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The Free Energies of Hydrolysis of Some Esters and Thiol Esters of Acetic Acid[1]

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The free energies of hydrolysis of ethyl acetate, methoxyethyl acetate, chloroethyl acetate, acetylcholine, and trifluoroethyl acetate to the free acid in aqueous solution at 25° (based on a water activity of 1.0) are −1660, −2180, −2840, −2940, and −4970 cal./mole, respectively. The equilibrium constants for acetyl transfer from acetylcholine to the thiol groups of N-acetyl-β-mercaptoethylamine and mercaptoacetate are 0.076 and 0.132, respectively. The free energies of hydrolysis (to free acetic acid) of N,S-diacetyl-β-mercaptoethylamine and S-acetylmercaptoacetate are, therefore, −4460 and −4140 cal./mole, respectively, in good agreement with a previously reported value for a thiol ester of acetic acid.

The measurements of the equilibria and free energies for the formation and hydrolysis of esters of acetic acid which are reported here are part of a more extensive investigation of the free energies of hydrolysis and other properties of "energy-rich" compounds.[2,3] Two factors, in particular, prompted the present investigation.

First, it was desired to measure the rate constants in both directions and the equilibrium constants for a series of acetyl-transfer reactions, in order to permit a more detailed consideration of the mechanism and the effect of structure upon reactivity in these reactions. These points are considered in more detail in the following paper.[4]

Second, it was desired to measure directly a series of nonenzymatic equilibria for acetyl-transfer reactions which would relate the group potential of such "energy-rich" compounds as acetylimidazole, acetyl phosphate, and acetyl coenzyme A to esters such as ethyl acetate and acetylcholine, the free energies of hydrolysis of which can be measured directly in aqueous solution. The equilibrium constants for group-transfer reactions which relate the former group of compounds to most other "energy-rich" compounds of biochemical importance are known. These data, therefore, would provide a new reference standard for the determination of the free energies of hydrolysis of "energy-rich" compounds, about which there has been some controversy, without the problems attendant upon the measurement of enzyme-catalyzed group-transfer equilibria, such as the binding of magnesium ion to polyphosphates. A somewhat similar series of values, which has been reported previously,[2] was based upon a value for the free energy of thiol ester hydrolysis in aqueous solution which was obtained from the equilibrium constant for the intramolecular S–O acetyl transfer reaction of the monoacetyl derivative of mercaptopropanol and the free energy of hydrolysis of mercaptopropyl acetate. The generality of this result is open to the objection, however, that the hydroxyl group of S-acetylmercaptopropanol might affect the stability of the thiol ester by hydrogen-bonding or polar effects. It appeared desirable, therefore, to determine the free energy of hydrolysis of a thiol ester which is structurally more closely related to acetyl coenzyme A.

(1) Supported by grants from the National Science Foundation and the National Cancer Institute of the National Institutes of Health (CA-03975).

(2) W. P. Jencks, S. Cordes, and J. Carriuolo, *J. Biol. Chem.*, **235**, 3608 (1960).

(3) (a) W. P. Jencks, C. Moore, F. Perini, and J. Roberts, *Arch. Biochem. Biophys.*, **88**, 193 (1960); (b) W. P. Jencks, M. Caplow, M. Gilchrist, and R. G. Kallen, *Biochemistry*, **2**, 1313 (1963); (c) J. F. Kirsch and W. P. Jencks, *J. Am. Chem. Soc.*, **86**, 833, 837 (1964).

(4) J. Gerstein and W. P. Jencks, *ibid.*, **86**, 4655 (1964).

