

METHIONINE FORMATION BY TRANSMETHYLATION IN VITRO

BY HENRY BORSOOK AND JACOB W. DUBNOFF

(From the William G. Kerckhoff Laboratories of the Biological Sciences, California
Institute of Technology, Pasadena)

(Received for publication, February 8, 1947)

We reported in a previous communication (1) that guanidoacetic acid is methylated to creatine by rat liver slices in the presence of choline and either homocysteine or homocystine. Choline, homocystine, or homocysteine alone had little or no effect. These observations closed the gap which had existed between the evidence from tissue slice experiments on the one hand (2) and that from experiments *in vivo* on the other (3-5) regarding the transfer of choline methyl in the formation of creatine.

Two transmethylation reactions, one in which methionine, the other in which creatine is formed, were thus exposed for study. We had observed that the rate of methylation of guanidoacetic acid by choline is as fast or faster with homocystine than with homocysteine as the methyl carrier (1). This was surprising, as it might have been expected, *a priori*, that homocysteine would be the more effective methyl carrier, and it suggested that the two transmethylation reactions may have different characteristics. We had found an indication earlier (2) that the methylation of guanidoacetic acid by methionine is not a simple methyl transfer between the two substances in that the reaction is inhibited when respiration is inhibited, whether by anaerobiosis or by oxidation inhibitors. We undertook a study of methionine formation by transmethylation in rat liver and kidney slices and homogenates to obtain more information on this question, and also because of the physiological significance of the reaction in itself, to which no *in vitro* studies had as yet been devoted.

Methods

The animals used were adult white rats which had been bred from the Wistar Institute strain. They were killed by stunning; the liver and kidney tissue were sliced free-hand with a straight edge razor to an average thickness of 0.4 mm. The slices were rinsed in Krebs and Henseleit (6) Ringer's solution without the bicarbonate and finally suspended in the same Ringer's solution with such a concentration of bicarbonate that the pH, when equilibrated at 38° with 5 per cent carbon dioxide, was 7.4. The reactants were dissolved in the latter version of the Krebs-Henseleit bicarbonate Ringer's solution.

The reaction vessels were 20 ml. beakers. They were placed in a closed chamber which was partially immersed in a water bath. The chamber was provided with a gas inlet tube through which the gas mixture was passed under slight pressure; the excess gas escaped under water through a slit in the side. The chamber could hold thirty beakers. When the latter were in place, the gas mixture was blown through vigorously for 10 minutes, after which it was slowed down. Through the period of gassing and afterwards the chamber containing the beakers was rocked at a rate of about 80 cycles per minute. By prior tests it was ascertained that the evaporation from the reaction vessels in a 4 hour run was negligible. The temperature of the water bath was 38°.

When homogenates were used, the tissue was removed and, without chilling, cut into small pieces and homogenized in the apparatus of Potter and Elvehjem (7) in isotonic phosphate-Ringer's solution (6) at pH 7.4. The homogenate was strained through two layers of cheese-cloth.

The lyophilized enzyme was prepared as follows. The tissue was sliced, the slices rinsed as described above, and then frozen on a block of solid carbon dioxide, transferred to flasks previously cooled to the temperature of solid carbon dioxide, and then dried under a high vacuum with the water trap kept in a bath of solid carbon dioxide in methyl cellosolve (Mefford). The thoroughly dried tissue was pulverized.

The first experiments were carried out with preparations of DL-homocysteine and of DL-homocystine, generously given us by Professor V. du Vigneaud.

Later it seemed desirable to use the natural isomers. The starting material for their preparation was L-methionine obtained from casein by the method of Hill and Robson (8) with the following modifications. After the usual acid hydrolysis, removal of excess HCl, adjustment of the pH to 2.4, decoloration with charcoal and separation of the tyrosine, the pH was adjusted to 6.0 and the iron (presumably in the HCl and NaOH used) was removed by treatment with H₂S. The removal of the iron clarified the solution further and facilitated the crystallization of the leucine and methionine. Methionine was separated from the leucine by the mercury precipitation procedure described by Hill and Robson; after removal of the mercury the solution of impure methionine hydrochloride was concentrated nearly to dryness, dissolved in alcohol, and the solution brought to pH 6.0 with concentrated NaOH instead of with pyridine, as recommended by Hill and Robson, and set away overnight in the ice box. Methionine which had crystallized out was removed, the mother liquor concentrated, alcohol added to 80 per cent concentration, the pH adjusted to 6.0 with concentrated NaOH, and set away in the ice box overnight. The second crop of methionine thus obtained was added to the first. The methionine

was purified further by a second mercury precipitation as described by Hill and Robson, and recovered from the mercury precipitate by the same procedure as after the first mercury precipitation. The methionine was purified finally by two precipitations with alcohol from water.

