Enthalpy Changes Accompanying the Transfer of a Methyl Group from *S*-Adenosylmethionine and Other Sulfonium Compounds to Homocysteine*

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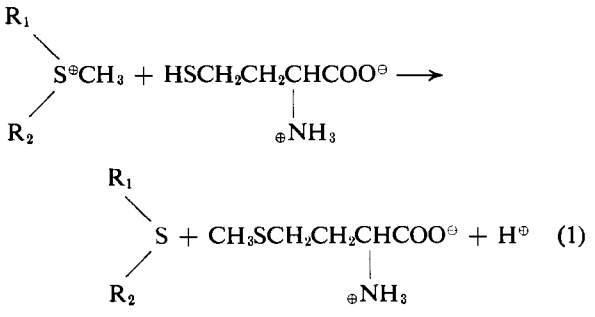
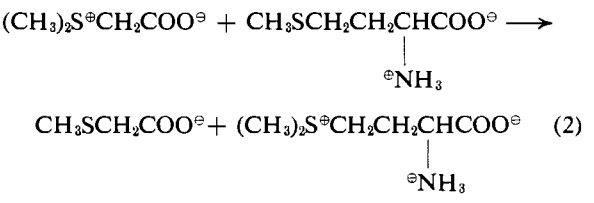
ABSTRACT: The enthalpy changes during methyl transfer from *S*-adenosylmethionine, dimethylpropiothetin, or trimethylsulfonium salts to homocysteine in neutral aqueous buffered solution have been measured calorimetrically. The observed values are compared to previously reported values for similar reactions. Transfers from these sulfonium compounds are all highly exothermic reactions, but there are relatively large differences in the changes observed within the present series. Possible reasons for these differences are discussed. It is suggested that differential hydration may play an important role.

The thermodynamics of reactions in which a methyl group is transferred from a sulfonium salt to a sulfhydryl-containing acceptor have not been studied extensively because experimental reversibility of such reactions has not been achieved. Direct calorimetric determinations of the enthalpy changes during transfer of the methyl group from either of two sulfonium methyl donors to the common acceptor, homocysteine, have been reported by Durell and Sturtevant (1957). They found an enthalpy change of −12.6 kcal/mole for the transmethylation from dimethylacetothetin to homocysteine (reaction 1, $R_1 = CH_3$; $R_2 = {}^{\ominus}OOCCH_2$) at 25° and neutral pH (Durell and Sturtevant, 1957).



Later this value was revised to −11.3 kcal/mole (Durell *et al.*, 1962). The value for the analogous methyl transfer from L-methylmethionine sulfonium salt (reaction 1, $R_1 = CH_3$; $R_2 = {}^{\ominus}OOCC({}^{\oplus}NH_3)HCH_2CH_2$) was found to be −7.7 kcal/mole (Durell *et al.*, 1962). Combination of these two reactions allows one to obtain the enthalpy change during methyl transfer from dimethylacetothetin to methionine.



$\Delta H = -11.3 - (-7.7) = -3.6$ kcal/mole (at pH 7.1).[1] Since it was estimated that there should be 0 entropy change in this reaction, the conclusion was drawn that dimethylacetothetin is unstable relative to methylmethionine sulfonium to the extent of 3–4 kcal/mole (Durell *et al.*, 1962).

With a view toward gaining further insight into structural factors which affect the enthalpy changes in methyl transfer reactions to the common acceptor, homocysteine, we have now carried out comparable studies on several other sulfonium compounds. Of particular interest are measurements in which *S*-adenosylmethionine was the methyl donor since this compound is by far the most important methyl donor in biological transmethylation reactions [evidence reviewed recently by Mudd and Cantoni (1964)]. This sulfonium compound is not a substrate for dimethylthetin:L-homocysteine *S*-methyltransferase (EC 2.1.1.3) (Durell *et al.*, 1957), and therefore could not be included in the studies mentioned above (Durell and Sturtevant, 1957; Durell *et al.*, 1962). Recently, an enzyme which catalyzes the transfer of the methyl group of *S*-adenosylmethionine to homocysteine was described (Shapiro, 1958; Shapiro *et al.*, 1964). This enzyme has made it possible to determine calorimetri-

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[1] ΔH will refer to enthalpy changes at the pH and ionic strength of the specified experiments.

cally the enthalpy change during transfer of a methyl group from *S*-adenosylmethionine to homocysteine and thus allow a comparison between *S*-adenosylmethionine and other sulfonium compounds. The present paper reports the results of these studies.