Experimental

Materials.—Commercial reagents were redistilled or recrystallized. Acetylcholine chloride and choline chloride were dried carefully over phosphorus pentoxide under vacuum and stored in a desiccator over phosphorus pentoxide. N,S-Diacetyl-β-mercaptoethylamine (N-2-mercaptoethylacetamide acetate) was prepared from β-mercaptoethylamine and acetic anhydride.[4] An aqueous solution of N-acetyl-β-mercaptoethylamine (N-2-mercaptoethylacetamide) was prepared by the addition of 4.4 ml. of 8.0 *M* potassium hydroxide to 2.66 g. of N,S-diacetyl-β-mercaptoethylamine. The solution was mixed with a stream of argon gas and was incubated for 10 min. at 25°. Portions of this solution were then added, with good stirring, to previously prepared reaction mixtures which contained the desired amount of hydrochloric acid for partial neutralization. Spectrophotometric examination of controls indicated that cyclization to a thiazoline did not occur under these reaction conditions.

Methods.—The concentrations of esters were determined by modifications of the alkaline hydroxylamine method of Hestrin[5] for oxygen esters and of the neutral hydroxylamine method of Lipmann and Tuttle for thiol esters.[6] Determinations of most oxygen esters were carried out by the addition, with good mixing, of 0.1 to 1.0 ml. of sample to 0.75 ml. of a freshly prepared mixture of 1 part of 4 *M* hydroxylamine hydrochloride, 2 parts of 3.5 *M* sodium hydroxide, and 1 part of water. Extra sodium hydroxide was added to this solution, if necessary, to neutralize concentrated acid in the aliquot. After approximately 1 min. the solution was brought to 2.0 ml. with water, 4 ml. of 10% $FeCl_3 \cdot 6H_2O$ in 0.7 or 0.3 *M* hydrochloric acid was added, the sample was shaken thoroughly, and the absorbance at 540 mμ was read against an appropriate blank after 30 to 60 min. Concentrations were determined by comparison with a standard solution of the appropriate ester which had been carried through the same procedure. Standard solutions of acetylcholine were prepared in solutions of acetic and hydrochloric acid similar in composition to the reaction mixtures and were analyzed immediately, because of a small effect of acid on the color yield of the assay with this ester. It was necessary to extract chloroethyl acetate from the reaction mixture before assay because of interference with the determination by chloroethanol. An aliquot of the reaction mixture was extracted with an equal volume of toluene, which was then washed with water; 2 ml. of the toluene solution of ester was added to 2 ml. of a mixture of 5 parts of 3.5 *M* sodium hydroxide, 4 parts of 4.0 *M* hydroxylamine hydrochloride, and 1 part of water, and the mixture was shaken vigorously for 5 min. One millimeter of aqueous layer was then added to 1 ml. of water and 4 ml. of 20% $FeCl_3 \cdot 6H_2O$ in 2.5 *M* hydrochloric acid and the absorbancy of the samples was read at 540 mμ. The results were corrected for the (small) amount of color produced by chloroethanol carried through the same procedure.

The concentration of thiol ester in the acetylcholine–thiol ester equilibrium experiments was measured by incubation of 0.1 ml. of sample with 1.9 ml. of water and 0.5 ml. of a mixture of 5 parts of 4 *M* hydroxylamine hydrochloride and 2 parts of 3.5 *M* sodium hydroxide for 2 min., followed by the addition of 2 ml. of 10% $FeCl_3 \cdot 6H_2O$ in 1.25 *M* HCl and measurement of the absorbance at 540 mμ within 5 min. The sum of acetylcholine and thiol ester concentrations in these experiments was measured by addition of 0.5 ml. of a diluted aliquot of the reaction mixture to 0.5 ml. of a freshly prepared mixture of 2 parts

(5) S. Hestrin, *J. Biol. Chem.*, **180**, 249 (1949).

