TRITIUM-LABELLING OF LIPIDS

V.P.Shevchenko and N.F.Myasoedov

Institute of Molecular Genetics,

USSR Academy of Sciences, Moscow

SUMMARY

Tritium-labelled lipid preparations are obtained by heterogeneous isotopic exchange with gaseous tritium in solution. After appropriate purification, the localization of the label was determined in the main fragments of $[^3H]$-compounds. The obtained data on specific radioactivity and the pattern of label localization allow an insight into processes taking place on labelling under particular experimental conditions.

INTRODUCTION

Labelled compounds are at present widely used in studies of biosynthesis, metabolism and localization of various classes of biologically active compounds in living cells. It is often required to label complex compounds isolated from natural sources. The use of traditional tritium-labelling methods[1,2] is often impeded due to complexity of structure and limited quantities of such compounds. In this respect the method of heterogeneous catalytic isotopic exchange with gaseous tritium in solution seems very promising[2]. This method offers a convenient means of quick preparation of labelled compounds, introduction of the label

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under relatively smooth conditions, dealing with relatively low quantities of gaseous tritium and minimal quantities of starting material. Compounds labelled by this method usually have a high chemical and radiochemical purity simplifying their purification. However using this method of tritium-labelling sometimes causes the reduction of unsaturated bonds. Therefore it is of interest to choose optimal conditions for isotopic exchange for each class of biologically active compounds.

The present study has been undertaken to examine some regularities of tritiation of unsaturated lipid preparations and its possible mechanisms. Such phenomena as cis-trans-isomerization, migration and reduction of double bonds are also studied. As a result some conclusions are made as to applicability of the method of heterogeneous isotopic exchange for preparation of labelled biologically active compounds.

MATERIALS

We used the following commercial products: stearic, oleic, linoleic, elaidic, 8, 11, 14-eicosatrienoic and arachidonic acids, prostaglandins $F_{1\alpha}$ ($PGF_{1\alpha}$) and E_2 (PGE_2), cholesterol. A number of preparations were isolated from natural sources according to standard techniques. Eqq phosphatidylcholine (eqq PC) from chicken eqq yolk[3] was further treated with phospholipase A_2[4] to obtain lyso-PC. We used sphingomyeline from bovine brain[5], phosphatidylinositol (PI) from baker's yeast[6]. Natural sphingolipids: galactosylceramide (GC)[7], N-acetylneuraminosyl-(α,2 → 3) galactosyl- (β,1 → 3)-N-acetylgalactosaminyl (β,1 → 4) galactosyl/ (α,2 → 3)-N-acetylneuraminosyl/ (β,1 → 4)

glucosyl- (1 → 1) ceramide (G_{D1a})[8] and galactosyl (β,1 → 3) N-acetylgalactosaminyl (β,1 → 4)/ N-acetylneuraminosyl - (α,2 → 8)N-acetylneuraminosyl (α,2 → 3)/ galactosyl (β,1 → 4)-glucosyl (1 → 1) ceramide (G_{D1b})[8] were isolated from bovine brain. N-glycolylneuraminosyl-(α,2 → 6) - glycosyl - (1 → 8) - N-glycolylneuraminosyl (2 → 6)-glycosul (1 → 1)-ceramide (G_1)[9] and 8-sulpho-N-glycolylneuraminosul--(α,2 → 6)-glucosyl-(1 → 8)-N-glycolylneuraminosyl-(2 → 6)--glucosyl(1 → 1)-ceramide (G_2)[9] from ovary cells of sea urchin Strongylocentrotus intermedius were provided by the Laboratory of lipids of the M.M.Shemyakin, Institute of Bioorganic Chemistry, Academy of Sciences of the USSR. Methyl esters of fatty acids (MEFA) were obtained by treating the above-cited fatty acids with diazomethane according to procedure described earlier[10]. Synthesis of DL-1-O-(1'-palmitoyl-2'-oleoyl-sn-glycero-3'-phosphoryl)-myo-inositol-4-phosphate (PIP) was performed according to procedures described in[11,12].

METHODS

Basic procedure

Unlike amino acids, peptides, proteins, nucleic acids and carbohydrates, lipids are easily soluble in non-aqueous solvents. Therefore solvents for reactions were chosen considering the solubility of each particular lipid and the preference was usually given to dioxane whose molecule contains no labile hydrogen atoms.

The best results (Tables 1,2) were obtained when saturated lipid compounds were tritiated using 5-7% palladium on barium sulphate as a catalyst, the substance-palladium molar ratio was

(1:2), the reaction time was 4-6 hours[13,14]. With unsaturated compounds we used Lindlar catalyst, the substance-palladium molar ratio was (1:1), the reaction time was 1-3 hours[15-19].