L-Homocystine was prepared from L-methionine by the method of Butz and du Vigneaud (9), as modified by du Vigneaud and Patterson (10).

L-Homocysteine was prepared from L-homocystine by reduction with sodium in liquid ammonia, following the procedure of Riegel and du Vigneaud (11).

Choline hydrochloride and betaine hydrochloride were used from several commercial sources. Dr. N. Horowitz kindly supplied specimens of the following compounds used: di- and monomethylethanolamine, monoethylmethyl- and diethylmonomethylhydroxyethylammonium chloride, arsenocholine, phosphorylcholine, and homocysteinethiolactone.

Methionine was determined by an adaptation of the method of McCarthy and Sullivan (12) which made the method more sensitive but somewhat less specific. The reduction in specificity was not significant under our experimental conditions, *i.e.* Ringer's solution in which tissue slices or liver homogenates had been incubated and in which the methionine did not exceed 10 mg. per cent and the total of other amino acids 10 mg. per cent. The procedure was as follows: to 4 ml. of Ringer's solution containing the slices were added 0.4 ml. of 30 per cent trichloroacetic acid; 2 ml. of filtrate were transferred to a 20 ml. beaker; 0.2 ml. of 5 N NaOH followed by 0.1 ml. of 1 per cent freshly made sodium nitroprusside were then added. The resulting solution was warmed with gentle rocking in a water bath at 38° for 8 minutes, after which it was cooled in ice water until the temperature was below 4°, and then 1 ml. of an acid mixture consisting of 9 volumes of concentrated HCl and 1 volume of 85 per cent H₃PO₄ was added with shaking. The beaker was again placed in ice water for 1 to 2 minutes, after which it was warmed to room temperature and then transferred to a vacuum desiccator in which the CO₂ was removed by 3 minutes suction. The color was then measured at 510 m μ in a Beckman spectrophotometer. Methionine standards were run simultaneously with the unknowns; the standard solution containing no methionine was used as the blank in the spectrophotometer. A straight line relation is obtained between the logarithm of the transmission and the concentration of methionine over the range, 0 to 10 mg. per cent.

The method was tested by incubating known amounts of added methionine in Ringer's solution with liver slices for 4 hours at 38°. The assay gave 98 per cent of the added methionine over a range of 0 to 10 mg. per cent after deducting the methionine liberated by the slices. The deficiency of 2 per cent was probably the result of oxidation of the added methionine.

The above modification of the McCarthy-Sullivan method for the determination of methionine may be used only when the total concentration of non-methionine amino acids is of the order of 0.001 M or less. When the latter are present in higher concentration, the original recipe of McCarthy and Sullivan is advisable.

*Results*¹

In Table I are summarized the results of experiments which show the formation of methionine by rat liver slices from homocysteine or homocystine and a suitable methyl donor. Larger yields of methionine were obtained under low oxygen tension (4 per cent O₂), or anaerobically, than under 95 per cent O₂, from homocysteine than from homocystine, whether under aerobic or anaerobic conditions, and with betaine than with choline as the methyl donor. The superiority of betaine over choline was greatest under anaerobic conditions and least under 95 per cent oxygen. The effect of oxygen tension on the yield of methionine was far less when choline was the methyl donor than when betaine was used.

The following facts offer a qualitative and at least partial explanation of the higher yields of methionine under nitrogen than under oxygen and of the greater relative effect of anaerobiosis with betaine than with choline as the methyl donor. Homocysteine is more stable under nitrogen than under oxygen, presumably because it is protected from oxidation.² Choline is rapidly oxidized by rat liver slices and homogenates to betaine on the alkaline (pH 7.8) and to betaine aldehyde on the acid (pH 6.7) sides of neutrality (13, 14). In its oxidation to betaine choline becomes a more effective methyl donor for methionine formation. Aerobiosis and anaerobiosis thus have opposite effects on the methyl donor and methyl acceptor when choline is the methyl donor; hence the small difference in the yield of methionine under the two conditions. Betaine is not oxidized by rat liver

¹ Nearly all the results reported here were first obtained with the DL forms of homocysteine and of homocystine. The experiments were repeated with the natural isomers, and, as there were no essential differences between the two sets of experiments, only those of the latter are reported.

