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THE SYNTHESIS OF METHIONINE BY ENZYMIC TRANSMETHYLATION*

III. MECHANISM OF THE REVERSIBLE POLYMERIZATION OF THETIN-HOMOCYSTEINE METHYLPHERASE AND ITS RELATION TO THE MECHANISM OF METHIONINE SYNTHESIS

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SUMMARY

1. Purified horse liver thetin-homocysteine methylpherase undergoes a reversible polymerization reaction in addition to catalyzing the synthesis of methionine by transmethylation from dimethylthetin and homocysteine.

2. The mechanism of the 2 reactions has been investigated in a variety of ways.

3. The reversible polymerization appears to be due to the formation and cleavage of intermolecular disulfide bonds. Separate sites of the protein molecule seem to be involved in the polymerization and the transmethylation reactions.

4. There is no evidence to suggest that a methylated enzyme functions as an intermediate in the transmethylation reaction.

INTRODUCTION

Thetin-homocysteine methylpherase has been purified from horse liver[1] and it invariably has been found to consist of a mixture of polymers of varying molecular weight. Treatment with any one of several thiols is required to transform all of the polymerized

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protein into the constitutive monomer units. The thiol-treated enzyme behaves as a homogenous protein on ultracentrifugal or electrophoretic analysis, and its molecular weight is approximately 200,000.

One feature of this phenomenon which is of particular interest is the fact that homocysteine, one of the substrates of thetin-homocysteine methylpherase, is itself a thiol, and as such capable of depolymerizing the enzyme. Furthermore, as will be shown below, several SH group binding reagents such as *p*-chloromercuribenzoate, N-ethylmaleimide and to a lesser degree iodoacetamide, are capable of inhibiting the activity of the enzyme and also of influencing the polymerization reaction. One question which arises from these observations is whether there are 2 separate sites on the protein molecule involved in these reactions, or whether, indeed, both the polymerization reaction and the catalysis of the transmethylation reaction are functions of the same part of the protein molecule. This communication is concerned primarily with this issue and with some aspects of the mechanisms of both of these reactions.

The data on the mechanism of the enzymic transmethylation do not support the hypothesis that a methylated enzyme functions as an intermediate in the reaction. Studies of the reversible protein polymerization indicate that this phenomenon depends upon the formation and cleavage of intermolecular disulfide bonds. Attempts to show a relationship between the catalytic activity and the polymerization reaction have led to the conclusion that the polymerization reaction involves a site on the protein molecule which is not required or active in the enzymic catalysis.

MATERIAL AND METHODS*

Protein determination

In a previous publication[1] the protein concentration of all crude and partially purified enzyme preparations were estimated by the u.v. absorption method of WARBURG AND CHRISTIAN[2]. Since the absorption spectrum of the purified enzyme indicates that it contains less than 0.5% nucleic. acid, it was thought more desirable to estimate its extinction coefficient at 280 mμ, and to use this value routinely to determine the protein concn. of solutions of the purified enzyme. The extinction coefficient (1 g/l; 1 cm lightpath) at 280 mμ was calculated from the u.v. absorption and the nitrogen content (determined by Dr. W. C. ALFORD, to whom the authors are greatly indebted) of several samples of purified THMenz; assuming a 16% nitrogen content for pure protein a mean value of 1.21 $\pm$ 0.04 was obtained. This value has been used throughout to estimate the protein content of purified THMenz solutions; the values obtained by this procedure are about 27% lower than those previously obtained using the WARBURG-CHRISTIAN method and the figures for specific activity of the purified enzyme are correspondingly higher.

Quantitative estimation of protein SH groups

2 methods were employed to determine quantitatively the SH content of protein

* The following abbreviations are used throughout: THMenz and THMenz* = thetin-homocysteine methylpherase; PCMB = *p*-chloromercuribenzoate; NEM = *N*-ethylmaleimide; DMAT = dimethylacetothetin; MMAA = methylmercaptoacetic acid; GSH = reduced glutathione; SH = sulfhydryl; TCA = trichloroacetic acid; Tris = tris hydroxymethylamino methane.

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solutions. One, the PCMB method of BOYER[3] was performed in potassium phosphate buffer (0.2 *M* and pH 7.0), and the O.D. measured in a Beckman Model DU spectrophotometer at 250 mμ. As the molecular extinction coefficient of the PCMB–SH complex differs in different proteins[4] it had to be determined with each sample of protein by adding to one aliquot an amount of PCMB insufficient to combine with all the SH groups and thus providing a known concentration of the complex. The increment in O.D. observed in the presence of excess PCMB divided by the molar extinction coefficient of the PCMB–SH complex allows calculation of the total concn. of –SH groups in the sample. It was observed that the absorption at 250 mμ rose abruptly upon mixing, but then continued to rise at a much slower and decreasing rate for several hours; increasing turbidity was at least in part responsible for the slow rise. Arbitrarily, therefore, the value obtained 2 min after mixing was used to calculate SH concn.