Reaction with gaseous tritium

Reaction tube was loaded with organic compound (1-50 mg) dissolved in 0,1-2 ml solvent, and the necessary amount of catalyst was added, the tube frozen with liquid nitrogen, evacuated up to the pressure of 1×10^{-3} mm of Hg and filled with tritium up to 250 mm of Hg. The reaction was carried out at 23°C with continuous stirring. Then the tube was again frozen and the excess of tritium was removed. The catalyst was filtered and labile tritium removed by evaporation using protic solvents in vacuum. The labelled products were purified according to standard chromatographic techniques. The products of reduction, cis- and trans-isomers were separated on argentinized carriers[20-22].

Localization of label

Localization of the tritium label in MEFA, fatty acids and prostaglandins was determined after periodate-permanganate oxidation[15,23-25] while in phospholipids and in sphingolipids - after acidic methanolysis[26,27] or hydrolysis[27,28] followed by chromatography on silica gel plates of the products of degradation[15,24,29] and measuring of radioactivity of the new-formed fragments. Migration of the double bond in methyl esters of octadecenoic acids was studied in the following way[15]. After periodate-permanganate oxidation of 1-2 mg of substance carboxylic acids were extracted from water solution containing sodium chloride (saturation aqueous NaCl - H_2O, 1:1) by ethyl acetate (3 x 1ml). The

organic layer was washed with saturated aqueous NaCl - 0,1 *N* HCl, filtered and evaporated to dryness at -30° in current of argon. The resudue was methylated with small excessive quantities of diazomethane and the resulting methyl esters were analyzed by gas-liquid chromatography (GLC) (Table 3).

Analytical methods

GLC method was used to analyse the purity of the starting material and labelled products before and after their purification, to analyse the products of periodate-permanganate oxidation, and to determine the quantities of the products of reduction. The Varian 2100 Chromatograph (U.S.A.) with a column of 2 x 2000 mm with 3% SE-30 and the W-HP Chromosorb (80-100 mesh) with helium consumption of 30 ml/min was used.The analysis of periodate-permanganate oxidation products, MEFA and cholesterol was performed in an isotherm at respectively 70°, 170° and 250°C. A fatty--acid fraction of tritium-processed phospholipids was analysed after their alkaline deacylation[12]. The radioactivity content was measured using a scintillation counter registering tritium with $\sim$ 12% efficiency in a dioxane scintillator[30].

RESULTS AND DISCUSSION

With tritium introduced into MEFA and prostaglandins (Table 1) the yield of compounds of natural structure was about 30%, the specific activities of labelled preparations were about 1 Ci/mmol. If the reduction of double bonds due to tritium was partial, the yields of labelled preparations (sphingo- and phospholipids) usually exceeded 50% and specific radioactivities were in the order of 10 Ci/mmol (Table 2). The ability of palladium to catalyze

TABLE I. Data on isotopic exchange between 80% tritium and methyl esters of unsaturated fatty acids and prostaglandins in dioxane on the Lindlar catalyst and localization of the label in these preparations as well as in fatty acids obtained by alkaline hydrolysis of the corresponding methyl esters.

compound	time of reaction (h)	reaction products	yield (%)	specific radioactivity (Ci/mmol)	Localization of the label in fragments after periodate-permanganate oxidation				
					double bond	dicarboxylic acids	monocarboxylic acids	fragments with a cyclopentane ring	malonic acid
		methyl stearate	8	26,3	-	-	-	-	-
methyl oleate	1	cis-isomer	30	3,96	45	55		-	-
		trans-isomer	56	5,94	49	51		-	-
methyl linoleate	3	cis-isomer	29	0,68	-	-	-	-	-
methyl eicosatrienate	-"-	-"-	26	0,92	-	-	-	-	-
methyl arachidonate	-"-	-"-	36	2,50	14	47		-	39
		PGE_1	4	25,4	-	-	-	-	-
PGE_2	1	cis-PGE_2	26	1,74	30	22	30	18	-
		trans-PGE_2	43	2,09	54	12	23	11	-
		PGE_1	7	25,4	-	-	-	-	-
PGE_2	3	cis-PGE_2	3	2,94	36	17	36	11	-
		trans-PGE_2	14	3,16	50	11	29	10	-
$PGF_{1\alpha}$	-"-	$PGF_{1\alpha}$	73	1,85	14	-	53	33	-
oleic acid	1	-	95	3,32	54	23	23	-	-
trans isomer from oleic acid	-"-	-	95	4,46	64	13	23	-	-
Linoleic acid	3	-	85	0,48	-	-	-	-	-
eicosatrienoic acid	-"-	-	89	0,63	-	-	-	-	-
arachidonic acid	-"-	-	94	1,34	27	12	28	-	33

