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

Isotopic Selection in the Neogenesis of Labile Methyl Groups from Monodeuterio-, Monotritio-, C^{14}-Labeled Methanol

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This study demonstrates that the mode of labeling a multiple hydrogen-bonded carbon in a biological precursor with deuterium or tritium has a pronounced effect on the quantitative results of a metabolic experiment designed to show the fractionation of the hydrogen isotopes *in vivo*. A fractionation factor of the order of 1.3 was obtained for tritium *versus* deuterium when methanol containing CH_2D, CH_2T and $C^{14}H_3$ as methyl groups is converted in the rat to the methyl groups of tissue choline and creatine. In an earlier study where methanol labeled with CD_3, CH_2T and $C^{14}H_3$ methyl groups was administered, the magnitude of the same fractionation factor was about 3. Formate, isolated from urine in the present study, showed a fractionation factor of about 1.7 between tritium and deuterium.

The usefulness of deuterium or of tritium as a label for carbon-bonded hydrogen in reactions where a cleavage of the carbon–hydrogen bond can occur depends on the magnitude of selection among the isotopes of hydrogen. In a previous study,[1] a test of the use of tritium in a metabolic experiment was carried out in order to establish whether tritium, with its desirable feature of detectability at high dilution, could be used as a label for hydrogen attached to carbon. Toward this end in view, methanol, triply labeled by admixture of CD_3OH, CH_2TOH and $C^{14}H_3OH$, was administered to rats. The choline and creatine of the tissues were isolated and were analyzed to measure the incorporation of the isotopic labels of the administered methanol in the neogenesis of labile methyl groups. It had been established previously[2] that, when methanol doubly labeled with deuterium and radiocarbon was administered, the carbon of the methanol appeared in the labile methyl groups with only one-quarter to one-third the deuterium. It appeared from this earlier experiment that methanol might be utilized for labile methyl synthesis through a series of reactions involving the rupture and re-formation of carbon–hydrogen bonds.

Since the cleavage of the carbon–hydrogen bond differs in energy requirements with the particular hydrogen isotope present, the experiment with the triply labeled methanol cited above served to compare the relative isotope effects of deuterium and tritium. The results of the experiment with the triply labeled methanol showed that there was about a threefold greater degree of incorporation of the tritium than there was of deuterium into the methyl groups of choline and creatine of the tissues. On the basis of this large selection between deuterium and tritium, it was conceivable that differing interpretations of possible biological pathways could result depending on whether deuterium or tritium were used in a metabolic experiment.

The possibility existed that the relatively large selection between deuterium and tritium could arise from the fact that the behavior of the CD_3 group was being compared with CH_2T group in the methanol. In order to answer this question, it was decided to prepare methanol containing CH_2D as the only deuterated species and to measure the selection between deuterium and tritium for a precursor mixture of methanol containing CH_2DOH, CH_2TOH and $C^{14}H_3OH$. In addition to the isolation and analysis of the methyl groups of choline and creatine, the isotopic content of the urinary formate was measured to obtain further information regarding isotopic selection.

Experimental

Preparation of Labeled Compounds: 1. Methyl-*d* Alcohol.—Monodeuteriomethanol, CH_2DOH, was prepared by modifying and extending a series of reactions developed by Bannard, Leitch and Morse[3,4] for the preparation of deuterioformaldehyde.[5] 1,2-Dibromoethane-1,2-d_2 was prepared by the method of Leitch and Morse[3] by allowing non-isotopic acetylene to react with deuterium bromide, generated from freshly distilled phosphorus tribromide and deuterium oxide (99.8 atom % D).[6] Deuterium analysis showed that the resulting purified ethylene bromide contained 49.6 atom % D.

The ethylene bromide was converted to the diacetate and the latter, then, to the glycol by the procedure described by Bannard, Morse and Leitch.[4] The resulting ethylene-1,2-d_2-glycol was dissolved in water. The solution of the glycol was chilled in ice and oxidized by the dropwise addition of an excess of a periodic acid solution. The mixture was allowed to stand for one hour at room temperature. Assay of the reaction mixture by iodometry showed a quantitative formation of formaldehyde. The iodic acid and excess periodic acid were neutralized and removed by treating the reaction mixture with a barium hydroxide solution, and excess barium ions were removed by passing carbon dioxide through the suspension. The resulting suspension was centrifuged, and the supernatant, containing the deuterioformaldehyde, CHDO, and the washings were combined.