The other method was the silver ion-tris buffer amperometric procedure of BENESCH AND BENESCH[5] modified only in details. The titration was performed in a 30 ml beaker in a liquid volume of 10 ml. The rotating electrode was powered by a Sargent synchronous motor; the standard electrode was the calomel electrode from a Beckman Model G pH meter. The electrodes were connected to a Leeds and Northrup Electrochemograph with the voltage set at —0.1 V; measured vols. of a standard $AgNO_3$ solution were added at fixed time intervals, and the flow of current recorded. It was found that when the endpoint was determined by the usual method, *i.e.* the point of intersection of the initial and final slopes, the values obtained for SH content of the protein were at least 50 % greater than that obtained by the PCMB method. To investigate this discrepancy standard cysteine solutions were titrated by the Ag^+-tris method, and it was likewise found that the standard method of determining the endpoint resulted in calculated values of the SH content approx. 50 % too high. It was found, however, that if the point at which the current–titrant curve broke from the initial slope was chosen as the endpoint, the values agreed within about 5 % with the known concn. of cysteine. When the latter method of determining the endpoint was applied to titrations of THMenz solutions, the values for SH content checked closely with the results of the PCMB method; thus all results reported herein were calculated in that way. This method of determining the endpoint results in uncertainties sometimes approaching 0.01 μmoles. To overcome this uncertainty multiple determinations were performed and aliquots containing at least 0.1 μmoles of SH groups were used for titration.

Enzyme preparations

2 types of preparations of thetin-homocysteine methylpherase have been used, THMenz and THMenz*. THMenz was prepared as previously described[1]; THMenz* denotes enzyme prepared by a variation of that method differing in the early stages of the procedure (Steps 2 and 3). Specifically the acetone powder was extracted with 0.01 *M* potassium phosphate buffer pH 7.4 instead of water and the dialysis was omitted. The extract was then diluted with 2 vols. of 0.01 *M* potassium phosphate buffer pH 6.1 and 0.03 mg $CaPO_4$ gel/mg protein added. The mixture was stirred, centrifuged, decanted, and the gel discarded. To the supernatant fluid an additional 0.32 mg $Ca_3(PO_4)_2$ gel was added/mg of protein in the original diluted extract. The gel was collected and washed in the usual manner but eluted with 0.2 *M* potassium

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phosphate buffer pH 7.8 at 3° instead of 0.5 *M* disodium phosphate as previously described. Before proceeding to the remainder of the purification procedure the eluate was carfully adjusted to pH 7.3 with dilute HCl. In all other details, the procedure was as previously described[1]. THMenz* preparations appear to be of the same purity as THMenz but differ in some other properties. Relevant to studies reported herein is the fact that THMenz* is uniformly more highly polymerized than THMenz and in addition more readily undergoes extensive repolymerization after conversion to the monomeric form.

Preparation of reagents and substrates

Except as indicated below, all substrates and reagents were commercially obtained, and recrystallized when necessary.

PCMB was purified and standardized spectrophotometrically according to the method of BOYER[3]. L-homocysteine, DMAT, and MMAA were prepared as previously described[1]. [^{14}C]methyl MMAA was prepared as above using $^{14}CH_3I$ obtained from Tracerlab. [^{14}C]methyl DMAT was prepared by first reacting $^{14}CH_3I$ with a small excess of thioglycolic acid in aqueous NaOH as for the preparation of MMAA. Then a 4 to 5-fold excess CH_3I was added and the mixture agitated at 25° for 4 days adding small amounts of conc. NaOH as required to hold the pH to about 6.0. The solution was then extracted repeatedly with ether, and the water evaporated, to yield an oil. To remove excess NaI the oil was dissolved in boiling alcohol, chilled to reprecipitate the DMAT hydroiodine and the alcohol removed by decantation. The oil was then dissolved in aqueous HCl and the water evaporated in vacuo. Finally DMAT was crystallized from hot ethanol (probably as a mixture of the hydroiodide and hydrochloride).

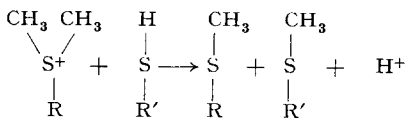
Measurement of radioactivity

Samples for estimation of radioactivity were plated directly in nickel planchets and counted with a Tracerlab thin window Geiger tube with the assistance of a Tracerlab automatic sample changer and superscaler. Correction for the mass of the sample on the plachets was not necessary.

RESULTS

Experiments on the mechanism of the transmethylation reaction

The overall reaction catalyzed by THMenz may be formulated as follows:



(1)

where R = —CH_2—COO^- and R′ = —CH_2—CH_2—$CHNH_3^+$ —COO^-.

The possibility that the reaction proceeds stepwise via the two reactions depicted in equations (2a) and (2b) was investigated experimentally.

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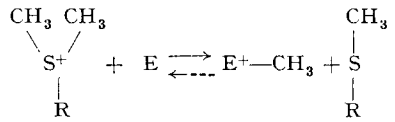
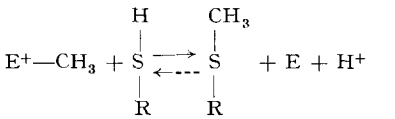


(2a)



(2b)

where E = THMenz.

The sum of equations (2a) and (2b) is identical with equation (1). Though the overall reaction has been shown to be essentially irreversible, this does not exclude the possibility that either of the postulated part reactions is freely reversible, (thus the broken arrows for the reverse reactions).