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

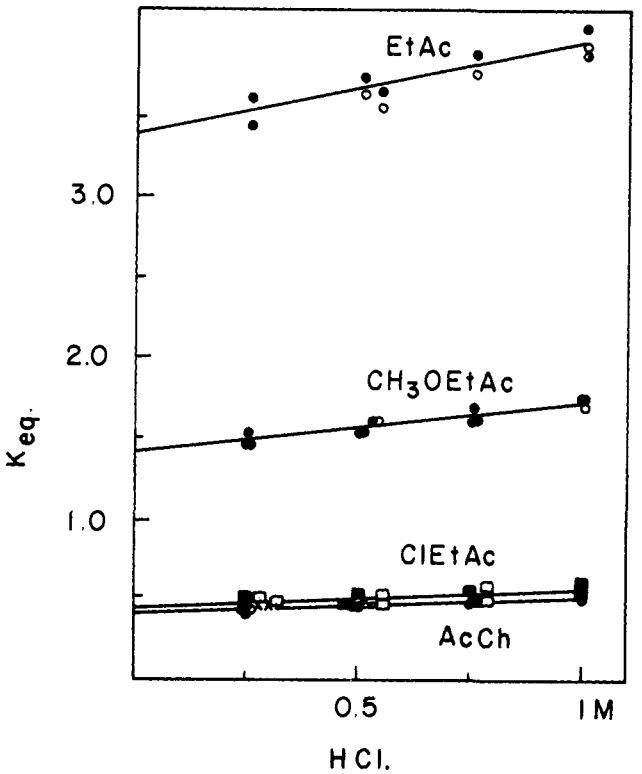

Fig. 1.—Equilibrium constants for the formation of acetate esters as a function of hydrochloric acid concentration at 25°: ● and ■, equilibrium approached from acid and alcohol; ○ and □, equilibrium approached from ester; □: data for chloroethyl acetate; △ (acetylcholine) and × (chloroethyl acetate), initial concentrations of acid or alcohol doubled; ethyl acetate and methoxyethyl acetate, 0.4 *M* acid, 0.3–0.5 *M* alcohol, 5–11 × 10^{-3} *M* ester at equilibrium; chloroethyl acetate, 0.8 *M* acid, 0.1–0.3 *M* alcohol, 1–3 × 10^{-3} *M* ester at equilibrium; acetylcholine, 0.4–0.8 *M* acetic acid, 0.4–0.8 *M* alcohol, 1.3–3.0 × 10^{-3} *M* ester at equilibrium.

of 3.5 *M* sodium hydroxide, 1 part of 4 *M* hydroxylamine hydrochloride, and 1 part of water, followed by incubation for 1 min., addition of 2 ml. of 10% $FeCl_3 \cdot 6H_2O$ in 1.25 *M* HCl, and measurement of the absorbance at 540 mμ after 10 to 30 min.

The total concentration of *N*-acetyl-β-mercaptoethylamine was determined in diluted aliquots of the reaction mixture by a modified nitroprusside method.[4] A standard was prepared by hydrolysis of 0.5 ml. of 0.05 *M* *N*,*S*-diacetylmercaptoethylamine in 5 ml. of 0.2 *M* sodium hydroxide for 30 min. at room temperature, followed by neutralization with 0.4 ml. of 2 *M* hyrochloric acid and dilution to 25 ml. The fraction of *N*-acetylmercaptoethylamine in the anionic form was determined by titration of aliquots of the reaction mixture to pH 7.0 with hydrochloric acid. Standard solutions of mercaptoacetic acid were prepared by weight and the concentration of free thiol in the reaction mixture was determined from the initial concentration of thiol, the amount of standard potassium hydroxide added, the amount of acid released by ester hydrolysis, and the amount of thiol formed from *S*-acetylmercaptoacetate in the reaction mixture at a given time.

Acetic acid concentrations were determined by titration. Alcohol concentrations were determined by weight or volume. The concentration of water in the reaction mixtures was estimated from the volumes or weights of the reagents and the amount of water required to prepare concentrated aqueous solutions of the reagents.

Determinations of pH were made with a Radiometer PHM4B pH meter and G-200B glass electrode. Measurements of absorbance were carried out with a Zeiss PMQ II spectrophotometer.

Reaction mixtures were incubated in glass-stoppered tubes sealed with stopcock grease at 25.0 ± 0.1° until successive determinations showed that equilibrium had been reached. Special care was exercised to prevent loss of the extremely volatile ester, trifluoroethyl acetate. In every case, equilibrium was approached from both directions. The experiments with acid catalysis required incubation for 2 to 10 days, while those with acetylcholine and thiol esters were incubated for 3 to 48 hr. Argon was bubbled through reaction mixtures containing thiols before addition of the thiol and before the tubes were stoppered. The acetylcholine–thiol ester experiments were carried out in the pH range 9.0 to 10.0 with the thiol as buffer.

