity which follows the ionization



(7)

of the carbanion. No general-base or -acid catalysis of the reaction could be detected. The transition state, therefore, is formed from the thiol ester and carbanion, and ionization of the carbon compound to the carbanion must occur in a rapid pre-equilibrium step. This is in contrast to the conclusion of Patai and Israeli that the ionization of malononitrile is rate determining in the condensation of malononitrile with aldehydes in water[34]; however, this conclusion is not tenable, in view of the fact that the over-all rate of the condensation is several orders of magnitude slower than the rate of ionization of malononitrile in water[35] and the over-all rate is different with different aldehydes.[34] The reverse reaction involves the elements of the thiol anion, which is also formed in a pre-equilibrium step, and the uncharged form of the acetylated carbon compound. An alternative, kinetically indistinguishable mechanism may be written in which the enolic form of the intermediate expels the thiol anion and, in the reverse reaction, the thiol anion attacks the uncharged, enolic form of the ketone.

The following argument leads to the conclusion that the rate-determining step in the reaction of mercaptoacetate with diethyl acetylmalonate is the breakdown of the hemimercaptal intermediate ($k_{-1}$), which follows a rapid, pre-equilibrium formation of this intermediate ($k_{-2}/k_2$). Conversely, $k_1$ must be the rate-determining step for the reverse reaction, by the principle of microscopic reversibility. The ratio of the rate constants for reaction with hydroxide ion to that for reaction with thiol anion is near one for reactions of *p*-nitrophenyl acetate and acetylimidazolium cation, but is 1400 for diethyl acetylmalonate (Table VI). The attack step is almost certainly rate determining for the reactions with *p*-nitrophenyl acetate and acetylimidazolium cation[36,37] (p$K$ of the leaving group = 7), and it has been shown that attack of hydroxide ion on the carbonyl group is the rate-determining step in the hydrolysis of diethyl acetylmalonate at pH values over 6.0.[28] The very low reactivity of thiol anion with diethyl acetylmalonate may, therefore, be ascribed to a change in rate-determining step such that expulsion of the carbanion ($k_{-1}$) becomes rate determining. Such a change in rate-determining step is not unexpected in view of the relatively low basicity of the thiol anion (p$K$ = 9.95) compared to that of the leaving carbanion (p$K$ of diethyl malonate = 15.2; see Table II, footnote *k*). The observed rate of reaction is expected to decrease precipitously when the attacking reagent is no longer sufficiently basic to cause expulsion of the leaving group every time the addition intermediate is formed.[36-39] The fact that no accumulation of the

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(35) R. G. Pearson and R. L. Dillon, *J. Am. Chem. Soc.*, **75**, 2439 (1953).

(36) J. Kirsch and W. P. Jencks, *ibid.*, **86**, 837 (1964).

(37) M. L. Bender, H. Matsui, R. J. Thomas, and S. W. Tobey, *ibid.*, **83**, 4193 (1961).

(38) K. B. Wiberg, *ibid.*, 77, 2519 (1955).

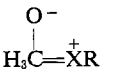
**Table VI.** Rates of Reaction of Thiolate and Hydroxide Ions with Carbonyl Compounds at 25°

| Carbonyl compd. | Thiol | $k$ with thiolate, $M^{-1}$ min.$^{-1}$ | $k$ with hydroxide, $M^{-1}$ min.$^{-1}$ | $k_{OH^-}/k_{RS^-}$ | Ref. |
|---|---|---|---|---|---|
| *p*-Nitrophenyl acetate | Mercaptoacetate | 2500 | 890 | 0.35 | 41 |
| | Mercaptoethanol | 640 | | 1.4 | |
| Acetylimidazolium | Mercaptoethanol | $10^8$ | $1.25 \times 10^8$ | 1.25 | 41 |
| Diethyl acetylmalonate | Mercaptoacetate | 130 | $1.8 \times 10^5$ | 1400 | 28 |

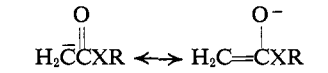
hemimercaptal addition intermediate can be detected indicates that the equilibrium constant for its formation, $k_{-2}/k_2$, is small. It is not known which step is rate determining in the reactions of the less basic carbanions with thiol esters.