To test for the reversibility of reaction (2a), a 2.0-ml reaction mixture containing 40 μmoles of DMAT, 6 μmoles of [^{14}C]methyl MMAA (1600 counts/min/μmole), 40 μmoles potassium phosphate buffer pH 7.4, 8 μmoles of neutralized GSH and 3.9 mg (1000 units§) of THMenz was incubated for varying times at 37°. The reaction was terminated by addition of 0.5 ml of 20 % TCA, the mixture centrifuged and the protein-free supernatant fluid decanted. To insure that the physical state of the precipitate was the same in all vessels a short incubation (0.5 min) was used as control instead of the usual zero time type of control in which the enzyme is added after addition of TCA. The protein-free supernatant fluid was mixed with 2 vols. of ammonium reineckate reagent§§, and kept at 2° overnight. The crystalline DMAT reineckate was collected by centrifugation, washed once with 1 % ammonium reineckate, and then with H_2O, decomposed as usual[6] and its radioactivity determined. Control expts. showed that when radioactive DMAT was added to the reaction mixture over 80 % of the added counts could be recovered. As can be seen in Table I incorporation of MMAA into DMAT was insignificant (less than 0.01 μmole). A small contamination (less than 1 %) is present at the earliest time and does not change significantly with incubation. Since MMAA is known to be an inhibitor for the enzyme an expt. identical

TABLE I

NON-INCORPORATION OF [^{14}C]METHYL-MMAA INTO DMAT

For conditions and method of isolation of DMAT see text.

Incubation time min	*Radioactivity of DMAT counts/min**	*% of total ^{14}C***
0.5	68	0.71
15	75	0.78
45	66	0.69

* Calculated for the original 2.0 ml reaction mixture

** Each sample contained 6 μmoles MMAA([^{14}C] methyl) with 9600 counts/min.

§ As previously defined[1] a unit corresponds to the formation of 0.54 μmoles of L-methionine in 15 min under the standard assay conditions.

§§ This was prepared by dissolving 0.15 g of ammonium reineckate in 10 ml of a solvent consisting of 16 parts 0.5 *M* K_2SO_4 and 9 parts 0.5 *M* H_2SO_4, and filtering. The addition of the buffered sulfates was found to favor complete precipitation of the DMAT reineckate.

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with the one just described except for the addition of 12 μmoles of DL-homocysteine was performed as a further control. In 45 min 3.5 μmoles of methionine were formed, indicating that the enzyme was active under these conditions. These data indicate that part reaction (2a), if it occurs at all, is essentially irreversible. Furthermore, no evidence could be obtained to support the possibility that reaction (2a) did in fact occur but was irreversible. This was investigated by attempting to isolate a stable methylated enzyme. The reaction mixture consisted of 2.0 mg of THMenz and 6 μmoles [^{14}C]methyl-DMAT (10,000 counts/min/μmole) in a final volume of 1.3 ml of 0.05 *M*, potassium phosphate buffer, pH 7.6; to half the vessels, 15 μmoles of neutralized GSH were added. After 15 min at 25° the protein was precipitated at 0° by the addition of ethanol to some vessels and of saturated $(NH_4)_2SO_4$ to others. The protein precipitate was redissolved and reprecipitated several times and by the fourth cycle it was essentially free of radioactivity, thus showing that a *stable* methylated enzyme is not formed.

Evidence for the formation of a methylated enzyme was sought also by investigating the reversibility of part reaction (2b). A 0.5-ml reaction mixture containing 5 μmoles of DL-[2-^{14}C]methionine, 2.5 μmoles DL-homocysteine, 10 μmoles potassium phosphate buffer pH 6.8 or 7.6 and 380 units of THMenz, (1.5 mg protein), was incubated at 37° for varying times and the reaction stopped with 0.5 ml of 5% TCA containing 5 μmoles DL-homocysteine. The homocysteine was then separated from methionine either by ascending chromatography in butanol–acetic acid–H_2O (60:15:25) or by precipitation as the silver mercaptide. The latter was accomplished by adding 0.2 ml of 0.10 *M* $AgNO_3$ to the TCA supernatant, which was then allowed to stand overnight at 2°. The precipitated mercaptide was isolated by centrifugation, washed by resuspending twice in 0.02 *M* $AgNO_3$, sedimented, suspended in water, and plated for counting. Neither procedure revealed incorporation of ^{14}C of methionine into homocysteine.

The data, therefore, does not support the reaction mechanism proposed in equations (2a) and (2b); it follows that methyl group transfer from DMAT to homocysteine occurs directly while one or both substrates are bound to the enzyme.

Effect of thiols and SH reagents on the degree of polymerization of THMenz

It has been demonstrated previously that purified THMenz is a mixture of various polymers of a unit with a mol. wt. about 200,000, and that treatment with glutathione or DL-homocysteine transforms all of the enzyme into the monomeric form[1]. It has now been established that a variety of other thiols, such as β-mercaptoethylamine, cysteine, and thioglycolic acid and certain other reducing agents, such as sodium sulfite and potassium cyanide are also effective in depolymerization, whereas methionine, glycine, hydroxylamine, DMAT, PCMB as well as a number of metal chelating agents such as 8-hydroxyquinoline and Versene, are without effect (Table II). A typical expt. is shown in Fig. 1 where the effect of incubating THMenz with 0.1 *M* sodium sulfite at 37° for 15 min is demonstrated.

Parallel results have been obtained in a series of expts. in which the polymerization phenomenon has been studied quantitatively by light-scattering techniques[5]. It may be postulated on the basis of all these results that in the polymer, the monomeric units are held together by intermolecular disulfide bonds. As many of the compounds which can depolymerize the enzyme are known to cleave disulfide bonds by disulfide

