The Biogenesis of Choline and Methionine Methyl Groups and of the Serine Hydroxymethyl Group from Intramolecularly Labeled Formaldehyde-\(^{14}\text{C},\text{D}_{2}\)*

Julian R. Rachele, Alan M. White, and Helmuth Grünwald

From the Department of Biochemistry, Cornell University Medical College, New York 21, New York

(Received for publication, July 1, 1963)

EXPERIMENTAL PROCEDURE

Preparation of Formaldehyde-\(^{14}\text{C},\text{D}_{2}\)—Barium carbide-\(^{14}\text{C}\) was prepared by fusion of barium carbonate-\(^{14}\text{C}\) (125 mg, 0.82 mc) with metallic barium in an atmosphere of helium, according to the procedure described by Ronzio (8). Intramolecularly labeled acetylene-\(^{14}\text{C},\text{D}_{2}\) was generated from the carbide by the action of deuterium oxide which contained 99.8% of deuterium. The labeled acetylene was converted to 1,2-dibromoethane-\(^{14}\text{C},\text{D}_{4}\) by reaction with deuterium bromide gas in a simple vacuum system, according to the methods of Leitch, Morse, and Banard (9, 10). At this point the doubly labeled ethylene bromide was diluted with 3.5 mmoles of 1,2-dibromoethane-\(^{14}\text{C},\text{D}_{4}\) prepared separately by similar procedures from acetylene-\(^{14}\text{C},\text{D}_{2}\) and deuterium bromide. The ethylene bromide was converted to the diacetate, and the latter to the glycol (9, 10). After removal of iodate and excess periodate as the barium salts, the formaldehyde-\(^{14}\text{C},\text{D}_{2}\) was obtained as a solution containing approximately 6 mmoles in 14 ml of water.

A solution of formaldehyde was prepared for injection into the animals as follows. A portion of the doubly labeled formaldehyde solution, containing approximately 3 mmoles, was added to a solution of about 40 mmoles of formaldehyde-\(^{14}\text{C},\text{D}_{2}\) in 48 ml of water, the deuterioformaldehyde having been prepared separately from 1,2-dibromoethane-\(^{14}\text{C},\text{D}_{4}\) by the steps outlined above. The resultant mixture was made isotonic by the addition of the appropriate amount of solid NaCl. The final solution was assayed gravimetrically for total formaldehyde by precipitation of the dimedon derivative from aliquots of the solution at the deuterium-\(^{14}\text{C},\text{D}_{2}\) was assayed for total formaldehyde by precipitation of the dimedon derivative from aliquots of the solution (1) and was found to contain 0.78 mmole of formaldehyde per ml. The formaldemethene obtained in the assay was used also for the deuterium and radiocarbon analyses of the administered formaldehyde. The isotopic contents are given in Table I.

Administration of Formaldehyde-\(^{14}\text{C},\text{D}_{2}\)—Four male rats of the Sherman strain (Rockland Farms), each weighing about 250 g, were allowed to become acclimatized for 2 days in individual cages, equipped for urine collection. Each animal then received a total daily dose of 0.54 mmole of unlabeled formaldehyde by two subcutaneous injections per day for 2 days. Subsequently formaldehyde-\(^{14}\text{C},\text{D}_{2}\) was administered in a daily dose of 0.58 mmole in a similar manner for a period of 10 days. Urine was collected from each animal, preserved with toluene, and stored in the refrigerator until used. Approximately 18 hours after the last injection, the animals were killed by ether anesthesia. The livers of the animals were removed for the subsequent examination of the liver protein amino acids. The animals had been maintained throughout the experiment on their accustomed pelleted diet (Rockland rat diet (complete)). Each animal had gained about 20 g in weight during the final 10-day injection period.

* Supported in part by Grant CA-03981-06 from the National Cancer Institute of the United States Public Health Service.
Methionine was isolated from the "protein" residue of the extracted carcasses of two of the animals as the methionine methylsulfonylum bromide by a method previously described by Stekol et al. (12). Both samples of the derivative had melting points of 137–138°C (capillary, uncorrected) in agreement with the published value of 136–138°C (12). The methionine methylsulfonylum bromide was demethylated with boiling hydriodic acid as described previously (13, 14) for methionine. The methyl iodide was trapped in a 5% solution of N,N-dimethylaminoethanol in ethanol, chilled in a Dry Ice-ethanol bath. Choline iodide, obtained after evaporation of ethanol and excess dimethylaminoethanol, was converted to the chloride by passage through a column of Dowex-1 in the chloride form. The choline chloroplatinate was precipitated from an ethanol solution of the chloride with chloroplatinic acid. The choline chloroplatinate samples were analyzed for their deuterium and radioactivity, and the results were used to calculate the isotopic concentrations of the methyl group of tissue methionine, as given in Table I. Trial experiments in which methionine with a known deuterium content was converted to the methylsulfonylum bromide and then converted to choline as outlined above, revealed that no labilization of deuterium took place during the entire procedure.

