The use of [7α-^{3}H]- and [7α,7β-^{3}H]cholesterol in the enzymic assay of cholesterol 7α-hydroxylase

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Abstract A tritium release method is described for following the enzymic conversion of cholesterol to 7α-hydroxycholesterol. Incubations of rat liver subcellular preparations (containing microsomes) with [7α-^{3}H]cholesterol or [7α,7β-^{3}H]cholesterol release the labeled hydrogen in the 7α position as ^3H_2O which, after counting, allows for the determination of the fraction of exogenous cholesterol converted to 7α-hydroxycholesterol. These findings document those recently reported by Van Cantfort, Renson, and Gielen (1975. *Eur. J. Biochem.* **55:** 23). Analysis of incubation mixtures containing both [4-^{14}C]cholesterol and either [7α-^{3}H] or [7α,7β-^{3}H]cholesterol demonstrate that one atom of hydrogen (from the 7α position) is incorporated into H_2O for every molecule of exogenous cholesterol that is converted to 7α-hydroxycholesterol. In the case of [7α-^{3}H]cholesterol no label is retained by the product. With [7α,7β-^{3}H]cholesterol, one atom is released as ^3H_2O and one is retained by the product in the 7β position. Microsomal incubations with [7α,7β-^{3}H]cholesterol were performed, followed by the acetylation of the steroid fractions with [^{14}C]acetic anhydride. If intermixing of exogenous with endogenous cholesterol were complete during the enzymic reaction, one would expect the ^{3}H: ^{14}C ratio of the isolated cholesterol acetate to be four times that observed in the 7α-acetoxycholesterol acetate. Average values of 4.23 in one series and 4.03 in a second series indicate that intermixing was sufficiently complete to use the tritium release method as an indicator of mass conversion.

Methods currently employed for measuring the enzymic conversion of cholesterol to 7α-hydroxycholesterol usually involve incubations of subcellular and microsomal fractions (1–3) or reconstituted systems (4) with [^{14}C]cholesterol followed by thin-layer separation of the steroids and strip counting to determine the fraction of added cholesterol that is converted to 7α-hydroxycholesterol.

Balasubramaniam, Mitropoulos, and Myant (5) have reported that the intermixing of exogenous with endogenous cholesterol could be incomplete, which would result in erroneous estimates of the specific activity of the actual cholesterol precursor pool. These workers described a double isotope dilution procedure capable of measuring the actual mass of the 7α-hydroxycholesterol formed (6). More recently, Shefer, Nicolau, and Mosbach (7) have described an enzyme incubation and assay procedure in which such problems of incomplete mixing are minimal. In an attempt to improve the convenience of this assay, we synthesized tritiated cholesterol labeled at the 7α and the 7α, 7β positions in order to ascertain the feasibility of a tritium release method for monitoring this hydroxylation process. During the preparation of this manuscript, a paper by Van Cantfort, Renson, and Gielen (8) appeared demonstrating that tritiated cholesterol with approximately 70% of its label designated as 7α can be utilized for such an enzymic assay. This paper confirms their findings. In addition we would like to report that our use of tritiated cholesterol with more specifically designated tritium markers allows for the direct and simultaneous determination of the stoichiometry of the two products, ^3H_2O and 7α-hydroxycholesterol. Also [7α,7β-^{3}H]cholesterol can be used to ascertain the degree to which the added cholesterol equilibrates with the total endogenous cholesterol. This determination makes possible an accurate and relatively convenient assessment of mass conversion (see discussion).

MATERIALS AND METHODS

[4-^{14}C]Cholesterol, tritiated water, and [^{14}C]acetic anhydride were purchased from New England Nuclear Corp., Boston, Mass. The 7α- and 7β-hydroxycholesterols and 7-keto-cholesterol were purchased from Steraloids, Inc., Pauling, N.Y. When gram quantities of high purity diacetyl 7α-hydroxycholesterol were required, the synthetic procedure of Johnson and Lack (9) was used. Questran, the bile salt binding polymer, is a product of the Mead Johnson Co., Evansville, Ind. All other reagents

TABLE 1. The conversion of [^{3}H,^{14}C]cholesterol to bile salts by individual rats bearing chronic common bile duct fistulas