Experimental Section

Materials. Dimethylacetothetin was a synthetic product (Brown and Getts, 1874). (−)-*S*-Adenosyl-L-methionine was prepared by use of a partially purified yeast enzyme eluted from calcium phosphate gel (Mudd and Cantoni, 1958). The sulfonium compound was precipitated as the reineckate salt (Cantoni, 1957) and, prior to use in the calorimetric experiments, converted to the chloride form (Cantoni, 1957). To obtain a preparation suitable for the potentiometric titration of *S*-adenosylmethionine, the nucleoside was subjected to preparative paper chromatography with ethanol, 160–HCl, 0.1 N, 80, as solvent. Other compounds were obtained commercially. L-Homocysteine thiolactone hydrochloride was converted immediately before use to the open form. To 15.4 mg of the thiolactone–HCl was added 0.40 ml of 1.0 N NaOH. After standing at room temperature for 1–2 min the solution was neutralized by addition of 0.60 ml of a solution made by combining equal volumes of 0.67 M potassium phosphate buffer, pH 7.07, and 0.87 N HCl.

Thetin:homocysteine methyltransferase was prepared as described (Durell and Cantoni, 1959). *S*-Adenosylmethionine:homocysteine methyltransferase was prepared from Baker's yeast and purified by ethanol fractionation and chromatography on DEAE-cellulose. The procedures were slight modifications of those described in detail by Shapiro *et al.* (1964). Air-dried Baker's yeast was the source of enzyme. The enzyme was assayed by a method similar to that of Shapiro and Yphantis (1959), with substitution of the NH_4^+ form of Dowex 50 for the Li^+ form. By use of radioactive substrates the final preparation was shown to be free of enzymes catalyzing the following possible side reactions: (a) conversion of *S*-adenosylmethionine to 5′-methylthioadenosine (Shapiro and Mather, 1958; Mudd, 1959); (b) hydrolysis of *S*-adenosylhomocysteine to adenosine (de la Haba and Cantoni, 1959); (c) hydrolysis of *S*-adenosylhomocysteine or 5′-methylthioadenosine to adenine (Duerre, 1962); (d) decarboxylation of *S*-adenosylmethionine (Tabor *et al.*, 1957, 1958).

Calorimetetric measurements were performed at 24° with a prototype model of the Spinco microcalorimeter which was essentially a direct copy of the heat-burst instrument described by Kitzinger and Benzinger (1960). The calibration of the instrument has been described (Ross and Scruggs, 1965). Glass reaction vessels with dimple recesses were used (Kitzinger and Benzinger, 1960). In the main compartment of both the control and experimental vessel were placed 15.0 ml of a solution of potassium phosphate buffer, pH 7.07, 0.035 M, NaCl, 0.025 N, and L-homocysteine, 0.004 M. The dimples of each vessel contained a total of 0.3 ml of sulfonium substrate, 0.3 M at pH 7 in potassium phosphate buffer, 0.033 M. Enzyme was present in the main compartment of the experimental vessel. The reaction was started after 1–2 hr of thermal equilibration. At the end of the calorimetric run an aliquot was brought to a final concentration of 4% in trichloroacetic acid. After centrifugation to remove precipitate, aliquots of the supernatant fluids were used either for colorimetric determination of methionine or for column chromatography to purify the methionine prior to determination of this compound. The extent of the reaction was calculated from the difference in methionine content between the experimental and blank vessels. Methionine was determined by a modification of the McCarthy–Sullivan procedure (Durell *et al.*, 1957). In analyses where both methionine and methylmercaptoacetic acid were present, the methionine content was calculated with the use of a chromogenic value for methylmercaptoacetate of 0.85 that of methionine (Durell *et al.*, 1957). In experiments in which the enzymatic reaction products included dimethyl sulfide or methylmercaptopropionic acid in addition to methionine, the latter compound was purified prior to colorimetric determination by chromatography on Dowex 50. An aliquot (8–11 ml) of the supernatant fluid from the deproteinized reaction mixture was passed through a column of Dowex AG 50-X4 (H^+), 200–400 mesh, 6.0 × 0.9 cm. The column was washed with 10 ml of water, then 5.5 ml of 1.0 N NaOH. The methionine was then eluted with 6–7 ml of 1.0 N NaOH. The volume of the final eluate was noted and an aliquot taken for determination of methionine. If dimethylpropiothetin was the methyl donor for the enzymatic reaction, the eluate was allowed to remain at room temperature overnight prior to colorimetric analysis to prevent color formation due to dimethylpropiothetin. Control experiments showed that methionine was recovered quantitatively from this column chromatographic procedure. Appropriate internal standards were used in each experiment to calibrate the color yield from methionine.

When *S*-adenosylmethionine was substrate, the above procedure was modified as follows: The substrate concentration in the dimples was 0.057 M. At the end of the reaction, aliquots of the reaction mixtures were deproteinized by addition of 0.1 volume of 30% perchloric acid. After removal of precipitate by centrifugation, aliquots of the supernatant fluids were neutralized and chromatographed upon IRC 50 (XE-64) to separate *S*-adenosylmethionine from *S*-adenosylhomocysteine (de la Haba *et al.*, 1959). The optical density of the column eluate containing *S*-adenosylhomocysteine was determined at 259 mμ and the concentration of this compound calculated on the assumption of a molar extinction coefficient of 15.4×10^3. The extent of the reaction was calculated from the difference between the value from the experimental vessel and that from a control vessel in which enzyme had been incubated with *S*-adenosylmethionine in the absence of homocysteine. The latter control did not give rise to any measurable heat uptake or evolution in the