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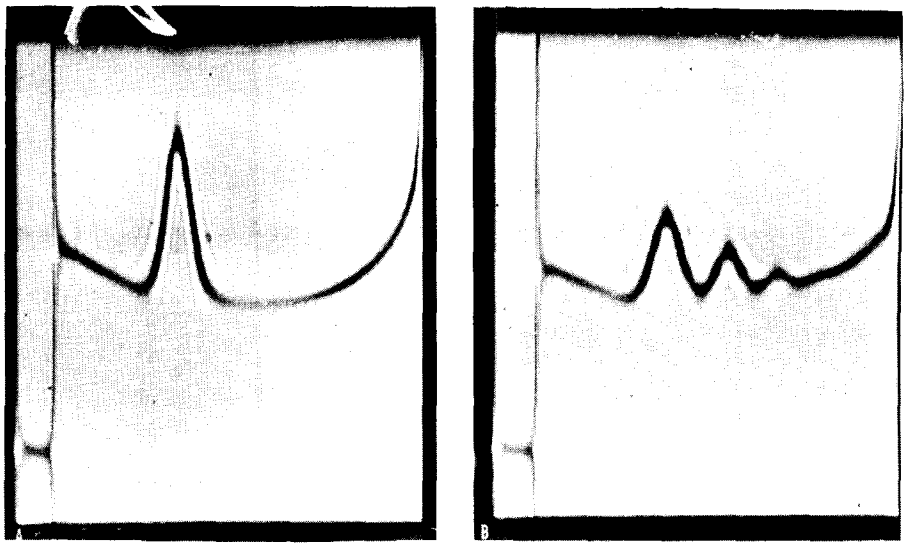

Fig. 1. Effect of sodium sulfite on sedimentation pattern of thetin-homocysteine methylpherase (a) 10 ml of THMenz dialyzed 16 h against 0.05 *M* potassium phosphate buffer, pH 7.4. (b) As above after treatment with 0.1 *M* sodium sulfite, 15 min at 37°. Both solutions were centrifuged in a Spinco Model E, ultracentrifuge at 59.780 rev./min for 32 min. Photographs were taken with a bar angle of 50°.

TABLE II

EFFECT OF VARIOUS REAGENTS UPON STATE OF POLYMERIZATION OF THETIN-HOMOCYSTEINE METHYLPHERASE

In all cases a partially polymerized solution of THMenz of about 1 % concn. was treated with the reagent and then examined in the ultracentrifuge under the conditions described in Fig. 1 and the Schlieren diagrams compared to those before treatment. (—) indicates no detectable change, (+) indicates partial depolymerization and (++) indicates almost complete depolymerization.

Reagent	*concn. (M)*	*pH*	*Minute incubation*	*Temperature*	*Depolymerization*
glutathione	0.016	7.4	20	37	++
DL-homocysteine	0.011	7.4	10	37	++
L-homocysteine	0.015	7.1	15	37	++
D-homocysteine	0.015	7.1	15	37	++
βMEA	0.050	7.4	10	37	++
L-cysteine*	0.005	7.0	10	25	++
sodium thioglycolate	0.015	7.1	15	37	+
$NaHSO_3$	0.100	7.4	15	37	++
KCN	0.011	7.4	10	37	+
methionine	0.018	7.4	10	37	—
glycine	0.20	8.6	120	25	—
DMAT*	0.10	7.0	30	25	—
8-OH quinoline	0.010	7.4	10	37	—
NH_2OH	0.067	7.4	10	37	—
Versene	0.011	7.4	10	37	—
PCMB	0.0006	7.4	10	37	—

* These 2 reagents were studied in collaboration with Dr. R. F. STEINER with light scattering techniques with more dilute protein solutions; the results, therefore, are not strictly comparable with the others but are included for the sake of completeness. The authors are indebted to Dr. STEINER for permission to include this data.

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interchange reactions, it may be postulated further that the depolymerization of the enzyme results from cleavage of these intermolecular disulfide bonds, as described in equation (3).

$$\text{Protein-S-S-Protein} + \text{RSH} \rightleftarrows \text{Protein-S-S-R} + \text{Protein-SH} \quad (3a)$$

$$\text{Protein-S-S-R} + \text{RSH} \rightleftarrows \text{R-S-S-R} + \text{Protein-SH} \quad (3b)$$

$$\text{Protein-S-S-Protein} + 2\ \text{RSH} \rightleftarrows \text{R-S-S-R} + 2\ \text{Protein-SH} \quad (3)$$

It was observed in earlier studies that the extent of polymerization varied in different