Results

Equilibrium constants for the formation of esters of acetic acid (eq. 1) were determined by analysis of ester

$$CH_3COOH + HOR \rightleftharpoons CH_3\overset{\overset{O}{\|}}{C}OR + H_2O \qquad (1)$$

concentration by the Hestrin alkaline hydroxylamine procedure[5] after the reaction mixtures had reached equilibrium over a period of 2 to 10 days at 25°. Use of this method permitted the determination of equilibria in relatively dilute solution for those esters with a favorable equilibrium constant and permitted the measurement of less favorable equilibria that would be difficult to determine by titration. In each case, incubation was continued until analysis of successive aliquots showed that equilibrium had been attained. Good agreement was obtained in experiments in which equilibrium was approached from both directions (Fig. 1, open and closed symbols). The equilibrium constants increase with increasing hydrochloric acid concentration[2,7] (Fig. 1) and the values summarized in Table I are extrapolated to zero hydrochloric acid concentration. The results are given in terms of equilibrium constants based on the molar concentration of water (K_1) and based on the convention that the activity of pure water is 1.0 (K_2). The value of 3.3 for ethyl acetate is 10% higher than the value reported previously at 39°, which was based on a longer extrapolation, and is in satisfactory agreement with previously reported values, in the range 3.5 ± 1.0, which were generally obtained in more concentrated solutions.[7-8] There is some evidence for an effect of solvent composition on the equilibrium constant for ethyl acetate formation,[9] but this effect is not large in the hands of most workers.[8] The value of 0.394 for the equilibrium constant for acetylcholine formation is in reasonable agreement with the value of 0.26 reported by Hestrin,[10]

TABLE I
EQUILIBRIA AND FREE ENERGIES FOR ESTER FORMATION AT 25°[a]

Ester	$K = \frac{[RCOOR'][HOH]}{[RCOOH][HOR']}$ K_1[b]	K_2[c]	$\Delta F°$,[c] cal./mole
Ethyl acetate	3.38	0.061	1660
Methoxyethyl acetate	1.42	.026	2180
Chloroethyl acetate	0.46	.0083	2840
Acetylcholine	.394	.0071	2940
Trifluoroethyl acetate	013	2.3×10^{-4}	4970

[a] Extrapolated to zero catalyst concentration; for experimental conditions see Fig. 1. [b] Based on the molar concentration of water; *i.e.*, pure water = 55.5 *M*. [c] Based on the convention that the activity of pure water = 1.0.

(7) W. J. Jones and A. Lapworth, *J. Chem. Soc.*, **99**, 1427 (1911).

(8) (a) O. Knoblauch, *Z. physik. Chem.*, **22**, 268 (1897); (b) H. Euler, *ibid.*, **36**, 405 (1901); (c) H. S. Harned and R. Pfanstiel, *J. Am. Chem. Soc.*, **44**, 2193 (1922); (d) R. J. Williams, A. Gabriel, and R. C. Andrews, *ibid.*, **50**, 1267 (1928); (e) R. C. Cantelo and R. D. Billinger, *ibid.*, **50**, 3212 (1928); **52**, 869 (1930); (f) H. M. Trimble and E. L. Richardson, *ibid.*, **62**, 1018 (1940).

(9) S. Poznanski, *Roczniki Chem.*, **8**, 377 (1928); *Chem. Abstr.*, **23**, 1559 (1929).

(10) S. Hestrin, *Biochim. Biophys. Acta*, **4**, 310 (1950).