The reduction of the deuterioformaldehyde in aqueous solution was accomplished by the method of Chaikin and Brown[7] with sodium borohydride which was added dropwise as a solution in 1 *N* NaOH. The reaction mixture was allowed to stand for 30 minutes at room temperature. Then excess borohydride was decomposed with 1:1 H_2SO_4 until the solution became acid to congo red.

The monodeuteriomethanol was removed as an aqueous solution from the reaction mixture by distillation. Methoxyl determination on aliquots of the distillate showed that the borohydride reduction of the formaldehyde and the distillation procedure were quantitative. The methyl-*d* *p*-nitrobenzoate was prepared from a small portion of the distillate. The m.p. of the ester, purified by vacuum sublimation, was 96–97° (micro, corrected). The deuterium analy-

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

(3) L. C. Leitch and A. T. Morse, *Can. J. Chem.*, **30**, 924 (1952).

(4) R. A. B. Bannard, A. T. Morse and L. C. Leitch, *ibid.*, **31**, 351 (1953).

(5) We wish to acknowledge our appreciation to Drs. Bannard, Leitch and Morse of the Defense Research Chemical Laboratories, Ottawa, Canada, for making available to us prior to publication their experimental details for the preparation of dideuterioethylene bromide, and its corresponding diacetate and glycol.

(6) We wish to thank Dr. Bertram Lowy for the preparation of the dideuterioethylene bromide.

(7) S. W. Chaikin and W. G. Brown, THIS JOURNAL, **71**, 122 (1949).

sis of the ester gave a value of 13.7 atom % excess D. The methanol from which the ester had been prepared was calculated, therefore, to contain 32.0 atom % excess of deuterium in the methyl group.

The main portion of the aqueous methanol solution, which was used to prepare the triply labeled methanol for injection, contained 0.186 g. of methyl-*d* alcohol per g. of solution as determined by methoxyl analysis.

2. **Methyl-*t* Alcohol.**—The tritiated methanol used in this expt. is the same preparation as employed in the previous study.[1]

3. **Methyl-C^{14} Alcohol.**—C^{14}-Labeled methanol,[8] with a specific activity of 1 millicurie per millimole, was diluted by distilling 29.72 mg. of the methanol through a vacuum manifold into 19.988 g. of water. To 9.165 g. of this aqueous C^{14}-methanol were added 230 mg. of unlabeled methanol. Therefore the resulting solution, which was used for preparing the methanol solution for administration, contained 0.815 mmole of methanol per g. of solution.

4. **Triply Labeled Methanol.**—The triply labeled methanol that was administered was prepared by admixture of the three isotopic methanols mentioned above. To 8.84 g. of the methyl-*d* alcohol solution was added 1.00 g. of the methyl-C^{14} alcohol solution. To this mixture, 0.13 g. of the tritiated methanol was transferred *in vacuo*. From a portion of the final aqueous solution of the three methanols, the methyl *p*-nitrobenzoate was made for the purpose of determining the isotopic ratios of the methanol solution administered to the rats.

Administration of the Triply Labeled Methanol.—The aqueous solution of the triply labeled methanol was injected subcutaneously three times daily for four days into two male rats, no. 526 (168 g.) and no. 530 (177 g.). During this period each animal received 23 millimoles of methanol. One hour previous to the first methanol injection, each rat received 500 μg. of folic acid and 10 μg. of vitamin B_{12}. The animals were kept on a diet previously described by du Vigneaud, Verly and Wilson.[9] The combined urine of both rats was collected during this period.

At the end of four days the animals were sacrificed in an atmosphere of chloroform. Rat 526 had lost 26 g., and rat 530, 20 g. During the latter part of the experimental period the animals refused the food which was given *ad libitum*. The loss in weight may have been due to this fact rather than to a specific action of the injected methanol. A necropsy was performed on each animal with no obvious signs of tissue damage.

Isolation of Compounds.—Choline was isolated as the chloroplatinate, and creatine as creatinine potassium picrate.[10] The isolated compounds were degraded, respectively, to trimethylamine and methylamine, which were obtained finally as the crystalline chloroplatinates.

Urinary formate was isolated as the lead salt without the addition of carrier formate according to the following procedure. The combined urine of both rats was acidified with phosphoric acid and distilled into 10 ml. of 5 *N* NaOH. The alkaline distillate was then concentrated to dryness on a boiling water-bath, volatile, non-acidic substances being removed by this process. In order to separate the low boiling methyl esters of organic acids, the dry white residue was dissolved in water, and to the solution, acidified with phosphoric acid, was added 5 ml. of methanol.[11] The mixture was distilled and all the material boiling at 65° and below was collected in a NaOH solution, chilled in an ice-bath. The alkaline solution which finally contained methanol and the sodium salts of the acids of the low boiling esters was concentrated to dryness on a water-bath.