² That homocysteine is protected by anaerobiosis was shown by the difference in the intensity of the transitory purple color after the addition of alkali and nitroprusside in the first stage of the methionine determination. In the experiments under 95 per cent oxygen with homocysteine the purple color was faint, and deep when they were conducted under nitrogen, even though more homocysteine was converted to methionine in the latter solutions. Similar results were found with homocystine; no purple color was seen after experiments under 95 per cent oxygen, a faint purple after the anaerobic experiments. In the latter case the color was less intense than when homocysteine was used under the same conditions. The purple color given by homocystine in the anaerobic experiments was qualitative evidence that it was reduced to homocysteine.

slices or homogenates (13, 15). The greater yields of methionine under anaerobic conditions with betaine are then to be ascribed to the greater stability of the methyl acceptor. This explanation is offered only pro-

TABLE I

Methylation of L-Homocysteine and of L-Homocystine by Choline and by Betaine

Rat liver slices; 4 hours, 37.5°. The results are expressed as micrograms of methionine per 100 mg. of tissue (dry weight).

Composition of gas mixture	95 per cent O ₂ + 5 per cent CO ₂			91 per cent N ₂ + 4 per cent O ₂ + 5 per cent CO ₂		95 per cent N ₂ + 5 per cent CO ₂	
	28.8	19.1	9.6	19.1	9.6	19.1	9.6
Initial concentration of methyl donor, mg. %							
Tissue alone	75	75	75	92	55	72	119
“ + homocysteine	104	104	104	259	207	183	223
“ + “ and choline	207	167	147	362	338	279	271
Tissue + homocysteine and betaine	254	215	163	716	470	1019	721
Tissue + homocystine	75		75	194	107	92	167
“ + “ and choline	107		107	239	211	139	215
Tissue + homocystine and betaine	115		115	279	235	633	525
Tissue + choline	75	75	75	95	55	72	119
“ + betaine	75	75	75	92	55	68	124

Each of the figures in the table is the average of a triplicate. L-Homocysteine and L-homocystine were used. The initial concentration of each was in every case 12.5 mg. per cent, and the volume of the reaction mixture was 4 ml. In any one experimental run the gas mixture and the concentration of the methyl donor were the same; the slices were obtained from the liver of one animal or from the pooled slices of the livers of two animals. The dry tissue weight in each reaction vessel was within the range, 15 to 30 mg. A number of experiments were carried out to observe the effect of varying the oxygen tension in the gas mixture, with slices from one animal. The results were essentially the same as those shown above; they were not selected for inclusion in Table I because, for physical reasons, the number of controls were limited, and the homocysteine and homocystine experiments had to be run separately in the latter experiment.

visionally; it needs the support of data on the rates of all the reactions and side reactions involved.

In most, but not all, experiments there was an increase in methionine over the tissue blank when only homocysteine or homocystine was added, *i.e.* not accompanied by an additional methyl donor. Presumably the methylation was effected by methyl donors in the liver slices; these were not removed even by washing the slices in Ringer's solution for $\frac{1}{2}$ hour.

In our studies on the methylation of guanidoacetic acid (1, 2) we found a considerable methylation by liver slices, even when no methionine was added. Until a sufficiently sensitive method for the determination of methionine was available, we were uncertain whether this "tissue" methylation might be ascribed to methionine liberated by the liver slices during the period of incubation or whether it was necessary to invoke some other methyl donor. The method for the determination of methionine used in this study was sufficiently sensitive. With it we have found that rat liver slices liberated methionine, under the conditions of the experiments on the methylation of guanidoacetic acid, in concentrations which ranged from 0.2 to 1.0 mg. per cent; in 4 hours the total free methionine increased usually to about 3 times the zero time value. As the "tissue" methylation of guanidoacetic acid could be accounted for by a free methionine concentration of 3×10^{-5} M, *i.e.* approximately 0.5 mg. per cent, the methionine liberated by rat liver slices is sufficient, therefore, to account for the rates of methylation of guanidoacetic acid observed when no methionine is added to the Ringer's solution.