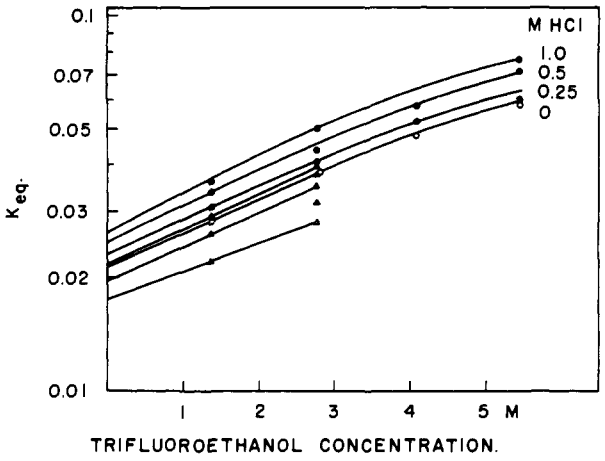

Fig. 2.—Dependence of the equilibrium constant for trifluoroethyl acetate formation upon the concentrations of trifluoroethanol, acetic acid, and hydrochloric acid at 25°: ●, 3.4 *M* acetic acid, 0.25, 0.5, and 1.0 *M* hydrochloric acid; ▲, 1.7 *M* acetic acid, 0.25 to 1.0 *M* hydrochloric acid; O and △, extrapolated to zero hydrochloric acid concentration.

which was measured in the presence of acetate buffers with catalysis by acetylcholinesterase and which is dependent upon the accuracy of pH determinations in concentrated solutions.

Determination of the equilibrium constant for trifluoroethyl acetate formation is complicated by a marked dependence of the equilibrium constant upon the composition of the reaction mixture. This may be partly because of the requirement for relatively concentrated solutions to measure this comparatively unfavorable equilibrium, but it is also attributed to an unusual sensitivity of the activity coefficients of the reactants to the nature of the solvent. The results are shown in Fig. 2. The equilibrium constant increases with increasing concentrations of trifluoroethanol, acetic acid, and hydrochloric acid. The results at a series of trifluoroethanol concentrations were first extrapolated to zero hydrochloric acid concentration (open symbols) and these values were then extrapolated to zero trifluoroethanol concentration. A final value of 0.013 was obtained by extrapolating these results, obtained at two different acetic acid concentrations, to zero acetic acid concentration. While this value is not of high precision, it is useful for comparative purposes.

The equilibrium constants for acetyl transfer between choline and thiols (eq. 2) were measured by

$$CH_3\overset{O}{\overset{\|}{C}}OCH_2CH_2\overset{+}{N}(CH_3)_3 + HSR \rightleftharpoons CH_3\overset{O}{\overset{\|}{C}}SR + HOCH_2CH_2\overset{+}{N}(CH_3)_3 \quad (2)$$

taking advantage of the fact that acetyl transfer to a thiol or a relatively acidic alcohol in moderately concentrated, slightly alkaline solution occurs more rapidly than hydrolysis of either the oxygen or thiol ester. The results of a typical experiment for acetyl transfer between acetylcholine, N,S-diacetyl-β-mercaptoethylamine, and the corresponding alcohol and thiol are shown in Fig. 3. Although there is a slow hydrolysis of the esters, acetyl transfer occurs much faster than hydrolysis and equilibrium constants determined at different times after the initial portion of the reaction