Formate in the residue was separated by a modification of the chromatographic technique for separation of organic acids described by Bulen, Varner and Burrell,[12] using a silicic acid column and 5:95 *n*-butanol–chloroform, equilibrated with 0.5 *N* H_2SO_4, as the eluting solvent. Each individual fraction of *ca.* 7 ml. of eluate was titrated with standardized 0.01 *N* NaOH, after the addition of 3 ml. of water and of phenol red indicator.

The formate fractions, which required a total of 1.16 milliequivalents of alkali, were combined and the butanol–chloroform layer was separated and discarded. The neutral aqueous layer was made slightly alkaline and concentrated to dryness under reduced pressure. The residue, colored red by the indicator, was dissolved in a small amount of water and the solution was filtered. Filtrate and washings were concentrated to a volume of about 1 ml. and made just acid with dilute HNO_3. Lead nitrate in slight excess to the amount equivalent to the formate as determined by the titration of the formate peak, in 1 ml. of water was added to the formate concentrate. Lead formate crystallized from solution immediately and was precipitated more nearly completely by addition of 10 ml. of absolute ethanol. After the mixture had been chilled in an ice-bath for a few hours, the lead formate was separated by filtration and washed with absolute ethanol. Recrystallization removed traces of lead sulfate and resulted in fine white needles of lead formate, which were washed with absolute ethanol and dried *in vacuo* over P_2O_5. Analysis for the lead content by ignition to PbO gave 75.5% PbO (theory: 75.3% PbO from $Pb(OOCH)_2$).

Isotopic Analysis.—All compounds were oxidized completely by combustion in a stream of oxygen. The water of combustion was collected in a Dry Ice–cellosolve chilled trap and the CO_2 in a liquid nitrogen trap. Water from nitrogen-containing compounds was passed over hot copper in the absence of oxygen in order to decompose the oxides of nitrogen. Hydrogen gas was produced by passing all the water vapor over zinc at 400°[13]; deuterium and tritium analyses were performed on this gas.[1] A measured amount of the trapped CO_2 was distilled into a Geiger–Müller counter tube by means of liquid nitrogen. The tube was filled to its counting pressure with non-isotopic CO_2, and CS_2 was added as a quenching gas.[14] The results of the isotopic analyses are recorded in Table I.

The analytical procedure described above provided for a quantitative estimation of the carbon content of the combusted compound by a manometric measurement of the CO_2 pressure in a calibrated volume. The recovery of CO_2 from a weighed sample, therefore, was an index of the purity of the sample combusted. In all the compounds analyzed, the recovery of carbon deviated not more than 2% from the calculated theoretical yield.

Discussion

The isotopic content of the methyl groups of choline and creatine are compared with the isotopic content of the methyl groups in the administered methanol in Table I.

The results in the last column of the table directly measure the extent to which the isotope of mass 3 is preferentially retained in the over-all transformation of the administered methyl group into the methyl groups of choline and creatine. If no selection had taken place between the two isotopically labeled methanols, or between labeled intermediates, the F_T/F_D value would have been expected to be unity. The results, ranging from 1.26 to 1.32, show that there exists a substantial isotopic selection or fractionation between D and T when the CH_2DOH, CH_2TOH and CH_3OH mixture is administered under conditions described in the experimental portion of this paper. However, the preferential retention factor for tritium relative to deuterium (F_T/F_D) is considerably closer to unity than the values ranging between 2.9 and 3.4 which were observed in the earlier experiment[1] when the deuterium-containing methyl precursor was mainly CD_3. Since the methanols ad-

(8) The radiocarbon labeled methanol (purchased from Tracerlab, Inc.) and the tritium gas used for preparing the tritiated methanol were obtained on allocation from the U. S. Atomic Energy Commission.

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

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(14) M. L. Eidinoff, *ibid.*, **22**, 529 (1950).