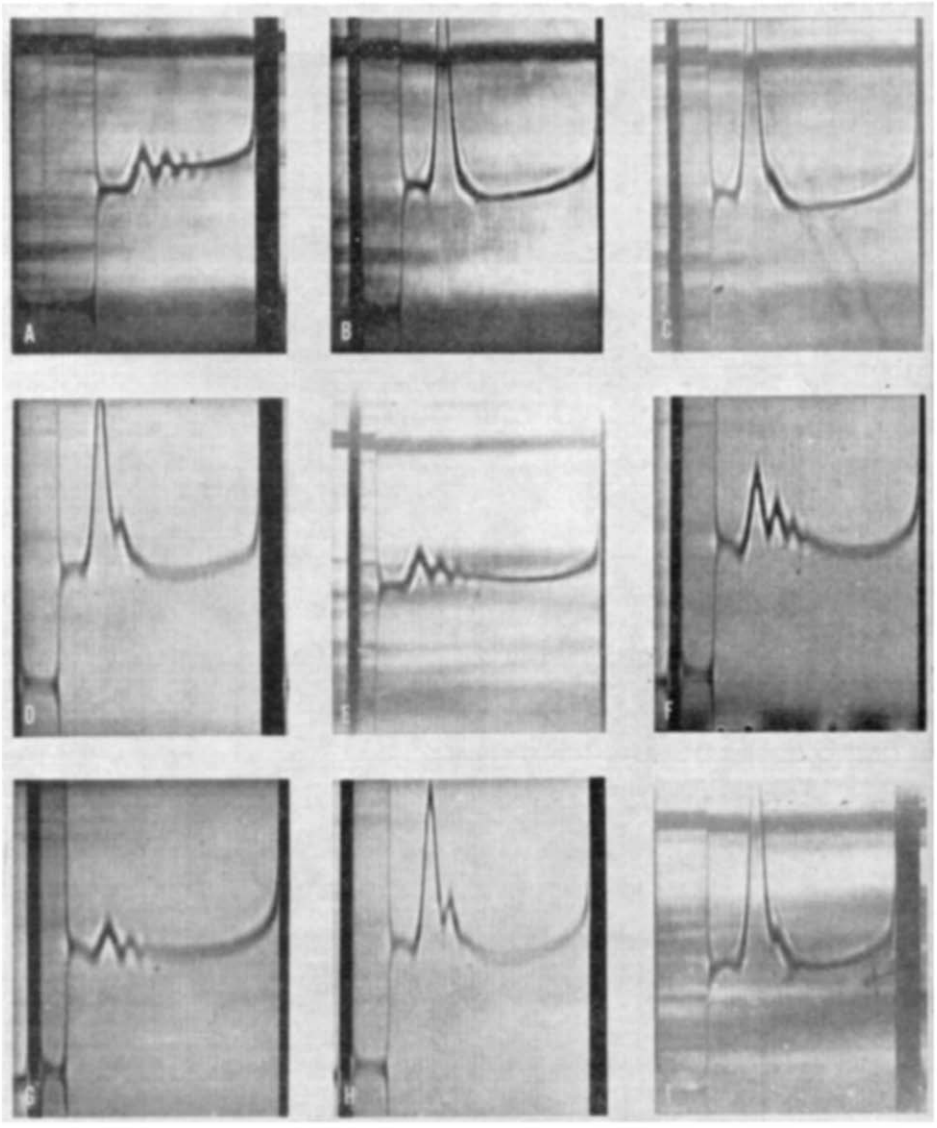

Fig. 2. Effect of various procedures on the sedimentation pattern of thetin-homocysteine methylpherase. See text.

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preparations of THMenz and increased on prolonged dialysis against neutral or slightly alkaline buffer or on reprecipitation of the enzyme by addition of 0.26 g dipotassium phosphate/ml of enzyme solution at approx. pH 9.0. When the latter operation was performed at room temperature, the polymerization sometimes proceeded so far that the bulk of the protein became insoluble.

To study further the conditions favoring polymerization a solution of THMenz* (about 10 mg/ml) (Fig. 2 A) was converted to the monomeric form by treatment with DL-homocysteine, (2 mg/ml at 37° for 14 min) (Fig. 2 B). Next, 10 ml of this preparation was dialyzed at 3° for 28 h in a rocking dialyzer against 0.02 *M* potassium phosphate buffer at pH 7.4 (4·800 ml). At the end of this period comparison with the original material showed that there was a small but definite increase in the amount of dimer present (Fig. 2 C). This solution (which will be referred to later as the *dialyzed monomer*) became only slightly more polymerized by standing 90 h, at 3° (Fig. 2 D). On the other hand when the *dialyzed monomer* was dialyzed as above for an additional 90 h, the enzyme became highly polymerized (Fig. 2 E). Another means of obtaining a considerable increase in polymerized enzyme consisted of incubating the *dialyzed monomer* for 15 min at 37° with $2 \cdot 10^{-3}$ *M* iodosobenzoic acid at pH 7.4 (Fig. 2 F) or with $5 \cdot 10^{-4}$ *M* I_2 (Fig. 2 G). On the other hand incubation of the *dialyzed monomer* under the same conditions of temperature and pH in the absence of any added oxidizing agents was essentially without effect on the sedimentation pattern. (Fig. 2 I; compare with Fig. 2 D). Protein repolymerized by precipitation with dipotassium phosphate or by extensive dialysis may again be depolymerized with mercaptans and retains enzymic activity.

It is particularly interesting that treatment of the monomeric protein with reagents that combine with SH groups blocks the repolymerization. In one expt., the repolymerization of the *dialyzed monomer* upon extensive dialysis was completely prevented by the addition of 10^{-4} *M* PCMB to the dialysis fluid (Fig. 2 H; compare with Fig. 2 E). The sedimentation pattern in this case was not appreciably different from that of the *dialyzed monomer* (Fig. 2 C).

It has also been shown that treatment of the *dialyzed monomer* with $3 \cdot 10^{-2}$ *M* iodoacetamide or NEM (pH 7.4, 30 min, 25°) renders it resistant to repolymerization upon extensive dialysis.

It might be considered that in the above expts., where repolymerization occurred it was due simply to the removal of homocysteine from solution. That this is not a valid interpretation of the results was shown in an expt. in which THMenz was depolymerized with L-homocysteine, dialyzed for 16 h against 0.02 *M* potassium phosphate buffer pH 7.1 (2·1000 ml) to remove excess homocysteine, and subsequently incubated with DMAT under conditions known to favor the utilization of the last traces of L-homocysteine. This treatment did not result in further repolymerization, which, however, was obtained upon extensive dialysis (72 h) against the same buffer.

It may be concluded from these observations that the essential feature required for repolymerization is oxidation of the SH groups on the enzyme. It is clear that treatment with iodosobenzoate or iodine or reprecipitation at alkaline pH favor SH group oxidation. It is not immediately obvious why dialysis should have this result; however, it appears likely that conditions prevailing during prolonged dialysis favor autooxidation of protein SH groups.