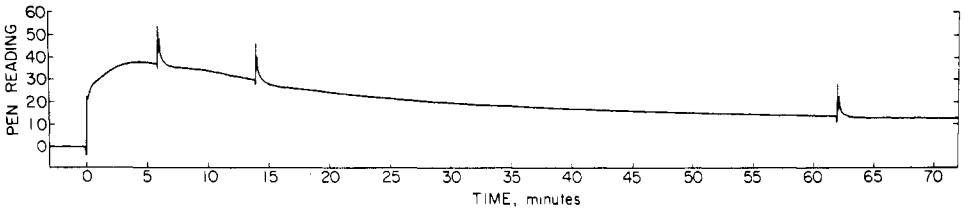

FIGURE 1: The record of a representative calorimetric experiment. In this experiment *S*-adenosylmethionine was the methyl donor. The experiment is no. 2 in Table I. Conditions are given in the text. The reaction was initiated by a stirring cycle at time 0. Subsequent stirs were carried out at 6, 14, and 62 min.

calorimeter. This control gave a slightly higher value for the optical density in the column eluate than did an alternate control consisting of reaction mixture incubated without enzyme.

The heat of ionization of the amino group of L-methionine was calculated from the Van't Hoff equation (Edsall and Wyman, 1958)

$$\Delta H = 2.303R \frac{\partial \mathrm{p}K}{\partial(1/T)}$$

using data derived from titration curves of L-methionine performed at an ionic strength of approximately 0.25 and at 21.8, 27.4, and 34.2°. The pK'[2] values were found to vary linearly with reciprocal temperature over this interval.

Results

The record of a typical calorimetric experiment is shown in Figure 1. In this case *S*-adenosylmethionine was substrate. The pen reading is a measure of the rate of heat evolution. The reaction was started at time 0 by inversion of the vessels in a mechanical stirring cycle. Heat evolution commenced immediately. The stir was repeated at 6 min. The rapid changes are artefacts caused by the stirring. The very slight increase in the rate of heat evolution after the second stir suggests that the enzymatic reaction is proceeding more rapidly due to more complete mixing. Third and fourth stirs at 14 and 62 min caused only the perturbation due to the stirring itself with no sign of an increase in the rate of heat evolution (Figure 1). In general we used a minimum of three to four stirs to ensure complete mixing. In Figure 2 is shown a plot of the integrator record as a function of time. This record gives a measure of the total heat evolved at any time and suffices to show that the effect of stirring is not discernible under the instrumental conditions employed. The heat due to stirring was evaluated from the change in rate of heat evolution caused by the final stirs.

It is apparent from Figures 1 and 2 that the enzymatic reaction had not reached completion when the experiment was terminated at 72 min. This change from the experimental design most often used for calorimetric determinations was dictated by the properties of the enzyme used to catalyze the reaction. That is, *S*-adenosylmethionine:homocysteine methyltransferase has rather low affinities for its two substrates and is inhibited by the two reaction products (Shapiro, 1965). Therefore, it was not practical to achieve complete consumption of either of the substrates. The experiments were designated to yield a moderately large total heat and a steady rate of heat production at the time the reaction was interrupted. Two small quantities could then be estimated relatively accurately and added to the measured heat evolution. (a) A quantity obtained by extrapolation and estimated to be equal to the heat evolved between the time the calorimetric record was

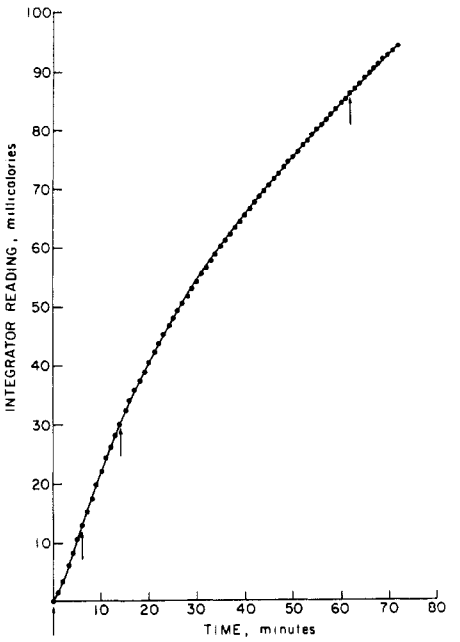

FIGURE 2: Heat production in a representative calorimetric experiment. The pen readings of experiment no. 2, Table I, with (−)-*S*-adenosyl-L-methionine as methyl donor have been integrated and converted to millicalories of heat evolved.

[2] Values of pK' in this paper are not extrapolated to 0 ionic strength and where not otherwise stated refer to values at 25°.

TABLE I: Observed Heats of Methionine Synthesis at pH 7.1.