TABLE 2. Data on isotopic exchange between 100% tritium, polar lipids and cholesterol. Localization of the label in these preparations (23°C, 3 hours)

compounds	catalyst	solvent	yield (%)	specific radioactivity (Ci/mmol)	localization of the tritium label	
					fatty acids	sphingosine bases
cholesterol	Lindlar catalyst	dioxane	65	0,045	-	-
egg PC	-"-	-"-	75	0,79	81	-
	5% $Pd/BaSO_4$	-"-	67	6,11*	94	-
	$(Ph_3P)_3RhCl$	-"-	87	14,60*	97	-
lyso-PC	5% $Pd/BaSO_4$	methanol	48	3,90*	92	-
yeast PI	-"-	chloroform	61	13,56*	89	-
	$(Ph_3P)_3RhCl$	-"-	94	0,91*	94	-
sphingomyeline	Lindlar catalyst	ethyl acetate	80	5,88*	64	35
PIP	5% $Pd/BaSO_4$	chloroform-methanol-water (5:5:1)	81	3,58*	95	-
	-"-	chloroform-methanol(2:1)	63	10,53*	97	-
ganglioside G_{D1a}	-"-	dioxane	36	3,10*	8	82
-"- G_{D1b}	-"-	-"-	31	3,70*	-	-
-"- G_1	$(Ph_3P)_3RhCl$	-"-	51	4,30*	-	-
-"- G_2	-"-	-"-	72	2,90*	80	19
GC	5% $Pd/BaSO_4$	-"-	74	52,40*	34	60
-"-	Lindlar catalyst	-"-	90	24,20*	67	33

* The final product contained products of tritium hydrogenation.

the exchange of protium for tritium appeared to increase in parallel with its ability to bind tritium via double bonds, the hydrogenation by protium being faster than the double bond reduction by gaseous tritium[18]. Investigation proved that the major part of the label in phospholipids is contained in their fatty acid moiety (Table 2) while in sphingolipids - in both fatty acid sphingosine moieties of molecules (Table 2).

We investigated the fatty acid composition of egg phosphatidylcholine (Table 2) before and after tritium treatment under identical conditions on Lindlar catalyst, 5% $Pd/BaSO_4$ and $(Ph_3P)_3$ RhCl. There was no tritium hydrogenation with Lindlar catalyst, whereas with 5% $Pd/BaSO_4$ and $(Ph_3P)_3RhCl$ the hydrogenation amounted to 15% and 35% respectively. Yet, partial hydrogenation of bonds in natural phospholipids has little effect upon their biological properties, for in a natural object these substances are already a mixture of more or less saturated products. The important thing is that even with no hydrogenation at all the bulk of the label was contained in the fatty acid moiety of egg PC (81%).

As a rule the above-mentioned degree of hydrogenation does not change the physiological properties of such lipid compounds. This may be demonstrated directly by biological experiments in vitro. For instance, sphingomyelin with a $\sim$10% content of tritium hydrogenation products was used in a study of the sphingomyelin-transferring activity of postmicrosomal proteins of rat hepatoma at the Shemyakin Institute of Bioorganic chemistry, USSR Academy of Sciences. In this sort of study the compound proved interchangeable with ^{14}C-sphingomyelin obtained biosynthetically. During isotopic exchange involving compounds like

methyl arachidonates, the major part of the tritium label tends to incorporate into allylic positions. A decrease in specific radioactivity on alkaline hydrolysis of methyl arachidonate is due mainly (to 78 per cent) to the exchange of tritium occupying allylic positions with the protons from the medium. The major part of the label in unsaturated compounds is likely to occupy allylic positions and double bonds (Table 1) except for the cases when adsorption of these regions is impeded ($PGF_{1\alpha}$, cholesterol).

Protium in organic compounds is known to be relatively easily exchangeable for tritium when it is found near the centres of high electron density[31]. In a substrate containing several functional groups with pronounced adsorptive properties, each group will compete to capture the catalyst's active centres[32]. All the above premises can explain why the specific radioactivity of polar lipids is lower than that of MEFA obtained under similar conditions. This is evidently due to the fact that the double bonds of unsaturated fatty acid residues in the case of phospholipids are adsorbed less readily due to a possibly increased adsorption of other functional groups. The same can be said about the specific radioactivity of gangliosides G_1 and G_2 (Table 2). These differ from each other by a sulphogroup found in ganglioside G_2. As a result the specific radioactivity of G_2 is reduced by comparison with G_1 (2,9 Ci/mmol and 4,3 Ci/mmol, respectively).

