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By Walter G. Verly, Julian R. Rachele, Vincent du Vigneaud, Maxwell L. Eidinoff  
and Joseph E. Knoll

[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, CORNELL UNIVERSITY MEDICAL COLLEGE, AND THE DEPARTMENT OF PHYSICS AND BIOPHYSICS, SLOAN-KETTERING INSTITUTE FOR CANCER RESEARCH]

## A Test of Tritium as a Labeling Device in a Biological Study

BY WALTER G. VERLY, JULIAN R. RACHELE, VINCENT DU VIGNEAUD, MAXWELL L. EIDINOFF AND JOSEPH E. KNOLL

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These experiments demonstrate that an isotopic selection takes place in the over-all direction of the greater retention of the carbon-tritium bond in the utilization of the methyl group of methanol in the biosynthesis of the labile methyl group. A mixture of methanols containing carbon-14, deuterium and tritium has been administered to rats and the isotopic contents of the methyl groups of the choline and creatine isolated from the tissues have been compared with those of the methyl group of the methanol administered. The ratio of deuterium to  $C^{14}$  in the choline methyl was 22% of that in the methanol, whereas the ratio of tritium to  $C^{14}$  in the choline methyl was 69 to 75% of that in the methanol. Hence one might arrive at quite different interpretations of the possible biological pathways of methanol depending on whether tritium or deuterium was used as the labeling device.

The use of tritium as a label for hydrogen attached to carbon in the study of the reactions of intermediary metabolism has definite attractions since tritium can be detected in high dilution. Both tritium and deuterium are generally considered to be satisfactory tracers for carbon atoms or for the fate of carbon-linked hydrogen atoms when the reactions that such a labeled group undergoes do not involve the cleavage of the carbon-hydrogen bond. When a cleavage of the carbon-hydrogen bond occurs, the possibility always exists that a fractionation of the three hydrogen isotopes may result, since the zero point vibrational energy is smallest for the carbon-tritium bond and largest for the carbon-protium bond (isotope effect). These differences result in a lower activation energy for the rupture of the carbon-protium bond and consequently an increased reactivity for the lightest isotope of hydrogen.

As a step in the direction of finding out whether tritium could be used as a label for hydrogen it occurred to us that a study in the rat of methanols labeled with tritium, deuterium and  $C^{14}$  in the methyl group as precursors of the labile methyl group would be of interest. It had already been found that when methanol labeled with deuterium and  $C^{14}$  was used as a precursor of the labile methyl group in the rat, approximately one-fourth to one-third of the deuterium appeared in the methyl group of choline, relative to the amount of  $C^{14}$  appearing in this group.<sup>1</sup> The utilization of methanol for methyl synthesis was therefore interpreted as occurring through an oxidation and subsequent reduction to the labile methyl group. This was in contrast to the results which had been obtained with doubly labeled methionine in which the deuterium to  $C^{14}$  ratio was the same in the choline

methyl as in the methyl group of the administered methionine.<sup>2,3</sup>

If in the experiments with methanols containing  $C^{14}$ , deuterium and tritium there were no differences between tritium and deuterium, one would expect to find approximately one-fourth to one-third of the tritium in the methyl group of the isolated choline. On the other hand, if more tritium than deuterium should be retained, it would be evidence that selection had occurred. Instead of comparing deuterium and  $C^{14}$  in one animal and tritium and  $C^{14}$  in another, we decided to test the occurrence of isotopic selection in one animal by the administration of triply labeled methanol, *i.e.*, a mixture of  $C^{14}$ -methanol, deuteriomethanol and tritium-labeled methanol. The latter method made it possible to eliminate the uncertainties introduced by the biological variations accompanying the separate employment of deuterium and tritium labels in different animals.