Substrate	Expt	Heat Evolved (mcal)	Methionine Formed (μmoles)	ΔH (kcal/mole)
Dimethylacetothetin[a]	1	200.2	16.8	11.9
Dimethylacetothetin[a]	2	158.9	12.5	12.7
Dimethylacetothetin[a]	3	162.1	14.3	11.3
Dimethylacetothetin[a]	4	410.3	36.7	11.2
			Mean ± SE	11.8 ± 0.3
Dimethylpropiothetin[a]	1	218.5	23.1	9.5
Dimethylpropiothetin[a]	2	233.9	22.7	10.3
Dimethylpropiothetin[a]	3	327.1	32.6	10.0
			Mean ± SE	9.9 ± 0.2
Trimethylsulfonium[b]	1	181.2	23.8	7.6
Trimethylsulfonium[b]	2	171.1	20.3	8.4
Trimethylsulfonium[b]	3	185.0	23.0	8.1
			Mean ± SE	8.0 ± 0.2
(−)-*S*-Adenosyl-L-methionine[a]	1	107.2	6.48	16.50
(−)-*S*-Adenosyl-L-methionine[a]	2	100.1	6.47	15.50
(−)-*S*-Adenosyl-L-methionine[a]	3	102.9	6.90	14.90
			Mean ± SE	15.6 ± 0.5

[a] Added as chloride salt. [b] Added as iodide salt.

discontinued and the time the enzymatic reaction was actually terminated by the addition of deproteinizing agent. This time was required to remove the reaction vessel from the calorimeter and to obtain an aliquot of the reaction mixture. Measured in each experiment, the time varied from 4 to 5 min for the series of experiments. Since the reaction rate was essentially constant when the calorimeter record was discontinued, the heat evolution for this 4–5 min period could be obtained by extrapolation with little uncertainty. (b) A quantity estimated to be equal to the unevolved heat; *i.e.*, heat given off by the chemical reaction which had not yet penetrated the area thermopile around the reaction vessel at the time the experiment was terminated. To obtain this quantity, the instrument was calibrated electrically at a series of constant pen readings. The increment in heat recorded by the integrator after electric current was discontinued, plotted *vs.* the pen reading, provided a calibration curve. This curve was used to determine the quantity of unevolved heat at any given constant rate of heat evolution. In practice the pen reading obtained by extrapolation to the time the reaction mixture was deproteinized was used to determine the actual correction used in each experiment.

The results of the experiment shown in Figure 1 [(−)-*S*-adenosyl-L-methionine, no. 2, Table I] were calculated as follows: heat evolution recorded by integrator, 95.07 mcal; heat due to stirring (4 × −0.19 mcal), −0.76 mcal; heat evolved from end of record until termination of reaction, +4.03 mcal; heat unevolved at termination, +1.71 mcal. The total was 100.05 mcal.

The corrections are seen to be small relative to the actual measured heat, in accord with the experimental design. Since each of these corrections may be estimated with good accuracy, the total error introduced by the corrections should be negligible. It should be noted that in our experiments a minimum of *ca.* 100 mcal was evolved. The minimum rate of evolution was *ca.* 1.3 mcal/min. This minimum was established to lessen the relative contribution due to erratic base-line shifts in the calorimeter. Such shifts may introduce an undetectable error into any single experiment and remain the major source of uncertainty in the present experiments. In our experience however the magnitude of these shifts are not such as to lead to major errors in our results.

The results of our experiments are presented in Table I. Dimethylacetothetin was studied in order to obtain a comparison between the results obtained by our methods and the results previously reported by Durell and co-workers (Durell and Sturtevant, 1957; Durell *et al.*, 1962) who studied the same compound by a different method. These workers found ΔH = 12.50 ± 0.35 kcal/mole with dimethylacetothetin as methyl donor. Our value for the same reaction is 11.8 ± 0.3. The different methods give the same answer within the limits of the combined standard errors of the means. In expt 1–3, the yield of methionine at the end of the calorimetric run was determined directly on the de-

proteinized reaction mixture without preliminary purification by chromatography on Dowex 50. In expt 4, this chromatography was performed, as it was in the series of experiments with dimethylpropiothetin and with trimethylsulfonium salts as substrates. The satisfactory agreement between the experiments with and without this purification step indicate that this step does not introduce significant error.

Discussion

Correction for Heat Due to Proton Uptake. During methyl transfer reactions such as those under discussion a proton is evolved (eq 1). The measured heat evolution is affected by subsequent reactions of this proton so that a correction must be made to obtain the heat due to the methyl transfer reaction itself. Since the reaction with dimethylacetothetin as substrate is carried out in phosphate buffer, the proton will react with the buffer, evolving 1.2 kcal/mole (Durell and Sturtevant, 1957). The measured heat evolution is decreased by this amount to obtain the value for the methyl transfer reaction. The same correction is applicable for our present experiments with dimethylpropiothetin and the trimethylsulfonium salt as substrates.