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Relation of the degree of polymerization to enzymic activity

The relationship between enzymic activity and the degree of polymerization of the enzyme was investigated in several ways. First it was reasoned that if the "site" involved in the polymerization were the same as the "active site" of the enzyme, substances which are able to depolymerize the enzyme might be expected to be competitive inhibitors of the transmethylation reaction. In fact it was found that GSH, L-cysteine, D-homocysteine and potassium cyanide all of which depolymerize the protein do not inhibit the enzymic synthesis of methionine. Secondly it may be assumed that if the "active site" in the enzymatic transmethylation reaction were involved in the polymerization reaction, it would be expected that the polymerized enzyme should have a specific activity lower than the monodispersed form. Determination of the specific activity of the polymer by direct assay is impossible, however, under the usual conditions of the enzymic assay, because the enzyme is very rapidly depolymerized by homocysteine. In order to circumvent this difficulty, 2 types of experimental procedures were employed; both depended on the finding that the rate of thiol-induced depolymerization, although rapid at 37° was markedly reduced at lower temperatures and at low SH concentration*. One type of experimental approach used depends upon an analysis of the kinetics of the reaction. If the polymer were less active than the monomer, it would be expected that the enzymic reaction catalyzed by highly polymerized enzyme should exhibit an initial lag phase. However, as shown in Fig. 3, no lag could be demonstrated even when the reaction was run at 1° with low homocysteine concn.

A kinetic approach of this type is open to the objection that the failure to demonstrate an initial lag in the reaction rate might have resulted from choice of inappropriate experimental conditions. A second type of expt. was therefore undertaken to compare more directly the enzymic activity with the degree of polymerization. A 0.8 % solution of highly polymerized THMenz* was used, and its activity for short-term incubations at 0° was determined both with and without a 10-min preincubation at 37° with L-homocysteine. To determine the activity of the highly polymerized enzyme, expts. were performed in 5 ml graduated cylinders containing 0.35 ml of enzyme solution, 0.2 ml of neutralized 0.2 *M* DMAT and 0.02 ml of 1.0 *M* potassium phosphate buffer pH 7.4 and the mixture was equilibrated in an ice bath. The reaction was started by the addition of 0.1 ml of cold 0.014 *M* L-homocysteine in phosphate buffer as above and the mixture was shaken in the ice bath; at the desired time the reaction was stopped with the addition of 0.4 ml 2.0 *M* NaOH. Under these conditions (low temperature, high enzyme and low homocysteine concns.) it would be expected that no appreciable depolymerization would occur during the course of the incubation. This expectation was borne out by ultracentrifugal analysis which showed that indeed very little depolymerization took place during the reaction. In parallel expts. 0.35 ml of enzyme were mixed with 0.12 ml of the same L-homocysteine solution (A larger quantity of homocysteine was used in the preincubated vessels to compensate for the oxidation of homocysteine which occurs during the preincubation period. That this more than compensates for the loss of homocysteine is demonstrated by comparing lines 3 and 6 of Table III. This shows that if the rection is allowed to proceed to completion (30 min), more methionine is formed in the vessel that has

* R. F. STEINER AND J. DURELL, (To be published).

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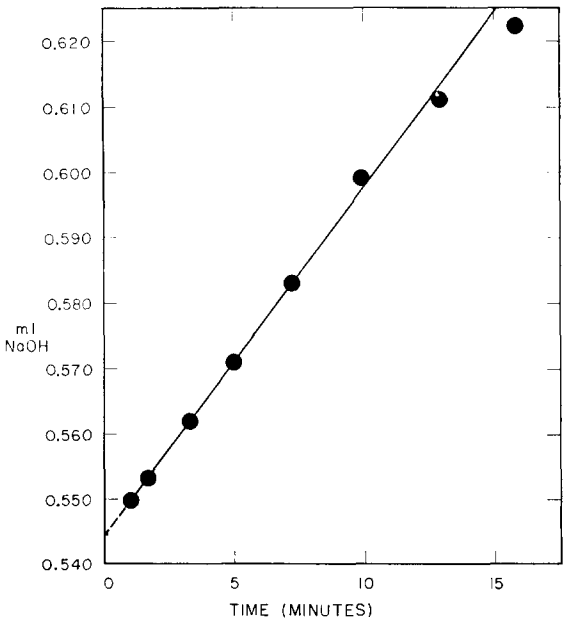

Fig. 3. Rate of methionine synthesis catalyzed by highly polymerized THMenz at 1° with low L-homocysteine concn. A 30-ml beaker containing 12.2 ml of reaction mixture was placed in a circulating ice bath which maintained a temperature of 1° within the beaker; the solution was stirred and the pH monitored with the external electrodes of a Beckman Model G pH meter. The reaction mixture contained 500 μmoles DMAT, 500 μmoles NaCl, and 7.0 μmoles L-homocysteine. At zero time 2 ml of highly polymerized THMenz was added; this had been dialyzed for 24 h. against 0.05 *M* NaCl and contained 5.5 mg of protein (1150 Units)/ml. The null point of the pH meter had been set at pH 7.7 and a small quantity of 0.041 *M* NaOH was added from a microburette to bring the pH of the solution slightly above the null point. The time was recorded when the needle passed the null point, and then a small quantity of NaOH was again added, the process being repeated until the rate of acid liberation slowed considerably (30 min). The data for the first 16 min are plotted above, and the first 12 min fit a straight line without significant deviation.

been preincubated, indicating that more homocysteine is present at the onset of the enzymic reaction.) used above and the mixture incubated 10 min at 37° before returning it to the ice bath; under these conditions appreciable depolymerization took place, the concn. of the monomer increasing considerably. In this case after equilibration at 0° the transmethylation reaction was started with the addition of 0.2 ml of the DMAT solution and then stopped as above at the desired times. The results, summarized in Table III, show that the initial rate of methionine synthesis not only was not appreciably faster following preincubation of the enzyme with homocysteine, but in fact was 10 to 15 % slower. A comparable decrease in enzyme activity (5–10 %) was also observed in control expts. in which after the 10-min preincubation at 37° with homocysteine an aliquot of enzyme solution was removed and assayed at 37° in the routine manner. Therefore it appears that the incubation with homocysteine which markedly alters the physical state of the protein has essentially no effect on its enzymic activity. The combined evidence from the expts. just described clearly indicates that the degree of polymerization has little or no effect on the enzymic activity.