### Experimental

**Synthesis of Tritium-labeled Methanol.**— $\beta$ -Naphthoic acid was dissolved in peroxide-free, dry dioxane, and tritiated water was added to the solution. Water and dioxane were then removed by distillation *in vacuo*. The carboxyl-tritiated  $\beta$ -naphthoic acid, dissolved in dry ether, was poured into an ethereal solution containing an excess of diazomethane prepared from nitrosomethylurea.<sup>3</sup> The excess of diazomethane and ether was then evaporated. The tritiated methyl  $\beta$ -naphthoate was then transferred to a glass tube; a slight excess of powdered, dry potassium hydroxide was added and mixed with the ester, and some ordinary methanol was added as a carrier. The sealed tube was heated in a boiling water-bath for several hours. The tube was then opened and the methanol was distilled *in vacuo* into a cooled trap.

The methanol so obtained was mixed with deuteriometh-

(2) E. B. Keller, J. R. Rachele and V. du Vigneaud, *J. Biol. Chem.* 177, 733 (1949).

(3) F. Arndt, *Organic Syntheses*, Coll. Vol. II, John Wiley and Sons, Inc., New York, N. Y., 1943, p. 165.

(1) V. du Vigneaud, W. G. L. Verly, J. E. Wilson, J. R. Rachele, C. Ressler and J. M. Kinney, *THIS JOURNAL*, 73, 2782 (1951).

TABLE I  
COMPARISON OF ISOTOPIC CONTENTS OF ADMINISTERED AND ISOLATED COMPOUNDS

Rat	Compound	Isotope content of methyl group of compound			D:C <sup>14</sup> in methyl group × 10 <sup>4</sup>	T:C <sup>14</sup> in methyl group	F <sub>D</sub> <sup>a</sup>	F <sub>T</sub> <sup>a</sup>	F <sub>T</sub> /F <sub>D</sub>
		Deuterium, atom % excess	Tritium, counts/min./std. filling × 10 <sup>-4</sup>	C <sup>14</sup> , counts/min./mM × 10 <sup>-4</sup>					
	Injected methanol	79.2	240	213	37.2	1.13			
J	TMCP <sup>b</sup>	0.550	5.7	6.77	8.12	0.84	0.22	0.75	3.4
	MCP <sup>c</sup>	.282	2.5	2.11	13.4	1.18	.36 <sup>d</sup>	1.05 <sup>d</sup>	2.9
F	TMCP	.458	4.33	5.6	8.18	0.77	.22	0.69	3.1
	MCP	.182	1.70	1.75	10.4	0.97	.28	0.86	3.1

<sup>a</sup> F<sub>D</sub> or F<sub>T</sub> are the fractions obtained by dividing the deuterium to C<sup>14</sup> ratio or the tritium to C<sup>14</sup> ratio, respectively, in the methyl group of the isolated compounds by the D:C<sup>14</sup> or T:C<sup>14</sup> in the methyl group of the administered methanol. <sup>b</sup> TMCP = trimethylamine chloroplatinate. <sup>c</sup> MCP = methylamine chloroplatinate. <sup>d</sup> The values for F<sub>D</sub> and F<sub>T</sub> for the methylamine chloroplatinate from animal J are probably too high, since a value for F<sub>T</sub> being greater than 1 would seem unlikely. The authors consider that this discrepancy may be due to contamination of the methylamine chloroplatinate with ammonium chloroplatinate. Calculation shows that a 10–20% contamination with ammonium chloroplatinate could exist without significantly affecting the platinum analysis, if the usual limits on a single determination of an elemental constituent are accepted. Calculation also shows, however, that the determination of C<sup>14</sup> is affected to a greater extent than the D or T analysis by the presence of ammonium chloroplatinate which would contain no C<sup>14</sup>, D or T. Calculation further shows that although the F<sub>D</sub> and F<sub>T</sub> values are considerably influenced by the respective isotope contents, the ratio of these values given in the last column of the Table is unaffected.

anol (CD<sub>3</sub>OD) and C<sup>14</sup>-methanol and after suitable dilution with ordinary methanol the isotopic analyses were performed.