It is evident from the experiments reported here and those of Eggerer, *et al.*,[9] that acyl transfer to and from carbanions occurs readily in aqueous solution in spite of the presence of a large molar excess of water. This is the case for many other acyl transfer reactions and is largely a reflection of the abnormally low nucleophilic reactivity of the hydroxide ion toward acyl groups.[40] This low reactivity is evidenced in these experiments by the fact that the malononitrile anion is more reactive than hydroxide ion toward N,S-diacetylcysteamine, although its basicity is only $10^{-4}$ that of hydroxide ion. A Brønsted plot of the rate constants in Table II shows that the reactivity of the carbanions increases with increasing basicity, but the points for the anions of ethyl cyanoacetate and N-acetyl-S-2-cyanoacetylcysteamine fall below those for malononitrile and cyanide anions, presumably because of the larger steric requirements of the former compounds. Cyanide itself is not a particularly effective nucleophilic reagent toward the acyl group of *p*-nitrophenyl acetate,[41,42] and the fact that some of the other compounds are even less reactive accounts for the fact that the yields of acetylated carbanion compounds are not large in experiments carried out in dilute solution. We had originally hoped to examine the condensation of a thiol ester with a monothiol ester of malonate as a more direct model for fatty acid synthesis. However, an extrapolation of the data obtained with other compounds suggests that this reaction would be difficult to observe, because of competing hydrolysis, at any pH value at which an appreciable concentration of the reactive carbanion would be formed. At the active site of the enzyme which catalyzes the reaction, this carbanion would presumably be bound adjacent to the thiol ester acyl donor, so that condensation could occur without interference from hydrolysis.

In view of the many biochemical condensations in which thiol esters are involved, it is of interest that thiol esters have a high reactivity in carbon–carbon condensations both as potential carbanions and as acyl donors. The high acidity of the α-hydrogen atom of a thiol ester is illustrated by the fact that the $pK_a'$ of cyanothiolacetic acid ethyl ester is 2.5 units lower than that of its oxygen analog, ethyl cyanoacetate; this is similar to the previously reported difference of 2.2 units between the $pK_a'$ values of ethyl acetoacetate and N-acetyl-S-acetoacetylcysteamine.[43] This difference presumably results from the smaller resonance contribution of the form



in the thiol ester than in the oxygen ester, which facilitates the formation of the carbanion



from thiol esters, compared to oxygen esters.[7b,44] The 45-fold faster base-catalyzed racemization of N-carbobenzoxy-β-cyano-L-alanine thiophenyl ester than of the corresponding phenyl ester shows that the rate as well as the equilibrium constants for carbanion formation are more favorable with thiol than with oxygen esters.[45] For the acetylation of the carbanion of N-acetyl-S-cyanoacetylcysteamine reported here it is the favorable equilibrium constant for ionization which favors the reaction, but for a number of enzymatic reactions in which proton removal to form the carbanion is more difficult and probably contributes to the energy barrier of the rate-determining step[46] the favorable rate constants for carbanion formation from thiol esters are of importance.

The high reactivity of thiol esters as acyl donors is illustrated by the fact that the ethyl cyanoacetate anion reacts with N,S-diacetylcysteamine at least $10^3$ times more rapidly than with N,O-diacetylethanolamine. Even more striking is the absence of an observable reaction of ethyl cyanoacetate anion with ethyl cyanoacetate, in spite of the expected activating effect of the cyano group. This high reactivity of thiol esters is presumably the result of the relatively small resonance stabilization of thiol esters, which facilitates addition to the carbonyl group, and the stability of thiol anions, which facilitates expulsion of the thiol anion. The latter effect would be expected to be of less importance with more basic nucleophiles, and it is known that the rates of alkaline hydrolysis of thiol esters and oxygen esters, for which attack of hydroxide ion on the carbonyl group is largely rate determining, are very similar.[39,47] Finally, thiol esters are better acyl donors than oxygen esters thermodynamically, as well as kinetically, as shown by their higher free energies of hydrolysis (ref. 13 and references therein).

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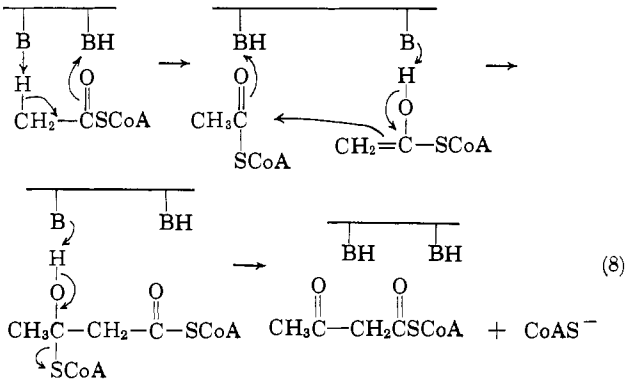
*Equilibria and Reversibility.* The equilibrium constant for the formation of acetoacetyl-S-CoA and CoA-SH from two molecules of acetyl-S-CoA, determined in the presence of the enzyme thiolase,[48] is unfavorable at neutrality, with a value of $K_f = 1.5 \times 10^{-5}$. At higher pH values the equilibrium shifts toward condensation because of ionization of the $\beta$-hydrogen of the acetoacetyl moiety of acetoacetyl-S-CoA ($pK = 9.45$)[49] and the sulfhydryl group of CoA ($pK = 9.6$).[50] It would be expected that the corresponding equilibria for the formation of acetylmalononitrile, N-acetyl-S-2-cyanoacetoacetylcysteamine, and ethyl 2-cyanoacetoacetate, which contain electron-withdrawing groups, would be equally or more unfavorable. The reason that the formation of these compounds proceeds to completion at pH values between 8 and 10 is that the reactions are pulled by ionization of the strongly acidic products; the $pK_a$ values of acetylmalononitrile, N-acetyl-S-2-cyanoacetoacetylcysteamine, and ethyl 2-cyanoacetoacetate are 1.0, 1.8, and 3.0, respectively. Claisen condensations of oxygen esters are driven to completion in strongly alkaline solutions for the same reason.[51]