Cyanide, azide, arsenite, and arsenate do not inhibit the methylation of homocysteine or of homocystine (Table II). In fact arsenite increased the yield of methionine even under 4 per cent oxygen. The non-susceptibility of this transmethylation to oxidation inhibitors is in accord with its independence of oxygen.

The possibility suggested itself, in view of the superiority of betaine to choline as a methyl donor in methionine formation, that the methylating action of choline may be via betaine; *i.e.*, that betaine is the immediate methyl donor to homocysteine or homocystine and that the methyl group lost by such a donation is replenished by choline. The possibility was tested in a number of experiments in which varying small amounts of betaine were added to the reaction mixture containing the methyl acceptor and choline. The choline concentration in these experiments was 3 times the molecular equivalent of the homocysteine and 6 times that of homocystine; the betaine concentration was varied from one-fifth to one-hundredth that of the choline. The results obtained argue against a carrier function of betaine. The effect of choline plus betaine was always less than the sum of their separate effects.

In addition to betaine and choline a number of other possible methyl donors were tested. As Table III shows at the end of the 4 hour incubation with liver slices at 38°, no more and in some cases less methionine was found than when the methyl acceptor alone was added to the Ringer's solution. The other possible methyl donors tested were dimethylethanolamine, monomethylethanolamine, diethylmethylhydroxyethylammonium chloride, monoethyldimethylhydroxyethylammonium chloride, arsenocholine, and phosphorylcholine.

These results illustrate one of the limitations of the tissue slice technique. The tissue slices contained significant amounts of methyl donors, even after being washed $\frac{1}{2}$ hour in large amounts of Ringer's solution. The failure of the five possible methyl donors other than betaine or choline to add, under our experimental conditions, to the rate of methylation effected by the methyl donors in the slices does not, in itself, indicate that they could not

TABLE II

Effect of Oxidation Inhibitors on Methylation of L-Homocysteine and of L-Homocystine by Choline and by Betaine

Rat liver slices; 4 hours, 37.5°. The results are expressed as per cent of the methionine found after addition of homocysteine plus choline in the absence of an oxidation inhibitor.

Composition of gas mixture Inhibitor	95 per cent O ₂ + 5 per cent CO ₂					91 per cent N ₂ + 4 per cent O ₂ + 5 per cent CO ₂			
	None	Cyanide	Azide	Arsenite	Arsenate	None	Azide	Arsenite	Arsenate
Tissue alone	46	50	44	28	35	21	15	4.5	19
" + homocysteine and choline	100	116	99	163	100	100	75	145	114
Tissue + homocysteine and betaine	115	121	103	324	119	112	87	155	121
Tissue + homocystine and choline	61	67	63	78	63	59	57	70	52
Tissue + homocystine and betaine	64	74	66	113	75	65	60	81	61

L-Homocysteine and L-homocystine were used at a concentration of 12.5 mg. per cent. The volume of the reaction mixture was 4 ml. In the experiments with 95 per cent oxygen the concentration of methyl donor was 28.8 mg. per cent and with 4 per cent oxygen, 9.6 mg. per cent. The oxidation inhibitors were used in the form of their sodium salts and at a concentration of 0.001 M. In every experiment the controls consisted of tissue alone, tissue plus homocysteine, and tissue plus homocystine; the controls and the different reaction mixtures, all in triplicate, were run simultaneously in the absence and in the presence of one of the above oxidation inhibitors.

do so *in vivo*. One possibility is that under our experimental conditions the above substances are not transformed to betaine or to choline fast enough to make their contribution to that of the methyl donors in the slices noticeable.

The results summarized in Table III are in accord with those on growth (4, 16) in that none of the compounds which were found to be incapable of supporting growth with homocystine exerted any additive methylating effect under our experimental conditions. Monoethyldimethylhydroxyethylammonium chloride and phosphorylcholine will support growth with homocystine presumably because they are converted to choline (or

betaine) *in vivo*; they exerted no significant additive methylating effects in our experiments because, presumably, they were not converted to betaine or to choline sufficiently rapidly to augment noticeably in short period experiments the effect of the methyl donors in the slices.

TABLE III

Relative Rates of Methylation of L-Homocysteine, L-Homocystine, and of Homocysteine-thiolactone by Various Possible Methyl Donors

Rat liver slices; 4 hours, 37.5°. Excess methionine over tissue blank with homocysteine alone taken as 100.