Material Injected	Injected Cholesterol ^{3}H/^{14}C	Methyl Cholate ^{3}H/^{14}C	% ^{3}H Lost
[4-^{14}C]Cholesterol and [7α-^{3}H]cholesterol	0.968	0.061	93.7
[4-^{14}C]Cholesterol and [7α,7β-^{3}H]cholesterol	1.12	0.57	49.1

were obtained from Calbiochem, San Diego, Cal.; Fisher Scientific Co., Pittsburgh, Pa.; Eastman Kodak Co., Rochester, N.Y.; Sigma Chemical Co., St. Louis, Mo.; and Aldrich Chemical Co., Milwaukee, Wis. Organic solvents used in extraction procedures were of reagent grade. They were stored over molecular sieves (Type 3A) in order to keep them anhydrous.

The [7α-^{3}H]cholesterol was synthesized by the method of Corey and Gregoriou (10), while the [7α,7β-^{3}H]cholesterol was prepared as follows.

6-Keto-cholesterol acetate

The 6-keto-cholesterol acetate was prepared by nitration of cholesterol acetate to 6-nitrocholesterol acetate (11) and conversion of the nitro compound to 6-keto-cholesterol acetate with zinc in an acetic acid–water mixture (12).

[5α,7α,7β-^{3}H]-6-Keto-cholesterol acetate

A mixture of the ketone (2.60 g, 5.85 mmoles), tritiated acetic acid [prepared by mixing T_2O (0.5 g, 500 mCi) with acetic anhydride (2.84 g) and heating at 50°C until homogeneity was achieved], and dry ethyl ether (25 ml) containing a drop of bromine was refluxed for 48 hr. The mixture was diluted with ethyl ether and washed with ice-cold 5% Na_2CO_3 solution until the washes were basic. After washing with water, drying with anhydrous $MgSO_4$, and filtering, the ether was removed in vacuo. The white solid was used without further purification.

[7α,7β-^{3}H]Cholesterol

The [5α,7α,7β-^{3}H]-6-keto-cholesterol acetate was converted to the tritiated cholesterol by the procedure of Corey and Gregoriou (10). All of the tritium at C-5 was lost after the formation of the double bond. Specific activity was 5.6 mCi/mmole.

Purification of [7α-^{3}H]cholesterol and [7α,7β-^{3}H]cholesterol

Both of the tritiated cholesterols were purified via their dibromides by the procedure of Fieser (13). In order to make certain that these cholesterol preparations had their tritium label at the sites designated by the methods of synthesis, they were injected together with [4-^{14}C]cholesterol into rats bearing a bile duct fistula of 3 days' duration as described by Bergstrom et al. (14). After hydrolysis of the conjugated bile salts, the cholic acid was identified, converted to its methyl ester and recrystallized from methanol to constant radioactivity. According to the findings of the above-cited workers, [7α-^{3}H]cholesterol would be expected to lose all its tritium label when undergoing biological conversion to cholic acid. The [7α,7β-^{3}H]cholesterol, however, would be expected to lose 50% of its tritium since the label at the 7β position is retained during this conversion. **Table 1** indicates that our cholesterol preparations were properly tagged with tritium.

Radioactive samples were counted on a Beckman LS-150 liquid scintillation counter containing three channels and an external standard source (Beckman Instruments, Fullerton, Cal.). Simultaneous radioassay of ^{14}C and ^{3}H was obtained by the discriminator ratio method of Okita et al. (15) with reference to quench curves. Protein was determined by the method of Lowry et al. (16). Cholesterol was determined by the procedure of Clark, Rubin, and Arthur. (17). Cholesterol obtained from the National Bureau of Standards, Washington, D.C., was used as a reference standard.