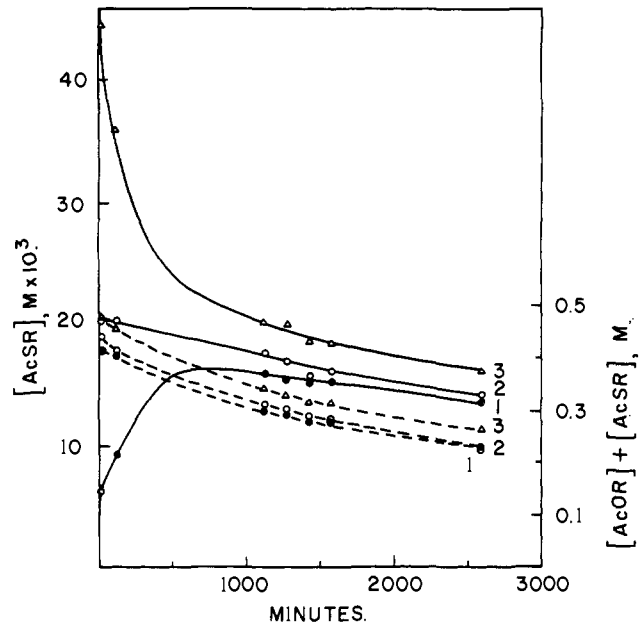

Fig. 3.—The concentration of N,S-diacetyl-β-mercaptoethylamine (solid line, left scale) and the sum of the concentrations of acetylcholine and N,S-diacetyl-β-mercaptoethylamine (dashed line, right scale) in a solution containing acetylcholine, N-acetyl-β-mercaptoethylamine, choline, and N,S-diacetyl-β-mercaptoethylamine at 25°. The experimental conditions are given in Table II. The pH of diluted aliquots at 1400 min. was 9.05 ± 0.03.

do not differ. The same results were found for reactions in which equilibrium was approached from both directions and in which the initial concentrations of reactants were near the equilibrium positions. The results of these experiments and of similar experiments with β-mercaptoacetate are summarized in Table II. The somewhat more favorable equilibrium constant for the formation of the thiol ester of mercaptoacetate than of N-acetyl-β-mercaptoethylamine presumably reflects the difference in the inductive effects of the carboxylate and acetamide groups.

Discussion

It was suggested previously that the enzyme-catalyzed synthesis of acetylcholine from acetyl coenzyme A should be reversible.[2] The directly measured value of 0.076 for the equilibrium constant for the transfer of an acetyl group from acetylcholine to N-acetyl-β-mercaptoethylamine, a model for coenzyme A, indicates that this reaction is readily reversible. Although the equilibrium constant for the enzyme-catalyzed reaction does not appear to have been measured directly, the equilibrium constant for the analogous transfer of acetate from acetylcarnitine to coenzyme A to form acetyl coenzyme A has recently been shown[11] to be 0.6.

Comparison of the equilibria for acetyl transfer from N,S-diacetyl-β-mercaptoethylamine to choline and for the hydrolysis of acetylcholine gives a value of −4460 cal./mole for the free energy of hydrolysis of this thiol ester to free acetic acid and thiol. This is in excellent agreement with the previously reported value of −4400 cal./mole for the free energy of hydrolysis of S-acetylmercaptopropanol.[2] The difference in the substituents on these two thiol esters, therefore, does not have an

(11) I. B. Fritz, S. K. Schultz, and P. A. Srere, *J. Biol. Chem.*, **238**, 2509 (1963).

TABLE II

EQUILIBRIA FOR ACETYL TRANSFER FROM ACETYLCHOLINE TO N-ACETYL-β-MERCAPTOETHYLAMINE AND MERCAPTOACETIC ACID AT 25°

	Initial concentrations				Equilibrium concentrations[c]				$K = \frac{[AcSR][ROH]}{[AcOR][RSH]}$
Run[a]	AcCh, *M*	Choline, *M*	AcSR, *M* × 10⁴	RSH,[b] *M*	AcCh, *M*	Choline, *M*	AcSR, *M* × 10⁴	RSH,[b] *M*	
				AcCh–AcSEtNHAc					
1	0.41	0.8	5.0	0.5	0.27	0.94	15.3	0.68	0.077
2	.42	.8	20.3	.5	.27	.95	16.0	.74	.075
3	.43	.8	44.4	.5	.30	.94	19.0	.78	.076
								Mean	0.076
				$AcCh–AcSCH_2COO^-$					
1	0.38	0.9	0	0.6	0.19	1.09	17.0	0.78	0.127
2	.39	.9	33.3	.6	.23	1.06	22.5	.81	.132
3	.39	.9	46.7	.6	.24	1.05	25.3	.82	.137
								Mean	0.132

[a] See Fig. 3. [b] Concentration of non-ionized thiol. [c] At approximately 1400 min. for the acetylmercaptoethylamine and 360 min. for the mercaptoacetic acid experiments.

