THE SYNTHESIS OF METHIONINE BY ENZYMIC TRANSMETHYLATION*

VIII. ENTHALPY CHANGES IN THE METHYL-TRANSFER FROM BETAINE AND S-METHYLMETHIONINE BROMIDE

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SUMMARY

The enthalpy changes accompanying the enzyme-catalyzed transfer of a methyl group from betaine and from L-methylmethionine sulfonium bromide to L-homocysteine at neutral pH and 25° have been determined calorimetrically. The heats and standard free energies of ionization of sarcosine and *N*,*N*-dimethylglycine have also been measured. The standard entropy values obtained from these data have been used to estimate the standard entropy changes, and hence the standard free energy changes, in the transmethylation reactions. The earlier data and calculations for the methyl transfer from dimethylacetothetin to L-homocysteine have been revised. It is concluded that all the reactions are strongly exergonic and essentially irreversible at pH 7.

INTRODUCTION

Purified THM catalyzes the synthesis of methionine from homocysteine and several sulfonium and quarternary ammonium compounds which act as methyl donors[1]. It has been impossible to detect reversibility of the reaction, and therefore, impossible to calculate the standard free energy of the methyl transfer[2]. We have undertaken calorimetric determinations of the enthalpy changes in three of these reactions in order to arrive at estimates of the free energies. In an earlier paper[3] experiments in which DMAT was the methyl group donor were reported. In the present communication similar experiments with betaine and MMS are described. Subsidiary data pertaining to the ionization of *N*-methylglycine (sarcosine) and *N*,*N*-dimethylglycine

Abbreviations: THM, thetin-homocysteine methylpherase; DMAT, dimethylacetothetin; MMS, *S*-methylmethionine sulfonium bromide.

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are also presented because of their relevance to the calculation of the entropies of the transmethylation reactions.

EXPERIMENTAL

The calorimetric method has been previously described[4] in detail. All experiments were carried out at 25.00 ± 0.05°. pH values were measured at 25° with a Radiometer and a glass electrode calibrated with Beckman standard buffers.

Sarcosine and sarcosine hydrochloride were purchased from Mann Research Laboratories, New York, and were used without further purification. Micro-Kjeldahl determinations gave 15.6% N (theory: 15.7% N) and 10.7% N (theory: 11.3% N) for the free base and the hydrochloride, respectively, without previous drying. The hydrochloride lost less than 1% of its weight on drying at 100°. Both substances had melting points in satisfactory agreement with values in the literature.

The sodium salt of dimethylglycine was a gift from Dr. C. G. MACKENZIE and had been prepared by the reaction of monochloracetic acid and dimethylamine[5]. Analysis by the micro-Kjeldahl method gave 11.0% N (theory: 11.2% N).

Betaine was purchased from Nutritional Biochemical Corporation of Cleveland.

MMS and L-homocysteine were prepared as described previously[1]. The enzyme was kindly supplied by Dr. W. A. KLEE, who prepared it according to the method described in an earlier communication in this series[1]. All other materials were reagent grade, and glass-distilled water was used throughout.

In the determinations of the heats of ionization of the amino acids, one half of each calorimeter was charged with 14.0 ml of a solution of the amino acid (0.04–0.05 *M* in the case of sarcosine, 0.003–0.006 *M* in the case of dimethylglycine) in 0.10 *M* NaCl, the pH of which had been adjusted to 9–10. The other half of each calorimeter was filled with 14.0 ml of 0.10 *M* NaCl containing an accurately known amount of HCl (prepared by dilution of constant-boiling HCl) sufficient to give a pH of the final solution no more than one unit below that of the amino acid solution. Thus essentially all the HCl was taken up by the amino acid. Care was taken to minimize absorption of CO_2 by the alkaline solutions.

The procedure for the transmethylation experiments, though similar to that previously described[3], was modified to compensate for the much slower rates of reaction with the present substrates. The concentration of either betaine or MMS was 0.05 *M* after mixing and 25 to 100 mg of THM were employed. Moreover, in order further to minimize interference due to autoxidation of homocysteine, the calorimeters were filled in a nitrogen atmosphere with solutions previously saturated with nitrogen.