Composition of gas mixture	95 per cent O ₂ + 5 per cent CO ₂	91 per cent N ₂ + 4 per cent O ₂ + 5 per cent CO ₂	95 per cent N ₂ + 5 per cent CO ₂
Homocysteine	100	100	100
“ + choline	217	220	270
“ + betaine	347	633	1905
“ + dimethylethanolamine	101	95	90
“ + monomethylethanolamine	65	99	93
“ + diethylmonomethylhydroxyethylammonium chloride	119	91	-20
Homocysteine + monoethyldimethylhydroxyethylammonium chloride	150	100	-120
Homocysteine + arsenocholine		76	
“ + phosphorylcholine		94	
Homocystine	55	51	60
“ + choline	123	102	104
“ + betaine	165	180	208
“ + dimethylethanolamine	-42		27
“ + monomethylethanolamine	-138		-240
“ + diethylmonomethylhydroxyethylammonium chloride	-138		0
Homocystine + monoethyldimethylhydroxyethylammonium chloride	54		-20
Homocystine + arsenocholine		21	
“ + phosphorylcholine		11	
Homocysteine-thiolactone		72	
“ + choline		92	
“ + betaine		254	
“ + arsenocholine		20	
“ + phosphorylcholine		49	

Table III also contains a summary of results obtained with homocysteine-thiolactone. It was transformed to methionine at a rate less than that of homocysteine (50 to 75 per cent) and slightly superior to that of homocystine. Betaine was in this instance also a more effective methyl donor than choline.

Homocysteinethiolactone can replace homocystine for rat growth (17). *Methionineless* mutants of *Neurospora* which can utilize homocystine as a substitute for methionine use homocysteinethiolactone better, and homocysteine still better.³ Our findings with homocysteinethiolactone and these are in accord.

Homogenized or lyophilized and dialyzed rat liver retain the ability to catalyze the methylation of homocysteine and of homocystine. Some results with lyophilized liver are given in Table IV. Some activity is lost

TABLE IV

Methylation of L-Homocysteine and of L-Homocystine with and without Choline and Betaine by Lyophilized Rat Liver

Nitrogen, 4 hours, 38°. The figures are mg. per cent of methionine in the trichloroacetic acid filtrates.

Reaction mixture	Undialyzed powder		Dialyzed enzyme used in each test equivalent to 100 mg. powder			
	25 mg.	100 mg.	Alone	Plus dialysate	Plus ash	Plus dialysate plus ash
Blank.....	0.6	2.5	2.7	3.1	2.7	3.2
Homocysteine.....	1.25	6.0	3.5	4.1	3.2	4.1
“ + choline.....	1.40	6.6	3.6	4.3	3.0	4.2
“ + betaine.....	3.00	9.8	6.4	5.5	5.7	6.1
Homocystine.....	0.6	4.2				
“ + choline.....	0.7	4.8				
“ + betaine.....	0.8	7.7				
Choline.....	0.55	2.6	2.7	3.0	2.8	3.2
Betaine.....	0.60	3.0	2.7	3.2	3.1	3.2

The powder and reactants were dissolved in 0.1 M phosphate buffer at pH 7.0. The final volume was 4 ml. Concentrations of homocysteine and of homocystine were 12.5 mg. per cent, of choline and betaine 37.5 mg. per cent.

in lyophilization and a further loss occurs in dialysis. The loss in activity indicates that there may be two mechanisms, one which uses choline, the other betaine; the former appears to suffer more loss in dialysis than the latter.

We have carried out a number of experiments with slices of rat kidney cortex. Methyl donors within (*i.e.* not washed out of) the kidney slices effected about the same amount of methylation of homocysteine and homocystine as did liver slices. Choline and betaine exerted only slight additive methylating effects. Unlike liver, kidney slices were more effective under aerobic than anaerobic conditions.

³ Horowitz, N., unpublished experiments, private communication.

