The Biogenesis of Choline and Methionine Methyl Groups and of the Serine Hydroxymethyl Group from Intramolecularly Labeled Formaldehyde-\(^{14}\text{C}, \text{D}_2\)

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The metabolism in the living rat of formaldehyde, doubly labeled with deuterium and \(^{14}\text{C} \), was investigated previously in this laboratory (1). In the aforementioned studies, the double labeling was accomplished by admixture of two preparations of formaldehyde, one labeled with deuterium, the other with \(^{14}\text{C} \). This type of double labeling has been referred to as \textit{intramolecular} (3, 4). The results obtained in this earlier investigation indicated that formaldehyde was incorporated into the methyl groups of choline and creatine via an intermediate at the oxidation level of formate, but it was pointed out that interpretation of the results was limited because of the possibility of isotope selection (1-7). A study of the biosynthesis of methyl groups with formaldehyde-\(^{14}\text{C}, \text{D} \) as precursor (6) showed that it is possible to minimize, in fact to eliminate, by \textit{intramolecular} double labeling, the disproportionating effects brought about by hydrogen isotope selection upon the deuterium to \(^{14}\text{C} \) ratios that had been observed with \textit{intramolecular} multiple labeling of compounds with \(^{14}\text{C} \), deuterium, and tritium (2-5, 7). Therefore, in order to arrive at less equivocal results with the doubly labeled formaldehyde, this compound has been prepared with the \(^{14}\text{C} \) bonded to deuterium intramolecularly, and the level of oxidation at which the carbon and hydrogen of formaldehyde are incorporated into the methyl groups of choline and creatine has been reinvestigated. Furthermore, the methyl group of methionine was also of interest in this regard, and it was also considered important to examine the level of oxidation of the formaldehyde carbon that occurred before incorporation into the hydroxymethyl group of serine. Since the urinary formate might have a bearing on the metabolism of formaldehyde, this substance was also isolated and examined for its isotopic content.

**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 3.3 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). The ethylene glycol-\(^{14}\text{C}, \text{D}_4\) was dissolved in water and treated with periodic acid (1, 3). 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 (1) and was found to contain 0.78 mmole of formaldehyde per ml. The formaldemethane 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.
Isolation and Degradation of Choline, Creatine, and Methionine

The isolation of choline and creatine from the carcasses of the animals, the degradation of these compounds, and the derivatization of the resultant trimethylamine and methylamine, respectively, as the chloroplatinate derivatives were carried out by procedures previously described (11). The chloroplatinate derivatives were analyzed for deuterium and radioactivity, from which analyses were derived the isotopic contents of the methyl groups of choline and creatine listed in Table I.

Methionine was isolated from the "protein" residue of the extracted carcasses of two of the animals as the methionine methylsulfonium bromide by a method previously described by Stekol et al. (12). Both samples of the derivative had melting points of 137–138° (capillary, uncorrected) in agreement with the published value of 136–138° (12). The methionine methylsulfonium 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 radioisotopic contents, 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 methylsulfonium bromide and thence degraded to give 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 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

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**Table I**

Incorporation of formaldehyde-14C,D2 into methyl groups

<table>
<thead>
<tr>
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<td>Rat 1</td>
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**Table II**

Incorporation of formaldehyde-14C,D2 into the hydroxymethyl group of serine

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a 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 2.05 X 10^6 c.p.m. per mmole, and hence had a D:14C value of 4.83 X 10^-3.

b The methyl groups of the choline isolated from each rat carcass were obtained and analyzed for their isotopic contents as the trimethylamine chloroplatinate derivative, and the latter had the following platinum contents (theoretical, 37.0%): from Rat 1, 32.3%; from Rat 2, 31.8%.

c The methyl group of the creatine isolated from the carcasses of Rats 1 and 2 was obtained and analyzed for its isotopic content as the choline 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 creatine isolated from the carcases of Rats 1 and 3 was obtained and analyzed for its isotopic content as the methylamine chloroplatinate derivative, which had the following platinum content (theoretical, 31.7%): from Rat 1, 32.3%; from Rat 2, 31.8%.

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b The methyl group of the creatine isolated from the carcases of Rats 1 and 2 was obtained and analyzed for its isotopic content as the methylamine chloroplatinate derivative, which had the following platinum content (theoretical, 31.7%): from Rat 1, 32.3%; from Rat 2, 31.8%.

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its isotopic content was determined on the crystalline lead formate and B-fold in the case of Rat 3 with normal sodium formate, and the isotopic contents are reported in Table III.

Table III. The diluted formate was obtained finally as the dioxide in a gas scrubber containing a solution of NaOH. The derivative, which had the following PbO residue analysis (theoretical, 75.1%): from Rat 2, 75.8%; from Rat 3, 75.5%.

The 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 content with a Consolidated-Nier isotope ratio mass spectrometer. BaCO3 was obtained by precipitating the CO2 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 as such by this system, the addition of cysteine permits utilization of formaldehyde for methyl biosynthesis, particularly as formaldehyde will combine quantitatively with tetrahydrofolate (32) and NS,N10-methylenetetrahydrofolate (33), both compounds of central importance in “l-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 formaldehyde 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

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