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TABLE III

EFFECT OF DEPOLYMERIZATION UPON ENZYMIC ACTIVITY OF THETIN-HOMOCYSTEINE METHYLPHERASE

THMenz in samples 1, 2 and 3 was highly polymerized and contained little monomer. The enzyme in samples 4, 5 and 6 was considerably less polymerized and the monomer was the largest single component. The experimental conditions are described in the text.

Sample No.	*Preincubation with homocysteine*	*Incubation time min*	*Methionine formed μmoles*	*Rate of methionine formation μmoles/min*
1	—	1.5	0.42	0.28
2	—	3.5	0.74	0.21
3	—	30	1.35	
4	+	1.7	0.42	0.25
5	+	3.5	0.64	0.18
6	+	30	1.51	

The estimation of SH groups of THMenz

Measurement of the SH groups of the same solution of THMenz* used in the expt. depicted in Fig. 2 yielded an average of 12.6 ± 0.3 μmoles SH/200 mg protein. Data to be reported subsequently indicate that the monomer mol. wt. of THMenz is in the order of 200,000. Therefore SH determinations are reported per 200 mg of protein, since this value is believed to approximate 1 μmole of protein. After treatment with DL-homocysteine and dialysis for 28 h against several changes of buffer there was an increase of the titrable SH to 13.8 ± 0.5 μmoles/200 mg protein. While the difference is greater than the experimental error it is difficult to know whether it is meaningful, especially since it has not been ascertained that homocysteine had been completely removed by dialysis. However, this expt. is significant in that it sets an upper limit of 2 moles of SH/mole of monomer to the difference in SH content of the polymerized (Fig. 2 A) and depolymerized proteins (Fig. 2 C).

Relation of enzymic activity to SH content of THMenz

In preliminary expts. it was established that preincubation of THMenz with $3 \cdot 10^{-3}$ *M* PCMB at 25° for 15 min and subsequent dilution 1:300 for assay resulted in total loss of enzymic activity. The PCMB treated enzymes could be reactivated by treatment with GSH and certain other thiols but not homocysteine. In more detailed studies, the SH groups of THMenz were titrated with PCMB, and the enzymic activity measured at various stages of the titration. The results, shown in Table IV indicate that the percentage of the enzymic activity retained is roughly equal to be percentage of SH groups remaining free.

The effect of other compounds known to react with SH groups through oxidation or alkylation, was also investigated. For this purpose THMenz* was incubated for 15 min at 25° with iodosobenzoate ($1 \cdot 10^{-3}$ *M* pH 7.4) or iodoacetate ($3 \cdot 10^{-2}$ *M* pH 7.0); there was no decrease of enzymic activity. With iodoacetamide ($2.5 \cdot 10^{-2}$ *M*, pH 7.4) there was a 15–20 % decrease in activity. A determination of the SH content, with the PCMB method, revealed that treatment with iodoacetamide or iodosobenzoate had resulted in a 15 % decrease of titratable SH groups. This change is equiv. to the loss of somewhat less than 2 μmoles SH/200 mg protein.

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TABLE IV

EFFECT OF PCMB ON ENZYMIC ACTIVITY OF THETIN-HOMOCYSTEINE METHYLPHERASE

Each sample contained 0.74 mg of THMenz in 4.0 ml of a solution of 0.05 *M* potassium phosphate buffer, pH 7.0.The required amount of PCMB was added and the final volume brought to 4.2 ml with H_2O. This operation was performed in an ice bath at 0°. All samples with the exception of 1 were immersed in a water bath at 25°, held at this temperature for 10 min, and then returned to the ice bath. For assay 0.05-ml aliquots were added to 0.6 ml reaction mixture containin DMAT, 0.033 *M*; DL-homocysteine 0.011 *M*; potassium phosphate buffer 0.022 *M*; pH 7.4 and NaCl 0.033 *M*, and incubated at 37° for 20 min.

Sample No.	*PCMB added µmoles*	*SH free µmoles*	*SH free %*	*Enzymic activity*	
				Units/ml*	*% of original*
1	0	0.046	100	33.1	
2	0	0.046	100	33.7	101
3	0.0139	0.032	70	20.2	61
4	0.0278	0.018	39	11.6	35
5	0.0418	0.004	9	5.9	18
6	0.0557	0	0	3.5	10

* 1 unit = 0.72 µmole of methionine in 20 min.

Since the enzyme appeared quite resistant to these SH reagents and since NEM in certain cases appears to be more potent as an alkylating agent, its effects were investigated. The protein was treated at pH 7.4 with $4 \cdot 10^{-2}$ *M* NEM. It was then incubated at 25° and at 37°, and aliquots were removed periodically; the extent of alkylation was determined by Ag^+ titration and related to enzymic activity. The results (Table V) clearly show that the enzyme is much more susceptible to NEM than to the other alkylating agents. It is also of interest that in contrast to the results obtained with PCMB, the enzymic activity decreases relatively more rapidly than the number of free SH groups.

