

# SYNTHESIS OF LABILE METHYL GROUPS BY GUINEA PIG TISSUE IN VITRO\*

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The formation of the methyl groups of methionine and choline from  $C^{14}$ -labeled formate has been demonstrated in the intact rat (1-3) and in rat liver slices (1). Du Vigneaud and his coworkers (2) have also shown the incorporation of the carbon of formaldehyde into the methyl groups of choline. It is conceivable that in rat liver the carbon of formate or formaldehyde is first incorporated into the methyl groups of choline or betaine, followed by transfer of the methyl groups to homocysteine. In the present report a study has been made of the possible synthesis of methionine methyl groups by a pathway not involving formation of choline methyl groups. In approaching this problem we have used guinea pig liver which has been reported to lack an active liver choline oxidase system (4), and shown to be unable to methylate homocysteine from choline (5).

In the present work it has been found that, while guinea pig liver does actually methylate homocysteine from choline, this reaction is blocked under anaerobic conditions, whereas synthesis of the methionine methyl groups from formate was relatively unaffected. These results indicate that labile methyl group synthesis may occur by some mechanism other than choline formation. In addition, evidence has been obtained that betaine methyl groups are not intermediates in the synthesis of the methyl groups of methionine from formate.

As part of an investigation concerning the conversion of the formate carbon to methionine, it was found that formaldehyde *per se* is not on the pathway of formate to methionine methyl groups.

## EXPERIMENTAL

The animal tissues used in this study were obtained from guinea pigs maintained on a diet of Purina rabbit food supplemented with carrots,

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and weighing between 250 and 400 gm. The animals were killed by stunning and exsanguination. The livers were quickly removed, washed with isotonic saline, and sliced with the Stadie-Riggs microtome. 1.5 gm. of slices were added to each of the chilled Warburg flasks containing the appropriate buffers and substrates. When homogenates were employed, the excised liver was quickly weighed, minced, and homogenized with 2 parts of buffer in a glass homogenizer. The flasks were gassed and then incubated for 2 hours at 38°. The exact experimental conditions have been included in Tables I to V. The reaction was terminated by adding 1 ml. of concentrated HCl to each flask. When it was not desired to isolate the formate, the acidified contents of each flask were lyophilized to remove excess formate, followed by the isolation of the methyl group of free and protein-bound methionine as methyl iodide (6). The methyl iodide was converted to tetramethylammonium iodide with trimethylamine (7) and the radioactivity determined.<sup>1</sup> One recrystallization from alcohol and water gave constant specific activity and a theoretical iodine titration.

When formate was isolated, the incubation was terminated with 1 ml. of 40 per cent trichloroacetic acid (TCA). Unlabeled formic acid carrier was added and the formic acid isolated by steam distillation. This treatment did not bring about any breakdown of trichloroacetic acid to formic acid. The formate was oxidized to CO<sub>2</sub> with mercuric ions (8) and counted after plating as BaCO<sub>3</sub>.<sup>1</sup>

In experiments in which formaldehyde was isolated, carrier formaldehyde and 1 ml. of 40 per cent TCA were added at the end of the incubation. The TCA filtrates were steam-distilled and the formaldehyde in the distillate was precipitated as the dimedon derivative (9), recrystallized from water and alcohol, and the radioactivity determined.<sup>1</sup>

Choline was isolated from a TCA filtrate by precipitation as the reineckate (10), decomposition of the reineckate by the method of Kapfhammer and Bischoff (11), and conversion to the chloroplatinate. The choline chloroplatinate was degraded to trimethylamine (12), which was subsequently converted to tetramethylammonium iodide (13) and the radioactivity determined.<sup>1</sup>

Betaine was isolated by precipitation as the reineckate (14). The

<sup>1</sup> The activity of the formate, formaldehyde, choline, and betaine was determined by conversion into barium carbonate and counting with an end-window Geiger counter. The tetramethylammonium iodide samples were plated on stainless steel planchets and counted directly, but for purposes of comparison the results have been calculated and expressed as the activities determined on barium carbonate. The formaldehyde-dimedon derivative was counted directly by using a barium carbonate self-absorption correction which had been found to be the same for a 10 mg. sample.