Isolation and Degradation of Serine—The livers of the animals were homogenized with absolute ethanol and the homogenates were extracted with hot ethanol and then with an ethanol-ether mixture. The extracted residue was boiled overnight with 10% NaCl and then treated with cold 6% trichloroacetic acid. The final extracted material was hydrolyzed for 24 hours with 6 N HCl under an atmosphere of nitrogen. The hydrolysate was decolorized with activated charcoal and evaporated to dryness under reduced pressure. Serine was isolated from the hydrolysate by ion-exchange chromatography with the method of Hirs, Moore, and Stein (15). The dimensions of the columns, the rates of flow, and the volume of the collected fractions were increased proportionally to accommodate about 2 g of protein hydrolysate. From each run, approximately 60 mg of serine were obtained; 10-mg portions of the serine were degraded with sodium metaperiodate by a procedure outlined and described by Sakami (16, 17). Since the isotopic content of hydrogen as well as of the carbon was of interest in this experiment, the formaldehyde derived from the serine β carbon in the periodate oxidation was isolated as formaldemethone. The deuterium and 14C contents corresponding to those of the serine β carbon were determined by the isotopic analysis of the isolated serine samples and of the formaldemethone derived therefrom. The analytical data are given in Table II.

Isolation of Urinary Formate—Formate was isolated from urine by a procedure involving the distillation of the volatile acids therein (3) and a chromatographic separation of these acids (3, 17). Nonisotopic formate was added as carrier to the

---

**Table I**

Incorporation of formaldehyde-14C, D4 into methyl groups

<table>
<thead>
<tr>
<th>Compound analyzed</th>
<th>Rat</th>
<th>D</th>
<th>14C</th>
<th>D:14C Ratio of D:14C in methyl to D:14C in formaldehyde</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>a</td>
<td>mole</td>
<td>14C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>0.113</td>
<td>0.69</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>0.131</td>
<td>0.79</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>0.149</td>
<td>0.96</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>0.132</td>
<td>0.80</td>
</tr>
<tr>
<td>Serineb</td>
<td>1</td>
<td>0.092</td>
<td>0.61</td>
<td>1.51</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.090</td>
<td>0.57</td>
<td>1.54</td>
</tr>
<tr>
<td>Methionined</td>
<td>1</td>
<td>0.187</td>
<td>1.31</td>
<td>1.43</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.187</td>
<td>1.29</td>
<td>1.44</td>
</tr>
</tbody>
</table>

* The administered formaldehyde was analyzed as its dimedon derivative and was found to have a deuterium content of 99.0 atom % excess and a specific radioactivity of 2.06 × 10⁹ c.p.m. per mmole, and hence had a D:14C value of 4.88 × 10⁻⁴.

b The methyl group of the creatine isolated from the carcasses of Rats 1 and 3 was obtained and analyzed for its isotopic contents as the trimethylamine chloroplatinate derivative, and the latter had the following platinum contents (theoretical, 37.0%): from Rat 1, 37.0%; from Rat 2, 36.9%; from Rat 3, 37.0%; from Rat 4, 37.0%.

c The methyl group of the creatine isolated from the carcasses of Rats 1 and 3 was obtained and analyzed for its isotopic contents as the trimethylamine chloroplatinate derivative, which had the following platinum content (theoretical, 41.4%): from Rat 1, 41.5%; from Rat 2, 41.6%.

d The methyl group of methionine isolated from the extracted "protein" residues of the carcasses of Rats 1 and 2 was obtained and analyzed for its isotopic contents as the dimethylaminoethanol derivative, which had the following platinum content (theoretical, 31.7%): from Rat 1, 32.3%; from Rat 2, 31.8%.

---

**Table II**

Incorporation of formaldehyde-14C, D4 into the hydroxymethyl group of serine

<table>
<thead>
<tr>
<th>Compound analyzed</th>
<th>Rat</th>
<th>D</th>
<th>14C</th>
<th>D:14C Ratio of D:14C in methyl to D:14C in formaldehyde</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>a</td>
<td>mole</td>
<td>14C</td>
</tr>
<tr>
<td>Serineb</td>
<td>1</td>
<td>0.672</td>
<td>2.01</td>
<td>2.31</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.662</td>
<td>2.70</td>
<td>2.45</td>
</tr>
<tr>
<td>Formaldemethonec</td>
<td>1</td>
<td>0.660</td>
<td>3.01</td>
<td>2.19</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.672</td>
<td>3.14</td>
<td>2.14</td>
</tr>
</tbody>
</table>

* The data in the isotope analysis of serine are calculated on the assumption that all the deuterium and 14C of serine reside in the hydroxymethyl group. This assumption is borne out by the analysis of the formaldemethone derivatives.

b The methyl group of the creatine isolated from the carcasses of Rats 1 and 3 was obtained and analyzed for its isotopic contents as the trimethylamine chloroplatinate derivative. This assumption is borne out by the analysis of the formaldemethone derivatives.

c The formaldehyde arising from the hydroxymethyl group of the serine of Rats 1 and 2 on periodate oxidation.
combined formate containing fractions in amounts indicated in Table III. The diluted formate was obtained finally as the lead salt (3), which was then analyzed for deuterium and 14C. The isotopic contents are reported in Table III.