DISCUSSION

The results of experiments reported here have established that there are at least two categories of transmethylation reactions: One, of which the methylation of guanidoacetic acid to creatine is an example, depends on oxygen and is inhibited by oxidation inhibitors; the other, represented by the formation of methionine from homocysteine or homocystine as the methyl acceptor and choline or betaine as the methyl donor, does not require oxygen and is not inhibited by oxidation inhibitors. In the first category methionine can act as a methyl donor but choline and betaine cannot unless homocysteine or homocystine is present to serve as a methyl carrier. Still another difference is that liver homogenates are unable, with or without added methionine, to methylate guanidoacetic acid; they retain their ability to catalyze methionine formation by transmethylation.⁴

In the first category the direction of the methyl transfer is from an S-methyl to an N-methyl and the reverse in the second category. This distinction may not be fundamental; it seems advisable to suspend judgment on this point at present.

Perlzweig *et al.* (18) reported that rat liver slices can methylate nicotinamide. The reaction is strictly aerobic, requires unbroken cells, is usually but not always accelerated by the addition of methionine, and the direction of the methyl transfer is from S-methyl to N-methyl, characteristics which place this methylation in the same category as the methylation of guanidoacetic acid. The two methylations are similar also in that they are restricted to the liver.

Stetten (19) proposed a scheme of choline formation from betaine in which betaine is demethylated to glycine, the latter is reduced to ethanolamine, which is then methylated to choline by methionine. The direct participation of methionine was not established and the pathway of betaine methyl was left open. The observations reported here show that a methyl group of betaine is available for choline formation by way of its incorporation into methionine and thus account for the physiological properties of betaine associated with its possessing a labile methyl group. Other possibilities are not excluded.

Chandler and du Vigneaud (20) found that there was a lag of several days before growth was resumed after betaine was added to a diet containing homocystine and devoid of or very low in methionine and choline, whereas growth was resumed immediately after the addition of choline. Even after the lag, betaine was somewhat less effective than equivalent amounts of choline. Griffith and Mulford (21) found that 3 to 4 mg. of methionine

⁴ After this manuscript was submitted for publication we found that the methylation of guanidoacetic acid by methionine occurs in guinea pig liver homogenates if adenosine triphosphate is provided.

or of betaine were required to give the same effect as 1 mg. of choline in preventing fatty liver or hemorrhagic kidney. As an explanation of the lesser efficiency of betaine than choline, the evidence Stetten obtained with N¹⁵ excluded direct reduction of betaine to choline. Our observations indicate that one route by which betaine methyl is made available for growth and for lipotropic purposes is via its incorporation into methionine, and hence its lower efficiency than choline for the latter function. None of the above observations excludes the possibility that ethanolamine may be methylated by betaine directly.

When choline is oxidized to betaine, at least one of its methyl groups remains labile via incorporation into methionine. There is considerable evidence that choline can be oxidized to betaine in the body. Rat liver slices and homogenates rapidly oxidize choline to betaine aldehyde and betaine (13, 14); betaine has been found in considerable amounts in animal tissues (22-25) and has been isolated from the urine of animals on a betaine-free diet after being fed large amounts of choline (26).

Although methyl transfer proceeds more rapidly from betaine than from choline, it seems unlikely from the evidence at hand that oxidation of choline to betaine is obligatory for this purpose. If it were, there should be little or no methionine formation with choline as the methyl donor under anaerobic conditions. Choline is effective under these conditions.

SUMMARY

1. Rat liver slices methylate homocysteine, homocystine, and homocysteinethiolactone to methionine. Choline or betaine can serve as the methyl donor.

2. The speed of the reaction with each of the three methyl acceptors is fastest with homocysteine and slowest with homocystine. It is faster with betaine than with choline as the methyl donor.

3. A number of possible methyl donors were tested; other than betaine and choline, none exerted a methylating effect which was additive to that of the methyl donors retained in the slices.

4. These transmethylation reactions are independent of oxygen and are not inhibited by oxidation inhibitors.

5. Catalytic activity is retained in homogenized and in lyophilized rat liver.

6. There are two (at least) categories of methyl transfer reactions. One is dependent on oxygen, is inhibited by oxidation inhibitors, and catalytic activity is lost by homogenization and is not restored by adding methionine, which accelerates the reaction with slices. In this category is the methylation by methionine of guanidoacetic acid to creatine and of nicotinamide to N¹-methylnicotinamide. Characteristics of the second category are

independence of oxygen, nonsusceptibility to oxidation inhibitors, and persistence of catalytic activity after cell structure is destroyed. In this category is methionine formation by the methylation of homocysteine, homocysteinethiolactone, or homocysteine by betaine or choline.

The authors wish to acknowledge the assistance of Miss I. Silberbach in the experiments reported here.

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