TABLE I

COMPARISON OF ISOTOPIC CONTENTS OF ADMINISTERED AND ISOLATED COMPOUNDS

Rat	Compound	Isotope content of methyl group of cpd.[a] Deuterium, atom % excess	Tritium, counts/min./std. filling $\times 10^{-4}$	C^{14}, counts/min./mmole carbon $\times 10^{-4}$	D:C^{14} $\times 10^{5}$	T:C^{14}	F_D[b]	F_T[b]	F_T/F_D
	Injected methanol	27.9	177.0	71.0	3.93	2.50			
526	TMCP[c]	0.367	2.92	1.46	2.51	2.00	0.640	0.804	1.26
	MCP[d]	.126	1.06	0.731	1.72	1.45	.439	.581	1.32
530	TMCP	.312	2.51	1.15	2.71	2.18	.690	.875	1.27
	Lead formate[e]	5.59	60.8	12.3	4.54	4.95	1.16	1.98	1.72

[a] In the case of the lead formate, the isotope content of the formyl moiety is listed. [b] F_D or F_T are the fractions obtained by dividing the deuterium to C^{14} ratio or the tritium to C^{14} ratio, respectively, of the isolated compounds by the D:C^{14} or T:C^{14} in the methyl group of the administered methanol. [c] TMCP = trimethylamine chloroplatinate. [d] MCP = methylamine chloroplatinate. [e] Formate isolated from pooled urine of both rats.

ministered in the two expts. differed qualitatively only in the principal deuterated species, *i.e.*, CD_3OH of the earlier study and CH_2DOH of the present, the difference between the selection factors, F_T/F_D, must be attributable to the kind of deuteriomethanol molecule involved in each expt.

The results for F_D and F_T in Table I represent the dilution of deuterium and tritium relative to the C^{14} content in the transformation from the methyl group precursor to the methyl group in the product compounds. As in the expt. using CD_3OH as precursor[1] the F_T values are always higher than the F_D values, a result in qualitative agreement with the interpretation that the tritium is preferentially retained with respect to the deuterium in the overall transformation. The F_D value of unity observed in the transfer of the methyl group of methionine to choline and creatine was interpreted as evidence for the transfer of the methyl group as a unit.[15] When *methanol* containing $C^{14}H_3$ and mainly CD_3 groups was used as a precursor[1,2] the F_D values in the range 0.22 to 0.36 were interpreted as evidence for the utilization of at least a part of the methyl group as a precursor of a biologically labile methyl group *via* an oxidized intermediate during which step deuterium was partially lost. Thus, for example, if oxidation to the intermediate involved the loss of one hydrogen atom, F_D and F_T would both be expected to equal 0.67 provided that all the isotopes of hydrogen were completely indistinguishable in their reactivity. Similarly, if the loss of two hydrogen atoms were involved in the formation of the intermediate (oxidation *via* "formate") then F_D and F_T would both be expected to be equal to 0.33.

It does not appear possible to calculate the isotope selection factors F_T/F_D from theoretical considerations, since the identity of the intermediates and the possible side reactions of methanol and of each intermediate are not known. A substantial fraction of the administered methyl groups was transformed to carbon dioxide[16,17] and to other metabolic products[18-20] while a relatively small fraction was converted to choline and creatine. Isotope fractionation effects in these processes would be reflected in the F_D and F_T values found for choline and creatine in a manner that would depend on the relative reaction rate constants and the extent of utilization of the precursor methyl groups in each of these pathways.[21]

The presence of two protium atoms in the precursor CH_2T and CH_2D groups may be considered a factor leading to the preferential retention of the heavier isotope, for the selective rupture of any C–H bonds results in intermediates enriched in C–D bonds and in C–T bonds. This is in contrast to the case for CD_3, since the cleavage of a C–D bond in CD_3 does not result in an intermediate enriched in deuterium. If these are the major considerations, then a larger selection factor between T and D could be expected when CD_3 is used as compared with the case when CH_2D is used. The result of these expts. are in qualitative accord with this argument.

Further evidence for the selection of the hydrogen isotopes is given by the results in the last line of Table I. The F_T/F_D ratio for the formate isolated from the urine following the administration of the labeled methanol is 1.72, representing a preferential retention of the mass 3 isotope. If C–H bonds are ruptured in preference to C–D bonds when the carbon of the labeled methanol is oxidized to any level of oxidation, *i.e.*, that of formaldehyde, of formate, or of CO_2, the precursors of formate or formate *per se* would be enriched with respect to deuterium. Since the C^{14} atoms of the labeled methanol are linked only to protium (neglecting the normal abundance of deuterium), the selection of C–H bonds for rupture would result in an F_D value greater than unity for the urinary formate if one assumes no significant isotope effect due to C^{14}. The observed F_D of 1.16 is in accord with this view and may be considered as evidence for the isotopic selection between protium and deuterium. Similarly the observed F_T value of 1.98 is evidence for selection between protium and tritium.