With *S*-methylmethionine the situation is more complicated. The pK' of the α-amino group of this compound is approximately 7.9 (Durell *et al.*, 1962). This pK' is lower than that usually found in an α-amino acid, presumably because of the effect of the positive sulfonium charge. At pH 7.1, the pH at which the experiments were carried out, the amino group of *S*-methylmethionine will then be 86% protonated and 14% unprotonated. The product of the reaction which contains this amino group is methionine, with pK = 9.2. Therefore, for each mole of *S*-methylmethionine which reacts, 0.14 mole of the proton product will be consumed by reaction with the resulting methionine. The remaining 0.86 mole of proton will react with the phosphate buffer. To allow for this correction, we have determined an approximate heat of ionization for the amino group of L-methionine: 8.5 ± 1.3 kcal/mole. This value may be compared with those previously reported for eight aliphatic (Smith *et al.*, 1937) and three hydroxy (Smith *et al.*, 1942) amino acids ranging from 10.0 to 11.6 kcal/mole with a mean value of 10.8 kcal/mole. Applying this factor, the measured enthalpy change during transmethylation from *S*-methylmethionine to homocysteine in phosphate buffer at pH 7.1 (Durell *et al.*, 1962) is corrected as follows: $-8.9 + (0.14 \times 8.5) + (0.86 \times 1.2) = -6.7$ kcal/mole. This value is 1.0 kcal/mole lower than that previously calculated without allowance for the protonation of methionine (Durell *et al.*, 1962).

When *S*-adenosylmethionine serves as methyl donor, the case is very similar to that of *S*-methylmethionine. We have titrated *S*-adenosylmethionine and found a pK' at 7.8. The corresponding pK' in the product *S*-adenosylhomocysteine is not known but may be presumed to be close to that of methionine. At pH 7.1, the α-amino group of *S*-adenosylmethionine will be 83% protonated, 17% unprotonated, and for each mole of proton product resulting from transmethylation, 0.17 mole will react with *S*-adenosylhomocysteine. To verify this point we determined the stoichiometry of this methyl transfer reaction, comparing enzyme- and homocysteine-dependent *S*-adenosylhomocysteine production to proton evolution (at pH 7.1 in a pH-Stat). The ratio of H^+ evolved/*S*-adenosylhomocysteine formed was found to be 0.85/1.0, in excellent agreement with the predicted ratio of 0.83/1.0. The measured enthalpy change during methyl transfer with *S*-adenosylmethionine as substrate at pH 7.1 in phosphate buffer is then corrected as follows: $-15.6 + (0.17 \times 8.5) + (0.83 \times 1.2) = -13.2$ kcal/mole. For this calculation the enthalpy of ionization of the α-amino group of *S*-adenosyl-L-homocysteine has been assumed to be 8.5 kcal/mole by analogy with the thioether methionine. If this value were assumed to be that of the average of eleven amino acids, 10.8 kcal/mole, the enthalpy change during the methyl transfer reaction from *S*-adenosylmethionine would be −12.8 kcal/mole. Therefore, only a small uncertainty is introduced by the fact that the heat of ionization of the α-amino group of *S*-adenosyl-L-homocysteine is not known.

Comparison of Enthalpy Changes. In Table II all the known enthalpy changes for methyl transfer from sulfonium compounds to the common acceptor, homocysteine, are presented. In the right-hand column these values are corrected for enthalpy changes in the reaction

TABLE II: Corrected Enthalpy Changes during Methyl Transfer Reactions

Methyl Donor	Heat Evolution (kcal/mole) Measured	Heat Evolution (kcal/mole) Corrected
$(CH_3)_2S^{\oplus}CH_3$ Trimethylsulfonium salt	8.0	6.8
$(CH_3)_2S^{\oplus}CH_2COO^{\ominus}$ Dimethylacetothetin	11.8	10.6
	12.5[a]	11.3[a]
$(CH_3)_2S^{\oplus}CH_2CH_2COO^{\ominus}$ Dimethylpropiothetin	9.9	8.7
$(CH_3)_2S^{\oplus}CH_2CH_2CHCOO^{\ominus}$ (with $^{\oplus}NH_3$) *S*-Methylmethionine	8.9[a]	6.7[b]
$CH_3S^{\oplus}CH_2CH_2CHCOO^{\ominus}$ (AR, $^{\oplus}NH_3$) (−)-*S*-Adenosyl-L-methionine	15.6	13.2

[a] Value taken from Durell *et al.* (1962). [b] Recalculated from value of Durell *et al.* (1962) as indicated in discussion. [c] AR is 5′-deoxyadenosine.