Isotopic Analyses—The deuterium and radiocarbon concentrations in all compounds were determined by way of complete combustion in oxygen and examination of the combustion products for their isotopic content. The water and carbon dioxide derived from the same weighed sample of substance were collected from the combustion gases by freezing the water in a trap chilled in a Dry Ice-ethanol bath and by trapping the carbon dioxide in a gas scrubber containing a solution of NaOH. The water was converted to hydrogen gas by passing the vapor over zinc at 400° (19). The hydrogen gas was analyzed for its deuterium with a Consolidated-Nier isotope ratio mass spectrometer. BaCO3 was obtained by precipitating the CO3 collected in the alkali scrubber. The BaCO3 was filtered and collected as a pad on a disk of paper. The 14C activity in the BaCO3 sample was determined with a thin mica window Geiger-Müller counter and was corrected for background and self-absorption.

RESULTS AND DISCUSSION

The values listed in the last column of Table I show that the D:14C ratios in the methyl groups of choline, creatine, and methionine derived from formaldehyde in the rat are near to one-third of the D:14C value in the administered formaldehyde. The conclusion now appears unequivocal that exogenous formaldehyde is utilized for methyl biosynthesis in the intact rat by prior oxidative removal of 1 hydrogen atom from the formaldehyde carbon to give an intermediate at the formate level. Therefore, a possible and probable explanation of our results is that the whole animal oxidative reactions of this kind make it impossible for formaldehyde to enter the various metabolic pathways which studies in vitro indicate may be open to it.

A similar argument can be used to explain the utilization of formaldehyde in this experiment for serine biosynthesis. Again the figures in the final column of Table II, comparing the isotope ratio in the β carbon of serine with that in the administered formaldehyde, show that a loss of hydrogen from the carbon of formaldehyde must have occurred before the latter is integrated into serine. Otherwise, the retention of both hydrogens of formaldehyde should have resulted in a final ratio of unity rather than the observed value of approximately 0.5.

Evidence of an early oxidation to a formyl level in the metabolism of formaldehyde is provided by the isotope concentrations in the urinary formate. Table III shows the ratio of D:14C in the urinary formate to have been about 90% of that in administered formaldehyde, whereas the specific isotopic contents of deuterium and 14C, corrected for dilution by carrier, were approximately 30% of the corresponding concentrations in the formaldehyde. The levels of these values suggest that the labeled formate in the urine arose extensively by a rather direct oxidative path from formaldehyde without involving a preliminary reductive conversion to other sources of formate such as methyl groups.

In contrast to our findings, conclusions drawn by others from experiments in vitro designed to investigate the incorporation of formaldehyde into methyl groups (20–23), the β-hydroxymethyl group of serine (26–33) and, more recently, prefollic A (34) generally imply that both hydrogen atoms of formaldehyde follow its carbon. However, only the formaldehyde carbon was labeled in these experiments. Under these circumstances the fate of the entire CH4 unit of formaldehyde remains equivocal. Although it is possible that certain systems in vitro do make use of the entire methylene group of formaldehyde for methyl group and serine biosynthesis, particularly as formaldehyde will combine nonenzymically with tetrahydrofolic acid to give hydroxymethyltetrahydrofolate (32) and N2,N6-methylenetetrahydrofolate (33), both compounds of central importance in “1-carbon” metabolism, these modes of utilization would appear to be of limited occurrence in the whole animal on the basis of the present experiment.

The work of Mackenzie, Abeles, and Harris (35,36) has shown that, although free formaldehyde arising from the oxidation of sarcosine methyl groups by rat liver mitochondria cannot be utilized as such by this system, the addition of cysteine permits the quantitative oxidation of formaldehyde, through the intermediate formation of thiazolidine-4-carboxylic acid, to the formyl level in N-formylcysteine. Moreover, liver alcohol dehydrogenase (37) and the formaldehyde oxidase of Strittmatter and Ball (38) quantitatively oxidize free formaldehyde to formic acid.

Therefore, a possible and probable explanation of our results is that in the whole animal oxidative reactions of this kind make it impossible for formaldehyde to enter the various metabolic pathways which studies in vitro indicate may be open to it.

SUMMARY

A study of the incorporation in the intact rat of both the carbon and the hydrogen of formaldehyde, intramolecularly doubly labeled, supports the conclusion that an obligatory loss of 1 hydrogen atom to give a formyl type of intermediate occurs before the utilization of formaldehyde for methyl group or serine biosynthesis.

REFERENCES

17. SAKAMI, W., Handbook of isotopic tracer methods. Western Reserve University School of Medicine, Cleveland, 1955, p. 72.
The Biogenesis of Choline and Methionine Methyl Groups and of the Serine Hydroxymethyl Group from Intramolecularly Labeled Formaldehyde-$^{14}$C,D$_2$
Julian R. Rachele, Alan M. White and Helmut Grünewald