Steric factors are also essential in affecting specific radioactivity of the labelled products. An evidence of it is localization of the label in PGE_2 and $PGF_{1\alpha}$. The 13,14-trans--double bond of PGE_2 is known to be hydrogenated with difficulty, unlike the 5,6-cis-double bond[33]. The effect is commonly associated with the influence of a cyclopentane ring located nearby,

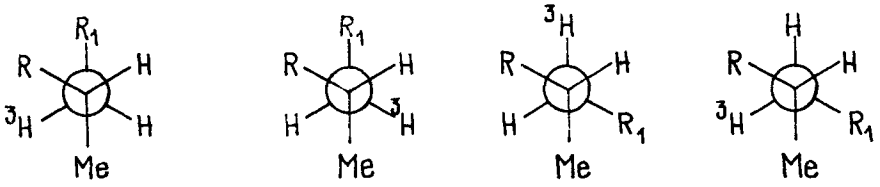
which hinders the adsorption of this double bond on a catalyst. This is just what explains why the 5,6-and the 13,14-double bonds are making different contributions to the value of the PGE_2 specific radioactivity[15]. The latter is estimated from the data on isotopic exchange at double bonds and hydrogen involving C-15 PGE_2 and isotopic exchange at one 13,14-double bond and hydrogen involving C-15 $PGF_{1\alpha}$. In the case of PGE_2 the periodate-permanganate oxidation causes a much greater loss of the tritium label (1070 mCi/mmol) than in the case of $PGF_{1\alpha}$ (260 mCi/mmol) indicating that tritium has probably incorporated into the PGE_2 5,6--double bond. The cholesterol double bond is even more screened. It is not hydrogenated on the Lindlar 5% $Pd/BaSO_4$ or $(Ph_3P)_3RhCl$ catalysts. This is likely to explain a low (45 mCi/mmol) specific radioactivity of this compound.

Thus the experimental results indicate that the reaction of heterogeneous catalytic isotopic exchange is highly perceptive to the structure of a substance, and this conforms to the data reported earlier[2].

The substrate absorbing on the catalyst's surface assumes a certain conformation depending on the structure and nature of functional groups, which determine polarity and stereospecificity of the particular molecule.

Two mechanisms are known to underlie the processes accompanying adsorption of unsaturated compounds on metallic catalysts - the associative and dissociative ones. The first of these underlies the isomeration of the cis-trans-double bond, while the second governs the migration of double bonds and introduction of the label into saturated molecule moieties. The catalyst's

reversible binding via the C=C bonds brings about formation of metal-alkyl intermediates[34], which assume delayed configurations I,II in the case of methyl oleate and I-IV in the case of methyl elaidate.

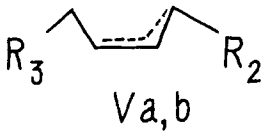


Me-metal

a $R_1 = C_7H_{14}COOCH_3$; $R = C_8H_{17}$

b $R_1 = C_8H_{17}$; $R = C_7H_{14}COOCH_3$

Formation of both cis- and trans- double bonds from I and II is equally possible; III and IV can only give rise to the trans-double bond, therefore the isomer's ratio should be 1:1 in the case of methyl oleate, while in the case of methyl elaidate the trans-isomer should predominate. This assumption is supported experimentally (Table 3). The fact that the products of the double bond migration have only the trans-configuration indicates that this process has assumed the dissociative rather than the associative mechanism. With proton leaving the α-position for the double bond, an intermediate radical of the π-allylic type (V) is formed, which after rearrangement and binding of tritium or protium produces a methyl ester of only trans-octadecenoic acid.



Va,b

a $R_2=C_6H_{12}COOCH_3$; $R_3=C_7H_{15}$

b $R_2=C_7H_{15}$; $R_3=C_6H_{12}COOCH_3$

It appears from the data of Table 3 that the both mechanisms are realized in the process of heterogeneous catalytic isotopic exchange with gaseous tritium. The predominance of either the associative or dissociative mechanism is evidently dependent on the structure of reacting molecules.

TABLE 3. Migration of the double bond by isotopic exchange in methyl oleate and methyl elaidate (dioxane, 1 hour, 23°C)

Isotopic exchange	the yield of methyl oleate (%)		the yield of methyl elaidate (%)	
	cis-isomer	trans-isomer	cis-isomer	trans-isomer
total	30	56	7	58
no shift	29	29	7	13
with a shift towards carboxylic group	<1	23	-	25
with a shift towards terminal methyl group	<1	4	-	20

All these findings suggest that the method of heterogeneous catalytic isotopic exchange is applicable to tritiation of not only saturated and aromatic compounds, but unsaturated products as well. The most convenient for labelling are compounds with

the cis-configuration of the double bond since they produce either saturated compounds or trans-isomers as by-products. These products can be separated from the starting material using well-known methods.

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