appreciable effect on the free energy of hydrolysis. The value for N,S-diacetyl-β-mercaptoethylamine may be accepted with some confidence as being essentially the same as that for acetyl coenzyme A, since acetyl coenzyme A is also a thiol ester of an N-acylated β-mercaptoethylamine and has properties very similar to those of other thiol esters.[2] The free energy of hydrolysis of these thiol esters at pH 7.0 is −7520 cal./mole, based on the thermodynamic dissociation constant for acetic acid, and −7700 cal./mole at ionic strength 0.2–1.0. The equilibrium constant for the enzyme-catalyzed formation of acetyl coenzyme A from acetyl phosphate has been determined[12] and, by comparison with the equilibria of acetyl phosphate[13] and N,S-diacetyl-β-mercaptoethylamine[4] with acetylimidazole, the free energy of hydrolysis of acetyl coenzyme A may be calculated to be −7200 to −7360 cal./mole at pH 7.0. In view of the several equilibria required for this calculation, not all of which were measured under the same experimental conditions, this is in reasonable agreement with the above value.

The free energy of hydrolysis of ATP (adenosine triphosphate) to ADP (adenosine diphosphate) and inorganic phosphate at pH 7.0 in the presence of excess magnesium ion has been reported to be approximately −7300 cal./mole.[14] This value is supported by comparison of the free energy of thiol ester hydrolysis with the equilibria for the reactions of acetyl phosphate with coenzyme A and ADP to form acetyl coenzyme A and ATP, respectively.[2] The present results provide further confirmation for this value. However, there is disagreement regarding the free energy of hydrolysis of ATP to AMP (adenosine monophosphate) and PP (inorganic pyrophosphate). The equilibrium constant for acetate activation by ATP to give acetyl coenzyme A, AMP, and PP (eq. 3) has been reported

$$ATP^{-4} + AcO^- + CoA \rightleftharpoons AMP^{-2} + PP^{-3} + AcCoA \quad (3)$$

to be 2.7 and 0.86 at pH 7.5 in the presence of excess magnesium ion.[15,16] Although the equilibrium constant for this reaction at pH 7.0 is not known exactly, it is certainly close to 1.0, and the free energy of hydrolysis of ATP to AMP and PP under these conditions should, accordingly, be on the order of −7700 cal./mole.[2] At about the same time that this estimate was reported, values of −10,300 cal./mole in the presence of excess magnesium ion[17] and ~−14,000 cal./mole in the absence of magnesium ion[18] at pH 7.5 were calculated for the same reaction through a series of interrelated equilibrium constants. One of the reasons for carrying out the equilibrium determinations reported here was to confirm the previously reported value for the free energy of thiol ester hydrolysis, in order to be certain that some peculiarity of the thiol ester used for the earlier equilibrium determinations did not result in an anomalously low value for this reaction. While a precise value for the free energy of hydrolysis of ATP to AMP and PP is not yet available, the present results confirm those previously reported and suggest that a value near −7700 cal./mole at pH 7.0 is more nearly correct than the larger values, which were based on less direct measurements.

(12) W. S. Sly and E. R. Stadtman, *ibid.*, **238**, 2639 (1963).

(13) E. R. Stadtman, "Mechanism of Enzyme Action," W. D. McElroy and B. Glass, Ed., The Johns Hopkins Press, Baltimore, Md., 1954, p. 581.

(14) (a) T. Benzinger, C. Kitzinger, R. Hems, and K. Burton, *Biochem. J.*, **71**, 400 (1959); (b) M. R. Atkinson, E. Johnson, and R. K. Morton, *Nature* (London), **184**, 1925 (1959); (c) E. A. Robbins and P. D. Boyer, *J. Biol. Chem.*, **224**, 121 (1957).

(15) M. E. Jones, *Federation Proc.*, **12**, 708 (1953).

(16) P. Hele, *J. Biol. Chem.*, **206**, 671 (1954).

(17) A. Schuegraf, S. Ratner, and R. C. Warner, *ibid.*, **235**, 3597 (1960).

(18) P. George and R. J. Rutman, *Progr. Biophys. Biophys. Chem.*, **10**, 1 (1960).