Enzymic incubations and assays

Subcellular fractions of rat (Sprague Dawley) liver were prepared by the procedure described by Shefer, Hauser, and Mosbach (18). Unless noted to the contrary the rats were fed regular rat chow supplemented with 5% Questran for 4 days prior to being killed. The conditions of incubation of microsomes are also those described by these authors (19) as noted in the legend to Table 3. In all of the studies reported, the final volume of the incubation mixtures was 2.5 ml. At the end of the period of incubation, the reaction was terminated by the addition of 12 volumes of dry methylene chloride. The aqueous phase was recovered after centrifugation (1500 rpm in a clinical centrifuge), washed again with 5 ml of methylene chloride, and centrifuged. The top layer (ca 2 ml of aqueous phase) was placed in the limb of a Rittenberg tube [Fig. 27–8 of (20)] preparatory to final purification by sublimation. After evacuation of the tube (mechanical pump) at room temperature the stopcock was closed. The closed and evacuated tube was set astride the rim of a Dewar flask filled with dry ice slurry, such that the empty limb was immersed in the cooling mixture while the

limb with fluid remained at room temperature. As the water evaporates from the compartment containing the aqueous phase, the cooling effect freezes the fluid and the sublimation of ice proceeds to completion. This usually takes between 50 and 80 min; however, any number of tubes can be handled simultaneously by this procedure. Aliquots of the thawed ice were assayed for tritium content and then related to the total water content (2.5 ml in these experiments).

In some experiments microsomes or postmitrochondrial supernatant (10,000 *g* supernatant) were incubated with both ^{14}C- and ^{3}H-labeled cholesterol. The percent conversion of [^{14}C]cholesterol to [7α-^{14}C]hydroxycholesterol, and the percent conversion of [^{3}H]cholesterol to $^{3}H_2O$ was determined simultaneously. At the end of the incubation period an aliquot (0.4 ml) of the incubation mixture was transferred to 15 volumes of methylene chloride–ethanol 5:1 and prepared for thin-layer chromatography as described by Shefer, Hauser, and Mosbach (19). The solvent system employed was ethyl acetate–benzene 13:7 in an unequilibrated tank. At the same time 12 volumes of dry methylene chloride were added to the remainder of the incubation mixture and processed for the radioassay of $^{3}H_2O$ as described above.

^{14}C Acetylation of sterols

The ^{14}C acetylation of the total cholesterol and 7α-hydroxycholesterol from [7α,7β-^{3}H]cholesterol incubations utilized the methylene chloride fraction from the incubation mixture. After separating the aqueous layer from the organic phase, and extracting the aqueous layer with chloroform, the combined organic fraction was washed with saturated NaCl solution, dried over anhydrous $MgSO_4$, filtered, and the solvent removed in vacuo.

The lipid residue was purified by thin-layer chromatography on silica gel with ethyl acetate–benzene 13:7 as solvent, in an unequilibrated tank. The plates were scraped in 1 cm segments and the segments containing the cholesterol and 7α-hydroxycholesterol were determined from standards that were visualized by spraying the plate with 1N H_2SO_4 in a separate lane and heating (21). After extracting the desired compounds from the silica gel with 10% methanol in chloroform, removing the solvent, and vacuum drying, the compounds were ready for acetylation.

To each tube of steroid fraction, 0.4 ml of dry benzene containing 4 mg of *N,N*-dimethyl-4-pyridinamine (22) and 1 ml of dry benzene containing 10 mg of [^{14}C]acetic anhydride were added. After heating at 37°C for 1 hr, acetylation was complete. The reaction mixture was quenched with saturated Na_2CO_3 and diluted with ethyl ether. The ethereal layer was washed first with Na_2CO_3 solution until basic, and then with cold dilute HCl until

TABLE 2. Fractional release of tritium from [7α-^{3}H]cholesterol, as $^{3}H_2O$ during the incubation of rat liver microsomes with cholesterol bearing ^{3}H radioactive label at the 7α position and ^{14}C at the 4 position

Experiment	CPM/0.1 ml H_2O	Total ^{3}H in H_2O^a I	Total ^{3}H in Cholesterol per Incubation II	% Conversion I/II × 100
		cpm	*cpm*	%
1	212	5300	493,000	1.08
2 (heat-treated)	1.8	45	493,000	0.01

[a] Volume of incubation 2.5 ml.