reineckate was split (11) and the betaine was separated from any choline by passing through a column of IRC-50 cation exchange resin (15). The filtrate was acidified with HCl, concentrated to dryness, the residue dissolved in a small amount of water, and the betaine hydrochloride precipitated with alcohol and ether (14). The betaine hydrochloride was oxidized to CO<sub>2</sub> by wet combustion (16), plated as BaCO<sub>3</sub>, and the radioactivity determined.<sup>1</sup>

The C<sup>14</sup>-formate used was prepared by catalytic reduction of potassium bicarbonate (17). C<sup>14</sup>-Methyl-labeled choline and betaine were prepared by modification of the methods of Ferger and du Vigneaud (14). C<sup>14</sup>-Formaldehyde was synthesized by reduction of C<sup>14</sup>O<sub>2</sub> with lithium aluminum hydride (18) and oxidation of the resulting methanol with dry air over a vanadium-molybdenum oxide catalyst (19).

TABLE I

*Synthesis of Methyl Group of Methionine from C<sup>14</sup>-Formate in Guinea Pig Liver Slices*

Substrate	Specific activity, c.p.m. per mg. methyl C
Formate + homocysteine.....	3032
“ .....	840
“ + homocysteine.....	8*

Final concentration, Na formate  $6 \times 10^{-3}$  M, homocysteine  $3.7 \times 10^{-3}$  M, dimethylaminoethanol  $5.8 \times 10^{-3}$  M. Activity of formate  $4.6 \times 10^4$  c.p.m. per  $\mu$ M. Final volume 5.0 ml. Suspending medium, that of Winnick *et al.* (24). Gas mixture 95 per cent O<sub>2</sub>-5 per cent CO<sub>2</sub>.

\* This preparation was heated at 100° for 5 minutes before the addition of formate and homocysteine.

#### RESULTS AND DISCUSSION

In Experiment I, guinea pig liver slices were equilibrated with C<sup>14</sup>-formate and with C<sup>14</sup>-formate plus homocysteine. The results of this experiment, summarized in Table I, demonstrate that guinea pig liver slices can synthesize the methyl group of methionine from formate and that this was promoted by addition of homocysteine. The synthesis observed when homocysteine was omitted was probably due to the presence of endogenous homocysteine.

Dubnoff has reported that net formation of methionine from choline could not be demonstrated *in vitro* with guinea pig liver homogenates (5). He has, however, isolated methyl-labeled methionine from the tissues of guinea pigs fed C<sup>14</sup>-methyl-labeled choline (20). We have investigated this reaction in slices and homogenates. These preparations were incubated with C<sup>14</sup>-methyl-labeled choline and homocysteine and the methyl group of methionine isolated and counted as previously described. The

results of this experiment, which are presented in Table II, indicate that both guinea pig liver slices and homogenates are able to methylate homocysteine to form methionine. The specific activity of the formed methyl group is undoubtedly many times higher, inasmuch as the method of degradation included total methionine.

To test the possibility that the methyl group of methionine can be synthesized from formate without first forming choline methyl groups, it was necessary to inhibit the methylation of homocysteine by choline. With rat liver Dubnoff (5) demonstrated that anaerobically the methylation of homocysteine is inhibited. From a representative set of data, summarized in Table III, it can be seen that formation of methionine

TABLE II  
*Methylation of Homocysteine by C<sup>14</sup>-Methyl-Labeled Choline by Guinea Pig Liver Preparations*

Tissue	Substrate	Specific activity, c.p.m. per mg. methyl C
Slices	Choline + homocysteine	3,988
	“ + “	57*
Homogenate	“ + “	10,550†
	“ + “	19,592‡

Final concentration, choline  $3 \times 10^{-3}$  M, activity  $7 \times 10^4$  c.p.m. per  $\mu$ M, homocysteine  $3.7 \times 10^{-3}$  M. Final volume 10 ml. Gas mixture 95 per cent O<sub>2</sub>-5 per cent CO<sub>2</sub>.

\* Slices inactivated with 1 ml. of concentrated HCl.

† With media described by Winnick *et al.* (24).

‡ With 0.015 M K-PO<sub>4</sub> buffer, pH 7.5.

from choline and homocysteine can be inhibited by anaerobiosis, while not affecting the conversion of formate to the methyl group of methionine.