*In Vivo* Experiment.—An aqueous solution of the methanol labeled in this manner was injected subcutaneously three times daily for a period of four days into two male rats, J (188 g.) and F (177 g.). The total amount injected was 23.3 millimoles and the molarity of the solution was 3.88 M. These animals were kept on the diet previously described,<sup>4</sup> and, prior to methanol injections, received intraperitoneally 10 μg. of vitamin B<sub>12</sub> and 500 μg. of folic acid.

The rats were then killed in an atmosphere of chloroform. Choline was isolated from the carcasses as the chloroplatinate, and creatine as the creatinine potassium picrate.<sup>5</sup> The choline chloroplatinates were degraded to trimethylamine, isolated as the chloroplatinate, and the creatinine potassium picrate was degraded to sarcosine, then to methylamine and isolated as the chloroplatinate.<sup>5</sup>

Rat	Choline chloroplatinate	Trimethylamine chloroplatinate	Creatine potassium picrate	Methylamine chloroplatinate
	<sup>3</sup> H <sub>2</sub> N <sup>+</sup> Or PtCl <sub>6</sub> <sup>-</sup> (Pt = 31.68)	C <sub>2</sub> H <sub>5</sub> N <sup>+</sup> PtCl <sub>6</sub> <sup>-</sup> (Pt = 36.96)	(% by Jaffe reaction)	C <sub>2</sub> H <sub>5</sub> N <sup>+</sup> PtCl <sub>6</sub> <sup>-</sup> (Pt = 41.35)
J	31.8	37.0	99	41.5
F	32.1	37.1	97	41.3

**Isotopic Analysis.**—For C<sup>14</sup> analyses, the chloroplatinates were decomposed with the Van Slyke–Folch oxidizing mixture<sup>6</sup> and the carbon dioxide was collected in a solution of sodium hydroxide. The methanol was burned in a current of oxygen and the carbon dioxide was collected in a similar manner. The carbon dioxide was precipitated as barium carbonate. The radioactivity was determined with a thin mica window Geiger–Müller tube in connection with a scaling unit. The results were corrected for background and self absorption.

For the deuterium analysis, the compounds were completely burned in a current of oxygen and the water of combustion was trapped in Dry Ice. In the case of the nitrogen-containing compounds, oxides of nitrogen were decomposed over hot copper in the absence of oxygen. The water vapor was converted completely to hydrogen over zinc at 400°. The deuterium determinations were made with a dual collector Nier type hydrogen mass spectrometer. Hydrogen gas samples of known deuterium content were used as standards in all deuterium determinations. In order to avoid memory effects in the determination of deuterium as well as tritium, three combustions and conversions were carried out for each analysis of isotopic content. The first combustion was used to season the glass lines and the hydrogen gas was discarded.

The gas obtained in the second and third combustion was analyzed. The reliability of the deuterium measurements is 1% of the measured deuterium content. The results in Table I are expressed as atom % excess, with 0.02% taken as normal abundance of deuterium.

In the analysis for tritium, the hydrogen gas from the combustion of the organic compound was obtained as described above. The Geiger–Müller counter tube was 18 mm. in diameter, had a silvered cathode and a 2-mil. diameter tungsten anode wire. The filling gas mixture contained the hydrogen gas to be analyzed (approximately 5 cm. pressure) and methane gas at approximately 65 cm. Measurements were carried out in the upper portion of the proportional counting region at approximately 3400 v. counting voltage.<sup>7</sup> A pulse amplifier and scaling circuit was used, the discriminator of which was set to accept all pulses greater than one millivolt. Under these conditions, the counting plateau is approximately 800 volts with a slope of less than 0.3% per 100 volts. The reproducibility of tritium determinations is better than 1%. In Table I, tritium activities are expressed as counts per minute per standard tube filling. The latter refers to a hydrogen gas pressure of 5.00 cm. at 298°K. in the single counter tube used throughout these experiments. This method of expressing tritium activities furnishes a precise measure of relative activity, since tube geometry factors remain constant. The approximate tritium activity in units of disintegrations per minute per millimole of hydrogen may be obtained by multiplying the tritium activities in Table I by the factor 5.7.