of the proton. In general this correction causes little uncertainty in the measured value. Clearly, methyl transfer from each of the sulfonium compounds studied is a highly exothermic process. More than 20 years ago, Toennies suggested that the sulfonium bond would be found to play an important role in methionine metabolism (Toennies, 1940). Subsequent work has supported this postulate and the general rule has emerged that in all cases where the methyl group is labile it is attached directly to an onium pole (Cantoni, 1952) (see recent review of Mudd and Cantoni, 1964).[3] The importance of the onium structure for kinetic or for energetic reasons should be clearly distinguished. Previous discussions of the energetics of methyl transfers from onium compounds have drawn attention to the role of ionization in driving these reactions (Cantoni, 1952; Durell and Sturtevant, 1957; Kalckar, 1954). For thermodynamic analysis, the reaction between a sulfonium donor and a sulfhydryl acceptor may be separated into hypothetical partial reactions as follows

$$(CH_3)_2S^{\oplus}R^1 + HSR^2 \longrightarrow CH_3SR^1 + CH_3S^{\oplus}HR^2 \quad (3a)$$

$$CH_3S^{\oplus}HR^2 \longrightarrow CH_3{-}S{-}R^2 + H^{\oplus} \quad (3b)$$

It has been suggested that the dissociation of a proton in neutral aqueous buffered solution from the very strong acid $CH_3S^{\oplus}HR^2$ may account for a great deal of the free energy evolved during the over-all reaction (Cantoni, 1952; Durell and Sturtevant, 1957). The results of the present study emphasize that at least for enthalpy changes other factors may play an important role. In all the cases reported here the methyl acceptor is the same, homocysteine. The strong acid formed in the first partial reaction (3a) of the over-all methyl transfer is thus the same and the ionization of this acid may then be disregarded as a cause of the observed differences in enthalpy changes. The basis of these differences must be sought in the structure of the methyl donor itself and the thioether formed from it.[4] Nevertheless, the fact that all sulfonium donors studied, even the simplest, trimethylsulfonium, participate in highly exothermic methyl transfer reactions suggests that the loss of this type of chemical bond supplies an important energetic driving force.

A more detailed examination of values in Table II may give some indication of the forces which modify the enthalpy during methyl transfer from a sulfonium to homocysteine. A pattern is apparent in the values for the first four compounds, those in which the substituents of the sulfur atom are relatively simple. Replacement of a methyl hydrogen in the parent compound, trimethylsulfonium salt, by a carboxylate anion to give dimethylacetothetin has the effect of increasing the heat evolution during transmethylation by approximately 4 kcal/mole. If this carboxylate anion is removed by a second methylene carbon from the sulfonium center to form dimethylpropiothetin, the enthalpy falls about halfway to the value for trimethylsulfonium salt. If the carboxylate anion is removed by yet another carbon atom (and a partially positively charged amino group is added) (*S*-methylmethionine) the enthalpy change is restored to the original value.

In comparing ΔH values obtained with the various substrates one must consider the enthalpy of both substrates and products. Our present results indicate that a carboxylate group in close proximity to the sulfonium center increases the enthalpy change in the transmethylation reaction. This effect is quite apart from any influence which the carboxylate group may have on the kinetics of the reaction. It is our present purpose to inquire whether the neighboring $COO^{\ominus}$ group primarily increases the enthalpy of the reactants or decreases that of the products, and to ascertain how this is accomplished. A number of factors may be considered: (a) Coulombic interaction between the positive and negative charges of dimethylacetothetin would be expected to stabilize this molecule relative to trimethylsulfonium salt which has no negative charge or to dimethylpropiothetin in which the charges are more widely separated.[5] Contrary to this expectation the observed enthalpy changes indicate a destabilization due to the α-carboxylate group. Since there is no reason to expect that the enthalpy of the thioethers produced by these reactions are very different, coulombic interaction is thought not to be the dominant effect. (b) Inductive effects of the carboxylate anion are known to be small (Cohen and Jones, 1963). Any inductive effects present

[3] A probable exception to this generalization is now known in the case of N^5-methyltetrahydrofolic acid. However the methyl transfer from this compound to homocysteine has not yet been studied in sufficient detail to prove the exact structure of the methyl donor.

[4] An interesting example of the effect of the structure of the methyl donor on the enthalpy of methyl transfer to homocysteine is found in the case of betaine. The heat evolved during reaction 4 has been estimated as merely 1.5 kcal/mole. The

$$(CH_3)_3{-}N^{\oplus}CH_2COO^{\ominus} + \text{homocysteine} \longrightarrow (CH_3)_2NCH_2COO^{\ominus} + \text{methionine} + H^{\oplus} \quad (4)$$

measured enthalpy of transmethylation at pH 7, -8.8 kcal/mole, is largely due to the fact that the proton formed in reaction 4 is taken up by the product, $(CH_3)_2NCH_2COO^{\ominus}$, with liberation of 7.3 kcal/mole. The enthalpy change in 4, *i.e.*, the reaction in which proton formation occurs, is relatively low (Durell *et al.*, 1962).