Further indication that choline *per se* is not an intermediate in the incorporation of formate to methionine methyl groups was afforded by dilution experiments. If choline methyl groups were intermediates in this conversion, incorporation of the radioactivity into the methyl group of methionine would be significantly decreased in the presence of a large pool of unlabeled choline. The results of such dilution studies necessitate the assumption that the intermediates formed in a reaction are in equilibrium with a pool of the same compound which has been added. While conclusive proof on this point, in the system employed, is lacking, the fact that choline does methylate homocysteine and is oxidized to CO<sub>2</sub> in the liver slice is taken as presumptive evidence that choline can penetrate the liver cells. C<sup>14</sup>-Labeled formate and homocysteine were incubated with liver slices in the presence of 100  $\mu$ M of unlabeled choline and the specific activity of the methyl group of methionine obtained, com-

pared to a control flask in which choline was omitted. To determine the size of the diluting pool remaining at the end of the experiment, 100  $\mu\text{M}$  of  $\text{C}^{14}$ -methyl-labeled choline were incubated in a system in which non-isotopic formate replaced the isotopic formate and the non-phosphatide choline was isolated at the end of the experiment. Of the 100  $\mu\text{M}$  of choline added initially, 72  $\mu\text{M}$  remained as free choline. The minimum dilution of the specific activity of the methionine methyl groups, if all of

TABLE III

*Comparison of Methylation of Homocysteine by Choline and Formate under Aerobic and Anaerobic Conditions in Guinea Pig Liver Slices*

Gas phase	Substrate	Specific activity, c.p.m. per mg. methyl C
95% O <sub>2</sub> -5% CO <sub>2</sub>	Choline + homocysteine	2276
	Formate + "	1212
95% N <sub>2</sub> -5% CO <sub>2</sub>	Choline + "	125
	Formate + "	2088

Final concentration, choline  $6.2 \times 10^{-3}$  M, activity  $2.6 \times 10^4$  c.p.m. per  $\mu\text{M}$  or Na formate  $6 \times 10^{-3}$  M, activity  $4 \times 10^4$  c.p.m. per  $\mu\text{M}$ , homocysteine  $3.7 \times 10^{-3}$  M. Final volume 5.0 ml. Suspending medium, that of Winnick *et al.* (24).

TABLE IV

*Methylation of Homocysteine by  $\text{C}^{14}$ -Formate in Presence of Unlabeled Choline and Betaine by Guinea Pig Liver Slices*

Substrate	Specific activity, c.p.m. per mg. of methyl C
Formate + homocysteine.....	3032
" + " + 100 $\mu\text{M}$ choline.....	1508
" + " .....	1872
" + " + 50 $\mu\text{M}$ betaine.....	875

Final concentration, Na formate  $6 \times 10^{-3}$  M, activity  $4 \times 10^4$  c.p.m. per  $\mu\text{M}$ , homocysteine  $3.7 \times 10^{-3}$  M. Final volume 5.0 ml. Suspending medium, that of Winnick *et al.* (24). Gas mixture, 95 per cent O<sub>2</sub>-5 per cent CO<sub>2</sub>.

the 30  $\mu\text{M}$  of the formate had passed through a pool of 216  $\mu\text{M}$  of the methyl carbons of choline, would be seven times. Actually, only a small portion of the formate is involved in the synthesis, as 60 per cent of the formate is oxidized to CO<sub>2</sub> and 8  $\mu\text{M}$  remained at the end. From Table IV it can be seen that the dilution found was considerably less than would be expected if choline were an intermediate. Similarly, when 50  $\mu\text{M}$  of betaine were used as the diluting pool, 49  $\mu\text{M}$  remained, but the dilution found was less than would be expected. The dilution observed in the presence of choline or betaine may be the result of independent formation of methionine by these known methyl donors, thus diluting the specific

activity of the end-product, or formation of formate from the choline and betaine and dilution of the formate available for synthesis.