## Results and Discussion

The isotopic content of the methyl groups of the choline and creatine following administration of the isotopic methanol are compared with the isotopic content of the administered methanol in Table I. The results on the comparison of the ratio of deuterium to C<sup>14</sup> in the choline and the administered methanol are in fairly good agreement with those obtained in the earlier experiments with methanol labeled with C<sup>14</sup> and deuterium.<sup>1</sup> In the earlier experiments, the ratio of deuterium to C<sup>14</sup> in the choline was 25 to 31% of the ratio in the methyl group of the administered methanol, and in the present experiment, the value was 22%. However, the ratio of tritium to C<sup>14</sup> in the methyl group of the choline was 69 to 75% of that in the methyl group of the administered methanol. Hence one might arrive at quite different interpretations of the possible biological pathways of methanol depending

(4) V. du Vigneaud, W. G. Verly and J. E. Wilson, *THIS JOURNAL*, **72**, 2819 (1950).

(5) V. du Vigneaud, C. Ressler, J. R. Rachele, J. A. Reyniers and T. D. Luckey, *J. Nutrition*, **45**, 361 (1951).

(6) D. D. Van Slyke and J. Folch, *J. Biol. Chem.*, **136**, 509 (1940).

(7) M. L. Eidinoff, *Proc. Am. Inst. Elec. Engrs.*, Second Joint Conference on Electronics in Nucleonics and Medicine, 65 (1950).

on whether tritium or deuterium was used as the labeling device.

Thus it may be noted from the last column of Table I that the deuterium and tritium were not equally diluted in the biological reactions but that relatively more deuterium was lost than tritium by the factors given in the table. Although certain interpretations of the results can be made, the mechanisms of the reactions involved are not understood. It is therefore impossible to account quantitatively for the results in Table I. However, if it were assumed that the three isotopes were indistinguishable completely in these reactions, then the results in the last column would be unity. Since this is not the case, we may conclude that selection is involved. It is planned to carry out similar experiments with methanol in which the deuterium-containing group is  $\text{CH}_2\text{D}$ , similar to the tritium-containing group  $\text{CH}_2\text{T}$ .

There are at least two important aspects of the problem of selection. On the one hand, there is the case in which the different isotopes of hydrogen are actually attached to the same carbon, and selection may occur in the cleavage of the carbon-hydrogen bond. On the other hand, there is the case in which one molecule contains deuterium or tritium and the other only protium. A particularly interesting case would be the one in which all the

hydrogen in one molecule is replaced by deuterium and in the other is entirely protium. There may be selection *metabolically* between these isotopically different *molecules*. In experiments in which a change in isotopic ratio occurs, as in the case of methanol, selection of both these types must be considered. It should, however, be pointed out that when no change in ratio is obtained, as in the earlier experiments in which the doubly labeled methyl group of methionine served as the source of the methyl group of choline, one may conclude that the cleavage of the carbon-hydrogen bond followed by reduction is not a significant pathway and that no appreciable selection between the molecular species  $-\text{S}-\text{C}^{14}\text{H}_3$  and  $-\text{S}-\text{C}^{12}\text{D}_3$  has occurred, unless the possibility obtains of fortuitous compensation.

It is of interest in connection with the work reported herein that Thorn<sup>8</sup> found that tetra-deuteriosuccinic acid and  $\alpha,\alpha'$ -dideuteriosuccinic acid were oxidized by the succinic acid oxidase system at only 40 and 70%, respectively, of the rate at which ordinary succinic acid was oxidized.

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(8) M. B. Thorn, *Biochem. J.*, **49**, 602 (1951).

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