RESULTS

Heats of ionization

The heats evolved on adding HCl to sarcosine and dimethylglycine are summarized in Fig. 1. The slopes of the least squared lines through the data can be taken directly as measures of the heats of ionization (with sign changed), since the dilution heats of the HCl solutions were completely negligible[6]. The heats of ionization obtained in this way are given in Table I. The uncertainty limits indicated are based on the

TABLE I

THE IONIZATION OF GLYCINE AND METHYLATED GLYCINES AT 25°

Substance	pK'_2	ΔH	$\Delta F°$	$\Delta S°$
		(cal/mole)	*(cal/mole)*	*(cal/degree/mole)*
Glycine	9.60*	10 710**	13 100	— 8.0
Sarcosine	10.10 ± 0.03	8870 ± 400	13 800	— 16.5
Dimethylglycine	9.54 ± 0.04	7350 ± 400	13 000	— 19.0

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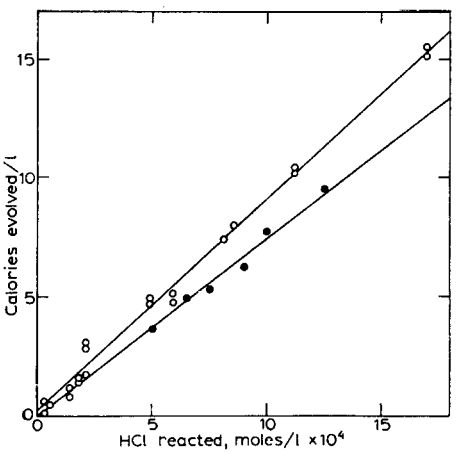

Fig. 1. Heat evolved on reacting sarcosine, O—O, and dimethylglycine, ●—●, with acid at 25° and 0.1 *M* ionic strength.

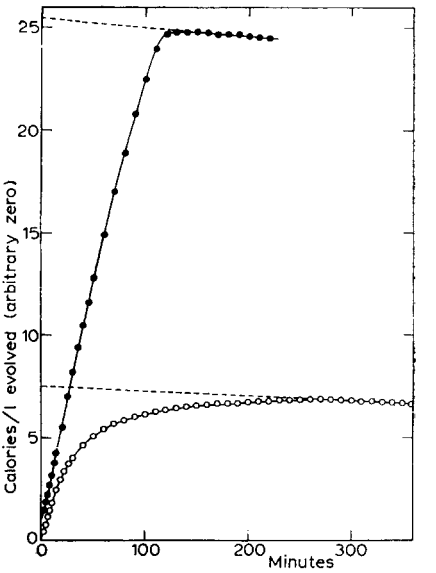

Fig. 2. Time course of the heat evolved in the transfer at 25° and pH 7 of a methyl group from betaine, ●—●, and methyl methionine sulfonium bromide, O—O, to L-homocysteine (see Table II and text).

standard errors of the slopes of the lines in Fig. 1, increased by an allowance for the uncertainty in the calorimeter calibrations. It should be noted that the method employed in these experiments leads to results which are relatively insensitive to the purity of the amino acid, since the extent of reaction is determined by the amount of HCl added.

The initial and final values of the pH in each experiment give measures of the apparent ionization constant of the amino acid. These quantities are listed in Table I, with the standard errors of the mean values obtained. They are in fair agreement with the values $pK'_2 = 10.01$ for sarcosine reported by LEVENE, SIMMS AND PFALTZ[7], and 9.86 for dimethylglycine reported by JOHNSTON[8]. Values at 0.1 *M* ionic strength of the standard free energy (calculated by the approximate relation $\Delta F° = 2.303\, RT(pK')$) and entropy of ionization are also listed in the table. In calculating the entropies,

the standard heats of ionization were assumed equal to the observed heats, a procedure which introduces negligible error since the actual amino acid concentrations were quite low.

Heats of transmethylation

The results obtained for the enthalpy changes in the transmethylation reactions are recorded in Table II, and curves showing the course of the heat liberation for typical runs are presented in Fig. 2. As pointed out in connection with the experiments on DMAT[3], there is a large thermal disturbance when the reactants are mixed, which precludes obtaining reliable thermal readings until 2–3 min after mixing; it becomes necessary, therefore, to extrapolate the curve back to the instant of mixing. The course of heat evolution with betaine and MMS does not follow any simple kinetic equation, so that direct extrapolation was employed to obtain the values in Table II. Because of the comparatively slow course of these reactions, this procedure is unlikely to introduce an error greater than 2 or 3%.

In the earlier experiments with DMAT[3], it appeared that the heat evolution during most of the reaction followed first order kinetics. On this basis we had decided to employ logarithmic extrapolation, although we had recognized that data establishing the first order kinetics of the initial period were not available. Now, for the sake of consistency with the other values reported herein, we have recalculated the data with DMAT employing direct rather than logarithmic extrapolation, and these results are included in Table II. Since the reaction with DMAT is faster than that with betaine and MMS, the uncertainty regarding the extrapolation procedure introduces a potentially larger error, and the recalculated heats included in Table II are approximately 10% smaller than those previously reported.