The reverse reaction, of N-acetylcysteamine with acetylmalononitrile, N-acetyl-S-2-cyanoacetoacetylcysteamine, and ethyl 2-cyanoacetoacetate, does not proceed at a detectable rate under the conditions of these experiments. By analogy with the thiolase reaction, the equilibrium constant for the reaction in this direction must be equal to or greater than $10^5$ for the uncharged species, and at the concentrations of reactants and the pH values of these experiments it was calculated that the reactions should proceed more than 85% to completion. The absence of a detectable reaction, therefore, may be ascribed to the low concentration of the uncharged, reactive species of the ketone which exists at any pH value at which an appreciable fraction of the N-acetylcysteamine ($pK = 9.4$) exists as the reactive anion. Evidently the effect of the electron-withdrawing substituents in lowering the $pK_a$ and the concentration of the reactive species of the acetyl donor more than offsets any activating effect on the rate of reaction of the uncharged form. This stabilizing effect of the cyano group is also illustrated by the fact that acetylmalononitrile, N-acetyl-S-2-cyanoacetoacetylcysteamine, and ethyl 2-cyanoacetoacetate are stable in neutral solution and in the presence of sodium hydroxide, whereas diethyl acetylmalonate undergoes hydrolysis to diethyl malonate and acetate in the pH range 0–14 with half-times of 1–10 hr.[28] The slow hydrolysis of these compounds in acid solution is probably a neutral (water) hydrolysis of the uncharged species, similar to that observed with diethyl acetylmalonate.[28]

In contrast to the above-described compounds, the reaction of diethyl malonate with N,S-diacetylcysteamine could not be detected at pH 10, but the reverse reaction of diethyl acetylmalonate with mercaptoacetate proceeds readily and was examined kinetically. The product of the forward reaction, diethyl acetylmalonate, is less acidic ($pK_a = 6.6$) than the products described above, so that ionization of the product will not drive the reaction in this direction as strongly. Conversely, the relatively high $pK$ means that an appreciable fraction of the acetyl donor will be in the uncharged, reactive form at pH values at which the thiol is partly ionized, so that the reverse reaction of eq. 7 proceeds readily and follows the expected bell-shaped pH–rate profile (Figure 3). The absence of a detectable reaction in the forward direction is not caused by a slow ionization of the diethyl malonate, because the half-time for the ionization of this compound is only about 1 min. in the presence of 1 $M$ acetate ion at pH 5.[52]

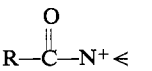
*Possible Enzymatic Mechanisms.* The formation of a free carbanion from a thiol ester is unlikely in most enzyme-catalyzed condensations. A possible mechanism for an enzyme-catalyzed reaction, which utilizes general acid–base catalysis by groups at the active site of the enzyme to avoid the formation of such an unstable intermediate, is shown in eq. 8. The enzymatic condensation of two molecules of acetyl-S-CoA




(8)

to form acetoacetyl-S-CoA and CoA-SH (the thiolase reaction) may proceed by general acid–general base catalyzed enolization of one acetyl-CoA molecule, followed by general acid–general base catalyzed attack of the enol upon the second acetyl-CoA molecule, followed by general base catalyzed expulsion of thiolate ion from the hemimercaptal intermediate.

Although imidazole catalysis of acyl transfer is known for a number of acyl donors and acceptors,[33] it has not previously been reported for acyl transfer from a thiol ester to a carbanion. The large rate enhancement reported here suggests that this catalysis could be an effective way for an enzyme to accelerate the rate of the reaction of a carbanion with a thiol ester. Cornforth[53] has suggested that the transfer of an acyl group from a thiol to a carbanion might be accelerated in an enzymatic reaction if it proceeds through an intermediate of the type




which subsequently reacts with the carbanion. It is of interest that the acyl carrier protein which carries the acetyl and malonyl residues that undergo condensation in fatty acid synthesis contains a histidine residue, as well as the single thiol group to which one of the reacting acyl groups is bound.[2]

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