Formaldehyde has been shown to be converted to labile methyl groups in the rat (2). We have investigated the possibility that formaldehyde is an intermediate in the conversion of formate to methionine methyl groups. Formate labeled with  $C^{14}$  was incubated, aerobically and anaerobically, with guinea pig liver slices in the presence of  $30 \mu M$  of unlabeled formaldehyde, and the specific activity of the methionine methyl groups determined. From Table V it can be seen that in both cases the dilution of the specific activity was about 50 per cent when compared to the controls. That a portion of the formaldehyde pool remained at the end of the incubation is demonstrated by an experiment in which  $26 \mu M$  of  $C^{14}$ -

TABLE V  
*Methylation of Homocysteine by  $C^{14}$ -Formate in Presence of Unlabeled Formaldehyde by Guinea Pig Liver Slices*

Gas phase	Substrate	Specific activity, c.p.m. per mg. methyl C
95% O <sub>2</sub> -5% CO <sub>2</sub>	Formate + homocysteine	3032
	" + " + 30 $\mu M$ formaldehyde	1304
95% N <sub>2</sub> -5% CO <sub>2</sub>	Formate + homocysteine	2900
	" + " + 30 $\mu M$ formaldehyde	1480

Final concentration, Na formate  $6 \times 10^{-3}$  M, activity  $4 \times 10^4$  c.p.m. per  $\mu M$ , homocysteine  $3.7 \times 10^{-3}$  M. Final volume 5.0 ml. Suspending medium, that of Winnick *et al.* (24).

labeled formaldehyde were incubated with the liver slices in the presence of formate. Approximately  $2 \mu M$  of free formaldehyde could be recovered aerobically, and  $3 \mu M$  of free formaldehyde anaerobically. These figures are based on the recovery of total radioactivity in the formaldehyde isolated at the end of the experiment and expressed in terms of the original formaldehyde. Essentially the same results were obtained when formaldehyde was precipitated as the dimedon derivative from the TCA filtrate by the method of Mackenzie (21). Actually the size of the formaldehyde pool may be larger, due to endogenous formaldehyde formation which is not determined by this procedure. From previous experiments it was estimated that 0.03 to 0.05 micromole of the original formate had been incorporated into the methyl groups of methionine under these conditions. This amount of formate, passing through a pool of at least 2 or 3  $\mu M$  of formaldehyde, would cause a dilution significantly greater than the 50 per cent dilution found. Siekevitz and Greenberg (22) have found that

rat liver slices, which yield formate from glycine labeled with  $C^{14}$  in the  $\alpha$ -carbon atom, do not convert formate to formaldehyde aerobically or anaerobically. Using pigeon liver homogenates for a study of purine synthesis from  $C^{14}$ -formate, Greenberg<sup>2</sup> has found no appreciable conversion of formate to formaldehyde. When  $C^{14}$ -formate was incubated in the guinea pig liver slice system with a pool of unlabeled formaldehyde, no significant conversion of formate to formaldehyde could be detected, as measured by the amount of radioactivity in the formaldehyde fraction recovered at the end of the incubation. The rapid oxidation of formaldehyde to formate and  $CO_2$  makes it seem likely that the pool of formaldehyde has entered the liver cells and thus is in equilibrium with any formed formaldehyde. The dilution of the specific activity of the methionine methyl groups by a pool of formaldehyde may be due in part to incorporation of the formaldehyde to methyl groups by another pathway and to conversion of a part of the formaldehyde to formate, thus diluting the formate pool available for the synthesis. These and other possibilities are now under investigation but preliminary experiments have been hampered by a non-enzymatic reaction between formaldehyde and homocysteine.<sup>3</sup>

Conversion of formate to methionine methyl groups in liver homogenates has not been observed with rat liver (23) and guinea pig liver. Recently we have found incorporation of the radioactivity of  $C^{14}$ -labeled formate into the methyl groups of methionine by using pigeon liver homogenates. A study of the mechanism and pathways involved in the synthesis is under investigation with this system.

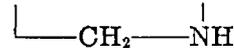
#### SUMMARY

The conversion of formate to the methyl group of methionine in guinea pig liver slices has been demonstrated. Inhibition and dilution studies indicate that the methyl groups of choline and betaine are not intermediates in this conversion. Formaldehyde *per se* does not appear to be an intermediate in the formation of the methyl group of methionine from formate.

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<sup>2</sup> Dr. G. R. Greenberg, personal communication.

<sup>3</sup> The non-enzymatic product has been isolated and shown to be different from methionine. Its structure has not been fully identified but preliminary data indicate that it is the heterocyclic structure *m*-thiazane 4-COOH acid,  $S-CH_2-CH_2-CH-COOH$ . Whether this compound is an intermediate in the



synthesis of methionine from formate is being investigated.

of Dr. W. Sakami and Dr. G. R. Greenberg throughout the course of this work.

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