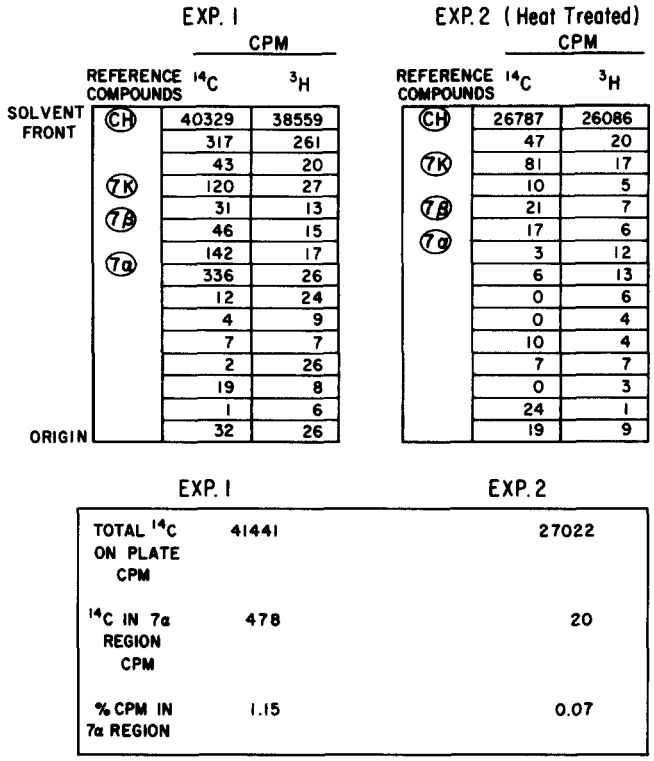

Fig. 1. Chromatographic separation and strip counting analysis of the sterol fractions following the incubation of rat liver microsomes with [4-^{14}C]- and [7α-^{3}H]cholesterol (both isotopic forms were present in each incubation flask). Each strip represents a 1 cm scraping of the chromatograms. The reference standards were visualized, after the removal of the 1 cm strips, by spraying with 1N H_2SO_4 and heating for several minutes above 120°C. The results are included with others in Table 2. Reference compounds: CH, cholesterol; 7K, 7-keto-cholesterol; 7β, 7-beta-hydroxycholesterol; 7α, 7-alpha-hydroxycholesterol. Solvent system: ethyl acetate–benzene (13:7) in an unequilibrated tank.

TABLE 3. Determination of enzymic conversion of added cholesterol to 7α-hydroxycholesterol by two methods

Expt. No.	Subcellular Fraction	Vol/ Incubation	Questran Prefeeding	Conversion of Exogenous Cholesterol to 7α-Hydroxycholesterol: ^{14}C Method (chromatographic) A: experimental	− heat treated control	= enzymic conversion	$^{3}H_2O$ Method (tritium release) B: experimental	− heat treated control	= enzymic conversion	A/B
		ml			%			%		
1	Microsomes	1.0	+	1.15	0.07	1.08	1.08	0.01	1.07	1.01
	Microsomes	0.67	+	0.71	0.09	0.62	0.61	0	0.61	1.02
2	Microsomes	1.0	−	0.23	0.05	0.18	0.20	0	0.20	1.11
	Microsomes	0.67	−	0.18	0.11	0.07	0.09	0	0.09	0.78
3	10,000*g* Supernatant	1.0	+	3.55	0.03	3.52	3.32	0.03	3.29	1.07
	10,000*g* Supernatant	0.67	+	2.40	0.11	2.29	2.06	0.03	2.03	1.13
4	10,000*g* Supernatant	1.0	−	0.63	0.06	0.57	0.58	0.03	0.55	1.04
	10,000*g* Supernatant	0.67	−	0.37	0.08	0.29	0.31	0	0.31	0.94

The methods of preparation of the various cellular subfractions and conditions of incubation were those described by Shefer et al. (16) (17). Each incubation contained both [4-^{14}C]cholesterol and [7α-^{3}H]cholesterol, 0.5 μmoles; potassium phosphate buffer (pH 7.4) 0.167 mmole; $MgCl_2$, 11 μmoles; $NaDP^+$, 3 μmoles; glucose-6-phosphate 6.0 μmoles; glucose-6-phosphate dehydrogenase, 5 IU; EDTA 2.5 μmoles. The cholesterol was "solubilized" with Cutscum in water such that 5.0 μmoles were present in each ml containing 0.1 ml of detergent. This cholesterol preparation (0.1 ml) was added to each incubation mixture to initiate the reaction.