These results indicate that at least some of the SH groups of the protein are required for enzymic activity. It should be pointed out, however, that the results

TABLE V

EFFECT OF NEM ON ENZYMIC ACTIVITY OF THETIN-HOMOCYSTEINE METHYLPHERASE

No.	*Time of incubation at*		*Titrated SH µmoles/ml*	*SH free %*	*U/ml × 10^{-3}*	*Initial activity %*
	25°	*37°*				
	min					
1	0	0	0.57	100	1.79	100
2	4	0	0.47	84	1.38	77
3	23	0	0.36	64	0.64	36
4	24	15	0.29	51	0.27	15

0.4 ml of 0.1 *M* NEM was added to a solution containing 9.0 mg of THMenz in 0.60 ml of 0.35 potassium phosphate buffer, pH 7.4. The solution was incubated for 24 min at 25° and then for 15 min at 37°. At the indicated times 0.20-ml aliquots were removed to 10 ml of buffer for SH group titration with the Ag^+ method. Almost simultaneously 0.05-ml aliquots were removed to 3.0 ml of an ice cold solution containing 5 µmoles of DL-homocysteine in 0.05 *M*, potassium phosphate buffer pH 7.6; these samples were kept at 0° until ready for assay, and then 0.05 ml aliquots were added to a reaction mixture as in Table IV. A control sample, not shown, incubated under conditions identical with above but without NEM, retained 93% of the activity and there was no decrease in titrable SH.

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obtained do not distinguish between loss of activity due primarily to the combination of thiol groups with the PCMB or NEM, and loss of activity resulting less directly from any changes in the structure of the protein that may be caused by the reaction with the free SH groups.

DISCUSSION

The data reported above indicate that the SH groups in this protein are of particular significance in both of the reactions under investigation. Although in a μmole or 200 mg of protein there are some 12 to 14 SH groups it is evident that they are not all equiv. since they differ both in their reactivity towards various SH reagents and in their influence upon the 2 reactions. As far as the reactivity of the SH groups is concerned, it is clear that different SH reagents affect them differently. For instance the results indicate that a few (and perhaps only 2) of the SH groups of the protein molecule are readily accessible to alkylating and oxidizing agents. It appears that the alkylation (or oxidation) of these particular SH groups has little or no effect on the enzymic acitivity although it prevents the subsequent repolymerization of the protein upon dialysis. With PCMB on the other hand, it is assumed that all of the SH groups undergo reaction, and in this case the decrease in enzymic activity roughly parallels the decrease in free protein SH groups; the reaction with PCMB also results in a loss of the capacity to undergo repolymerization on dialysis. Finally with NEM, a more subtle differentiation between the various SH groups has been obtained since conditions under which about 50 % of the titratable SH could be alkylated, (the remaining 50 % being resistant to NEM) were sufficient to cause almost complete inactivation of the enzyme.

Any interpretation of these results requires a number of extrapolations which may or may not be justified. As suggested by MADSEN AND GURD[8] in their study of interaction of PCMB with muscle phosphorylase "all thiol groups of a given protein molecule cannot be equally reactive because the first one, or the first few ones which react facilitate the reaction of the remainder". Thus, one possible interpretation of the results obtained with PCMB, essentially analogous to that suggested for muscle phosphorylase, would propose that the partially titrated protein consists of a mixed population of completely combined and completely uncombined molecules; at various stages in the titration, the mixture would differ only in the relative proportions of the 2 species.

With regard to the polymerization phenomenon the combined evidence presented above favors the hypothesis that the polymerization occurs through the formation of intermolecular disulfide bonds. The formation of such bonds in soluble proteins has been described only rarely under circumstances approaching physiological conditions[9–11]. Studies on mercaptalbumin[12] indicated that intermolecular disulfide bonds could be formed only by oxidation of the mercury dimer; attempts at direct oxidation of the serum albumin in the absence of mercuric ion did not result in the formation of intermolecular disulfides. Gelation of various proteins through the formation of intermolecular disulfide bonds by disulfide interchange reactions has been described by other workers[13,14] but only when experimental conditions were designed to denature the protein (6–8 *M* urea). It is not yet known whether the polymerization of THMenz occurs through the direct oxidative formation of *inter-*

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molecular disulfide bonds, or through disulfide interchange reactions following the formation of *intramolecular* disulfide bonds. In either case, however, the relative ease of polymerization of THMenz as compared to other soluble proteins might be due to the presence of one or more specially reactive SH groups. Because the SH group of an N-terminal cysteine residue would be expected to be considerably more reactive than the SH groups of other cysteine residues[15] it is possible that the polymerization phenomenon depends upon one or more such cysteine residues. The same SH groups would be expected to combine most readily with alkylating agents, perhaps explaining why iodoacetamide can block repolymerization while alkylating only 15 % of the proteins SH content.

Since the polymerization reaction appears unrelated to the enzymic activity, its possible biological significance remains obscure. At present it is not possible to say whether the enzyme is present in the cell as monomer or polymer. Assuming that this reversible polymerization may occur intracellularly, one wonders whether it could be of any structural significance. As noted earlier[1] the THMenz isolated from horse liver represents in the order of 1 % of the liver proteins, suggesting that this large amount may serve some cytostructural function. It is interesting to note that the spindle apparatus of sea urchin eggs appears to be held together by intermolecular disulfide bonds formed *in vivo* by a similar disulfide exchange reaction[16].

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