[5] The possibility that in solution the charge centers of dimethylpropiothetin are actually closer to one another than in dimethylacetothetin due to increased conformational adaptability may be dismissed upon the basis of data for dipole moments (Wyman, 1936) and ionization constants (Cohn and Edsall, 1943) in the analogous amino acids series which clearly establish that opposite charges separated by increasing chain lengths are in fact more separated spatially in aqueous solution. In addition, the pK values of the carboxyl groups of dimethylacetothetin, dimethylpropiothetin, and *S*-methylmethionine are consistent with the expected increase in charge separation of these compounds as chain length increases (W. A. Klee, and S. H. Mudd, unpublished observations).

in the reactants would, of necessity, tend to stabilize these compounds and thereby lower the enthalpy change. It is difficult to visualize carboxylate-induced electron displacements in the thioether products which could stabilize these compounds to the degree necessary to explain our results. (c) Solvation of dipolar ions such as dimethylacetothetin and dimethylpropiothetin will increase as the charges become more separated because the electrostatic fields around the two charge centers will interact to a lesser extent. This effect is demonstrated by electrostriction data for the amino acid series (Cohn *et al.*, 1934). Upon conversion of a thetin to the corresponding thioether the interaction between the charges will be lost because the charges are removed to separate ionic species. Therefore, the transmethylation from dimethylacetothetin will involve a net gain of hydration with respect to transmethylation from dimethylpropiothetin since there is less charge interaction to hinder hydration of the latter compound. Since the charge centers of *S*-methylmethionine are even further removed from one another, there should be even less interaction and, therefore, less of an enthalpy change due to hydration.[6]

Accordingly we suggest that the 4 kcal difference in the enthalpies of transmethylations from trimethylsulfonium ion as compared to dimethylacetothetin is supplied by the energy of hydration of that amount of water which is not bound to dimethylacetothetin due to the interaction between the two charge centers, but which would be bound if the charges were completely independent. The contribution of this differential effect of hydration will be in the proper direction to fit the trend of observed results. It has been estimated that the transfer of a molecule of water from the liquid state to the inner hydration shell of an ion liberates approximately 1–3 kcal of heat (Robinson and Stokes, 1959). Therefore, changes of a very few moles of bound water could account for differences of the magnitude of those observed.

Clearly *S*-adenosylmethionine is a much more complicated molecule than are the others listed in Table II. Replacement of a methyl group in *S*-methylmethionine by an adenosyl moiety leads to *S*-adenosylmethionine and increases the heat evolution during methyl transfer by 6–7 kcal/mole. It is of considerable interest that the reaction involving the biological methyl donor is the most highly exothermic of all those which have been studied to date. Further work now being carried out in this laboratory is aimed at finding an explanation for this interesting fact.

[6] The sulfonium atom of trimethylsulfonium ion would be hydrated to the same extent as the sulfonium atom of a compound with a carboxylate so far removed as to be without interaction. (Such a compound is represented to a first approximation in this series by *S*-methylmethionine. Clearly this treatment of *S*-methylmethionine in which the role of the α-amino group is disregarded is only an approximation. Further studies of other substrates, *e.g.*, γ-butyrothetin, could clarify the contribution of the amino moiety). *S*-Methylmethionine differs from trimethylsulfonium ion in that the former possesses a carboxylate anion and an amino group. However, hydration of these groups in the substrate will be balanced by equivalent hydration in the product so that these moieties will not contribute a difference in the enthalpy of transmethylation from *S*-methylmethionine compared to trimethylsulfonium. (We are neglecting in the case of *S*-methylmethionine the effect of the pK' shift which results in the somewhat more complete ionization of the amino group of methionine than of *S*-methylmethionine at pH 7.1).

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References

Brown, C., and Getts, E. A. (1874), *Ber. 7*, 695.

Cantoni, G. L. (1952), *in* Phosphorous Metabolism, Vol. II, W. D. McElroy and B. Glass, Ed., Baltimore, Md., Johns Hopkins, p 129.

Cantoni, G. L. (1957), *in* Biochemical Preparations, Vol. V, D. Shemin, Ed., New York, N. Y., Wiley, p 58.

Cohen, L. A., and Jones, W. M. (1963), *J. Am. Chem. Soc. 85*, 3402.

Cohn, E. J., and Edsall, J. T. (1943), Proteins, Amino Acids and Peptides, New York, N. Y., Reinhold, p 122.

Cohn, E. J., McMeekin, T. L., Edsall, J. T., and Blanchard, M. H. (1934), *J. Am. Chem. Soc. 56*, 784.

de la Haba, G., and Cantoni, G. L. (1959), *J. Biol. Chem. 234*, 603.

de la Haba, G., Jamieson, G. A., Mudd, S. H., and Richards, H. H. (1959), *J. Am. Chem. Soc. 81*, 3975.

Duerre, J. A. (1962), *J. Biol. Chem. 237*, 3737.

Durell, J., Anderson, D. G., and Cantoni, G. L. (1957), *Biochim. Biophys. Acta 26*, 270.

Durell, J., and Cantoni, G. L. (1959), *Biochim. Biophys. Acta 35*, 515.

Durell, J., Rawitscher, M., and Sturtevant, J. M. (1962), *Biochim. Biophys. Acta 56*, 552.

Durell, J., and Sturtevant, J. M. (1957), *Biochim. Biophys. Acta 26*, 282.

Edsall, J. T., and Wyman, J., Jr. (1958), Biophysical Chemistry, Vol. I, New York, N. Y., Academic, p 451.

Kalckar, H. M. (1954), *in* The Mechanism of Enzyme Action, W. D. McElroy and B. Glass, Ed., Baltimore, Md., Johns Hopkins, p 675.