TABLE II

OBSERVED HEATS OF METHIONINE SYNTHESIS AT pH 7.1, 25°

Methyl donor	*Methionine formed (μmoles)*	*Calories evolved*	*$-\Delta H$ (cal/mole)*
DMAT	8.1	0.105	13000
DMAT	30.8	0.417	13500
DMAT	34.0	0.381	11200
DMAT	40.9	0.528	12900
DMAT	41.8	0.495	11800
DMAT	44.8	0.569	12700
		Mean ± std. error	12500 ± 350
Betaine	35.3	0.288	8200
Betaine	52.4	0.403	7700
Betaine	62.5	0.600	9600
Betaine*	71.2	0.684	9600
		Mean ± std. error	8800 ± 500
MMS*	22.2	0.204	9200
MMS	25.3	0.236	9300
MMS	28.6	0.251	8800
MMS	40.1	0.330	8200
		Mean ± std. error	8900 ± 300

* See Fig. 2.

DISCUSSION

Ionization of amino acids

The data in Table I show that methyl substitution on the nitrogen atom of glycine lowers the heat of ionization by approx. 1700 cal/mole. This large effect is counterbalanced by a decrease in the standard entropy of ionization so that the standard free energy of ionization is relatively unaffected by methyl substitution. As might be expected, the first methyl group has a disproportionately large effect on ΔH and especially on $\Delta S°$. If we assume that the observed entropy differences result primarily from differences in the hydration of the substituted ammonium groups, we may conclude that the first methyl substitution increases the partial molar entropy of the dipolar ion by 8.5 units, and the second substitution causes a further increase of 2.5 units. A reasonable extrapolation suggests that the partial molar entropy of the betaine dipolar ion is approximately 1 unit above that of the dimethylglycine dipolar ion.

Transmethylation from DMAT

In view of our increased knowledge of the thermodynamics of the ionization reactions shown in Table I, it is possible to improve our earlier estimate[3] of the standard free energy of this reaction which is represented by the equation:

$$(CH_3)_2\overset{+}{S}\,CH_2COO^- + HS(CH_2)_2\,CH\overset{+}{N}H_3COO^- \rightarrow$$
$$CH_3SCH_2COO^- + CH_3S(CH_2)_2CH\overset{+}{N}H_3COO^- + H^+ \qquad (1)$$

Using the value obtained by direct extrapolation for the heat evolution, *i.e.* 12500 cal/mole, and subtracting 1200 cal for the heat evolved in the uptake of one mole of hydrogen ions by the phosphate buffer[3], the value for the enthalpy change of reaction (1) is $\Delta H_1 = -11300$ cal.

Following the argument previously employed, reaction (1) can be separated into two parts:

$$(CH_3)_2\overset{+}{S}CH_2COO^- + HS(CH_2)_2CH\overset{+}{N}H_3COO^- \rightarrow$$
$$CH_3SCH_2COO^- + CH_3H\overset{+}{S}(CH_2)_2CHNH_3COO^- \qquad (2)$$

$$CH_3H\overset{+}{S}(CH_2)_2CH\overset{+}{N}H_3COO^- \rightarrow CH_3S(CH_2)_2CH\overset{+}{N}H_3COO^- + H^+ \qquad (3)$$

Whereas we previously assumed that $\Delta S_3°$ would be of the order of the entropy change for the ionization of glycine, it appears more appropriate to assume that it is of the order of the entropy change for the ionization of sarcosine which is given in Table I; therefore, $\Delta S_3° = -16.5$ e.u. Moreover, whereas we formerly disregarded the entropy change of (2) as small, we can now estimate $\Delta S_2° = -2.5$ e.u. by analogy to the difference in partial molar entropy between the dipolar ions of sarcosine and dimethylglycine (see above). Therefore, $\Delta S_1° = \Delta S_2° + \Delta S_3° = -19.0$ e.u. On this basis we obtain $\Delta F_1° = -5600$ cal/mole.

Transmethylation from betaine

Since the reaction at pH 7 in this case is

$$(CH_3)_3\overset{+}{N}CH_2COO^- + HS(CH_2)_2CH\overset{+}{N}H_3COO^- \rightarrow$$
$$(CH_3)_2H\overset{+}{N}CH_2COO^- + CH_3S(CH_2)_2CH\overset{+}{N}H_3COO^- \qquad (4)$$

no buffer correction is to be applied, and $\Delta H_4 = -8800$ cal/mole. As suggested above,

the entropy change in this reaction is probably approximately —1 cal/degree/mole, so that $\Delta F_4^\circ = -8500$ cal/mole. For direct comparison with reaction (1), we may consider the process

$$(CH_3)_3\overset{+}{N}CH_2COO^- + HS(CH_2)_2\overset{+}{C}HNH_3COO^- \rightarrow$$
$$(CH_3)_2NCH_2COO^- + CH_3S(CH_2)_2\overset{+}{C}HNH_3COO^- + H^+ \quad (5)$$

By adding to the thermodynamic changes for reaction (4) those contained in Table I for the ionization of dimethylglycine, we obtain $\Delta H_5 = -1500$, $\Delta F_5^\circ = +4500$ and $\Delta S_5^\circ = -20$; this reaction is thus seen to be strongly endergonic.