The results of the present experiment are significant in at least two respects. A considerable degree of fractionation, although not as great as in the previous experiment with triply labeled methanol, was still observed between molecules of metha-

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(21) The authors wish to acknowledge a personal communication from Dr. L. Kaplan, of the Argonne National Laboratory, concerned with assumptions that could in theory be used to evaluate F_D or F_T.

nol *comparably* labeled with deuterium or tritium. Thus, in metabolic studies involving reactions where such fractionation may occur, conclusions regarding the possible pathways of a compound depend on the particular isotope of hydrogen chosen as the tracer, since the data obtained under identical conditions with a compound labeled identically with either deuterium or tritium allow different interpretations.

The second significant point, related closely to the isotope effect, is concerned with the ambiguity that can arise from replacing different numbers of equivalent hydrogen atoms in the individual molecules of a metabolite with the same labeling isotope of hydrogen. If one allows that the change in the $D:C^{14}$ ratio observed between the methyl of methanol and the methyl of tissue choline and creatine is due primarily to a *metabolic* change in the hydrogen–carbon ratios during intermediary steps, then the results of the previous experiments[1,2] utilizing the CD_3-species of methanol indicate the possibility that "formate" is an intermediate in the neogenesis of methyl from methanol whereas the present experiment with the CH_2D- species shows that labile methyl arises *de novo* from a "formaldehyde" intermediate without passage through a "formate" step. On the other hand, it can be seen that the deuterium content of formate isolated in this experiment from the total pooled urine has been increased relative to C^{14}. The ratio of $D:C^{14}$ in the methyl of tissue choline, that is suggestive of a "formaldehyde" precursor, could be a reflection of deuterium enrichment at all intermediary steps including a "formate" level. Thus "formate" as a required intermediate would not be ruled out. In view of the large degree of selection among the hydrogen isotopes during these metabolic reactions, it is not possible, from the type of experimentation employed in the present or the previous isotopic studies, to make an unequivocal deduction with regard to a particular intermediate.

It should be pointed out that the multiple labeling of carbon and hydrogen employed in the above mentioned experiments was intermolecular as a result of admixture of separately labeled species of methanol. The double labeling of a carbon and its bonded hydrogen within the same molecule, *i.e.*, intramolecularly, should minimize the influence of hydrogen isotope selection on the ratio of isotopic hydrogen to isotopic carbon. Any change in this last named ratio should be more an indication of the nature of intermediates and less a reflection of isotopic selection. However, if more than one hydrogen atom is linked to the carbon-labeling atom, *e.g.*, C^{14}, in a molecular grouping under study, the possibility of fractionation between the lighter and the heavier isotopes of hydrogen within the molecular grouping, and therefore between labeled carbon and labeled hydrogen, still can exist. The ideal case would be that in which the carbon-labeling atoms *only* are directly linked to only hydrogen-labeling atoms in pertinent carbon–hydrogen bonds. At the present time, this objective in labeling can be approached most closely with the available concentrations of deuterium, tritium and C^{13}, and to a lesser degree with the available C^{14}. Experiments in which metabolites are doubly labeled with isotopes of carbon and hydrogen within the same molecule have been undertaken in one of these laboratories (CUMC).

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The Polarography of Vitamins B_{12r} and B_{12a}

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Unlike vitamin B_{12}, which undergoes a single two-electron, polarographic reduction, B_{12a} undergoes two one-electron reductions corresponding to changes in valence of the cobalt from three to two and from two to one. Vitamin B_{12r}, in which the cobalt is bivalent, shows one anodic and one cathodic wave agreeing in position and height with the waves of B_{12a}.

The catalytic hydrogenation of an aqueous solution of vitamin B_{12} yields a brown solution containing a reduced cobalt compound[1] which has been designated as B_{12r}.[2] Potentiometric titration of B_{12r} with potassium ferricyanide in a completely oxygen-free atmosphere has indicated that one equivalent is required to oxidize the B_{12r}; the cobalt atom is thus in the bivalent state.[2] During hydrogenation the cyanide group of B_{12} is reduced to methylamine.[3] The polarography of vitamin B_{12r} has been reported briefly[4] and there also has been described the polarographic behavior of a related but distinctly different product resulting from the action of chromous ethylenediaminetetraacetate on B_{12r}.[5]

In the present paper the polarographic behavior of B_{12r} is given in further detail. In particular the polarogram has been extended into the region positive to the saturated calomel electrode by using potassium sulfate as the supporting electrolyte and another wave has been found having a half-

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