Kitzinger, C., and Benzinger, T. H. (1960), *Methods Biochem. Analy. 8*, 309.

Mudd, S. H. (1959), *J. Biol. Chem. 234*, 87.

Mudd, S. H., and Cantoni, G. L. (1958), *J. Biol. Chem. 231*, 481.

Mudd, S. H., and Cantoni, G. L. (1964), *in* Comprehensive Biochemistry, Vol. XV, M. Florkin and E. H. Stotz, Ed., New York, N. Y., Elsevier, p 1.

Robinson, R. A., and Stokes, R. H. (1959), Electrolyte Solutions, 2nd ed, New York, N. Y., Academic, p 56.

Ross, P. D., and Scruggs, R. L. (1965), *Biopolymers 3*, 491.

Shapiro, S. K. (1958), *Biochim. Biophys. Acta 29*, 405.
Shapiro, S. K. (1965), *in* Transmethylation and Methionine Biosynthesis, S. K. Shapiro and F. Schlenk, Ed., Chicago, Ill., University of Chicago, p 200.
Shapiro, S. K., and Mather, A. N. (1958), *J. Biol. Chem. 233*, 631.
Shapiro, S. K., and Yphantis, D. A. (1959), *Biochim. Biophys. Acta 36*, 241.
Shapiro, S. K., Yphantis, D. A., and Almenas, A. (1964), *J. Biol. Chem. 239*, 1551.
Smith, P. K., Gorham, A. T., and Smith, E. R. B. (1942), *J. Biol. Chem. 144*, 737.
Smith, P. K., Taylor, A. C., and Smith, E. R. B. (1937), *J. Biol. Chem. 122*, 109.
Tabor, H., Rosenthal, S. M., and Tabor, C. W. (1957), *J. Am. Chem. Soc. 79*, 2978.
Tabor, H., Rosenthal, S. M., and Tabor, C. W. (1958), *J. Biol. Chem. 233*, 907.
Toennies, G. (1940), *J. Biol. Chem. 132*, 455.
Wyman, J., Jr. (1936), *Chem. Rev. 19*, 213.

Respiration-Dependent Proton Movements in Rat Liver Mitochondria*

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ABSTRACT: Rat liver mitochondria were found to extrude protons into the medium at a constant rate when incubated in various media. Proton extrusion was measured with a sensitive pH electrode system. The addition of adenosine diphosphate (ADP) to mitochondria respiring in a medium containing magnesium and phosphate caused an immediate halt in the liberation of protons and, as expected, H^+ concentration decreased during the phosphorylation of the ADP. In all media the extrusion of protons was dependent on respiration. It did not occur in the presence of antimycin or cyanide or in the absence of oxygen. It was not noticeably affected by the presence of other ions such as Mg^{2+}, $H_2PO_4^-$, Na^+, or K^+. The rate of proton extrusion varied between 10 and 100% of the rate of electron transport under varying conditions. Uncoupling agents increased the rates of both respiration and proton extrusion but substantially decreased the ratio of the rate of proton extrusion to the rate of electron transport. In the presence of cyanide and ferricyanide the rate of proton extrusion was approximately equal to the rate of electron transport. These results suggest that proton extrusion is a normal mitochondrial process and that the protons probably arise as direct products of respiration.

During the past few years a considerable number of reports have been published dealing with the translocation of various ions in mitochondrial preparations. While most of these reports have dealt with the respiration-dependent movement of Ca^{2+}, $H_2PO_4^-$, Mg^{2+}, Mn^{2+}, Na^+, and K^+, in a number of cases it was shown that there was an extrusion of protons from the mitochondria as other ions were taken up. It has been proposed that protons may be released from the mitochondria due to the deposition of insoluble salts within the mitochondria (*e.g.*, Brierley *et al.*, 1962) or by an exchange reaction with other cations (*e.g.*, Chappell and Greville, 1963; Chance, 1965). Such an exchange reaction for K^+ or Na^+ apparently requires the presence of an uncoupling agent (Moore and Pressman, 1964; Chappell and Crofts, 1965). These movements of protons are generally considered to be secondary reactions resulting from the movement of other cations. However, Chappell and Crofts (1965) suggested that a respiration-dependent H^+ pump mechanism might provide the underlying mechanism for mitochondrial ion movements. Mitchell (1963) has suggested that the separation of protons and OH^- ions on opposite sides of the mitochondrial membrane during respiration provides the driving potential for the synthesis of ATP.[1]

Thus in view of these suggestions and the fact that protons, either free or bound, are presumably produced during mitochondrial respiratory chain activity, it appeared important to study proton movements in respiring mitochondria *per se*.

Methods and Materials

Rat liver mitochondria were isolated by the method of Hogeboom (1955) as described by Myers and Slater

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[1] Abbreviations used: ATP, adenosine triphosphate; ADP, adenosine diphosphate; TMPD, tetramethyl-*p*-phenylenediamine; DPN, 2-4-dinitrophenol.