Transmethylation from MMS

Here, as in the case of DMAT, hydrogen ions are formed in the reaction

$$(CH_3)_2\overset{+}{S}(CH_2)_2\overset{+}{C}HNH_3COO^- + HS(CH_2)_2\overset{+}{C}HNH_3COO^- \rightarrow$$
$$2\ CH_3S(CH_2)_2\overset{+}{C}HNH_3COO^- + H^+ \quad (6)$$

so that the buffer correction must be applied. Therefore, $\Delta H_6 = -7700$. The entropy change in this reaction is expected to be the same as in reaction (1), so that $\Delta F_6^\circ = -2000$.

Comparison of the transmethylation reactions: DMAT and MMS

It is of interest to consider the energetics of transfer of methyl from DMAT to methionine. Substraction of (6) from (1) gives

$$(CH_3)_2\overset{+}{S}CH_2COO^- + CH_3S(CH_2)_2\overset{+}{C}HNH_3COO^- \rightarrow$$
$$CH_3SCH_2COO^- + (CH_3)_2\overset{+}{S}(CH_2)_2\overset{+}{C}HNH_3COO^- \quad (7)$$

$\Delta F_7^\circ = \Delta H_7 = -3600$.

The estimate of zero entropy change in this reaction should be quite reliable, so that we may conclude that the methyl carbonium ion in DMAT is unstable relative to that in MMS to the extent of 3000 or 4000 cal/mole. Comparison of the ionization constants of dimethylglycine ($pK_2' = 9.54$) and the trimethylammonium ion[9] ($pK = 9.79$) suggests that there is very little electrostatic interaction in DMAT between the positive charge and the carboxylate ion. Similarly, the ionization constants[9] of γ-aminobutyric acid ($pK_2' = 10.4$), the ε-ammonium group of lysine ($pK_3' = 10.8$) and the butylammonium ion ($pK' = 10.7$) indicate that in MMS electrostatic interaction between the positive charge and the carboxylate ion may be neglected. On the other hand, we should expect electrostatic repulsion between the positive charges on sulfur and nitrogen in MMS ($pK_1' = 8.6$ for $\overset{+}{N}H_3(CH_2)_3\overset{+}{N}H_3$) to make a positive contribution to the free energy of reaction* (7). Thus, it is difficult to understand the appreciable negative free energy of this reaction.

DMAT and betaine

Substraction of (5) from (1) gives

$$(CH_3)_2\overset{+}{S}CH_2COO^- + (CH_3)_2NCH_2COO^- \rightarrow$$
$$CH_3SCH_2COO^- + (CH_3)_3\overset{+}{N}CH_2COO^- \quad (8)$$

$\Delta F_8^\circ = -10100$.

* Titration of MMS at 0.1 *M* ionic strength and 25° gave a value of $pK_2' = 7.9$. Compared to the value reported for methionine, $pK_2' = 9.21$, this confirms the electrostatic repulsion between the positive charges on N and S.

Thus transfer of a methyl carbonium ion from DMAT to anionic dimethylglycine is strongly exergonic. In this case there should be no significant electrostatic contribution to the free energy. In aqueous solution at neutral pH, dimethylglycine is principally in the form of the zwitterion, and the reaction would be:

$$(CH_3)_2\overset{+}{S}CH_2COO^- + (CH_3)_2H\overset{+}{N}CH_2COO^- \longrightarrow$$
$$CH_3SCH_2COO^- + (CH_3)_3\overset{+}{N}CH_2COO^- + H^+ \qquad (9)$$

This reaction, though endergonic at pH 0, would be exergonic to the extent of 6600 cal/mole at pH 7.

The thermodynamic data for the transmethylation reactions are summarized in Table III. Under approximately physiological conditions (pH 7), all the reactions are strongly exergonic. In particular, the transfer of methyl from DMAT to homocysteine has an unusually large negative standard free energy. Moreover, even the least exergonic reaction, the methyl-transfer from betaine, would appear to be essentially irreversible.

TABLE III

THERMODYNAMIC FUNCTIONS FOR THE TRANSFER OF METHYL GROUPS TO L-HOMOCYSTEINE AT 25° AND 0.1 *M* IONIC STRENGTH

Refer to text for equation of reactions designated by number below. $\Delta F°$ and ΔH in cal/mole; $\Delta S°$ in cal/degree/mole.

Reaction	*pH* = 0			*pH* = 7		
	$\Delta F°$	$\Delta S°$	ΔH	$\Delta F°$	$\Delta S°$	ΔH
(1)	— 5600	— 19.0	— 11 300	— 15 100	+ 12.7	— 11 300
(4)	— 8500	— 1	— 8800	— 8500	— 1	— 8800
(6)	— 2000	— 19.0	— 7700	— 11 500	+ 12.7	— 7700

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