The heat-treated controls were identical to the parallel experimental tubes except that the cellular subfractions were placed in boiling water for 1 min prior to addition to the incubation tubes. Final volume 2.5 ml.; incubation temperature 37°C; gas phase air. Time of incubation 30 min, with shaking.

Each experiment number is a separate rat liver preparation; therefore, comparisons of enzymic activity between microsomal and postmitochondrial supernatant incubations are not appropriate.

acidic. After washing with saturated NaCl solution, the ethereal solution was dried with anhydrous $MgSO_4$, filtered, and the ether removed in vacuo. The radioactive cholesterol acetate was diluted with 100 mg of carrier and crystallized to radiochemical purity from acetone. The radioactive 7α-hydroxycholesterol diacetate was diluted with 100 mg of carrier and further purified via an Act. III alumina column, using increasing concentrations of ethyl ether in hexane as eluant (7). The 7α-hydroxycholesterol diacetate was eluted in the 5% ethyl ether–hexane fraction. The diacetate had to be recrystallized up to seven times from acetone–water to obtain radiochemical purity.

RESULTS

Table 2 and **Fig. 1** demonstrate the total data from one incubation of rat liver microsomes and one heat-treated control. The added cholesterol was labeled with ^{14}C at C-4 and with tritium in the 7α position. Fig. 1 shows how the percent conversion of added cholesterol to 7α-hydroxycholesterol based on the label was obtained. Table 2 demonstrates how the percent conversion of the 7α-hydrogen (of the added substrate) to H_2O based on the tritium label was obtained. **Table 3** summarizes the results of eight such incubations. In addition, results from incubations employing the 10,000 *g* supernate instead of microsomes are also given.

Table 4 summarizes the results of additional double label experiments. This time the tritium label was present equally at the 7-α and the 7-β positions. Note that the fractional conversions calculated from the ^{14}C data are twice those values obtained by the tritium release method. In addition, the $^{3}H/^{14}C$ ratios observed in the region of 7α-hydroxycholesterol on the thin-layer chromatograms are one-half those of the cholesterol.

Microsomes were incubated with [7α,7β-^{3}H]cholesterol. The water was assayed for tritium as described above. In addition, the sterol fraction was chromatographed by thin-layer chromatography, and the materials present in the 7α-hydroxycholesterol and cholesterol regions were eluted and acetylated with [^{14}C]acetic anhydride (see Methods). After addition of the proper carrier, acetylated steroids, and column chromatography, the material was recrystallized to constant $^{3}H/^{14}C$ ratios. The isotopic ratios of cholesterol acetate were then compared with those of 7α-acetoxycholesterol acetate for each incubation. The results of 12 such experiments are given in **Table 5**.

TABLE 4. Summary of double label experiments with [4-^{14}C]cholesterol and [7α,7β-^{3}H]cholesterol

No.	Subcellular Fraction	Vol/ Incubation	Conversion of Exogenous Cholesterol to 7α-Hydroxycholesterol			^{3}H/^{14}C Ratio		
			A ^{14}C Method Chromatographic	B $^{3}H_2O$ Method Tritium Release	A/B	C Cholesterol Region of Chromatogram	D 7α-Hydroxycholesterol Region of Chromatogram	C/D
		ml	%	%				
1	Microsomes	1.0	2.05	1.09	1.90	2.35	1.24	1.90
	Microsomes	0.67	0.86	0.49	1.76	2.32	1.29	1.80
	Microsomes	0.45	0.64	0.33	1.94	2.44	1.15	2.12
2	10,000*g* Supernatant	1.0	3.68	1.69	2.17	2.42	1.21	2.00
	10,000*g* Supernatant	0.67	1.63	0.77	2.11	2.38	1.18	2.02
3	10,000*g* Supernatant	1.0	3.04	1.51	2.01	2.30	1.15	2.00
	10,000*g* Supernatant	0.67	1.75	0.82	2.13	2.31	1.15	2.01
4	10,000*g* Supernatant	1.0	2.80	1.50	1.93	2.26	1.16	1.95
	10,000*g* Supernatant	0.67	0.81	0.40	2.02	2.35	1.21	1.94

Incubation conditions are those previously described, Table 3, as cited. All values have been corrected for that activity observed in the heat-treated controls. Each experiment number represents an experiment with a separate rat liver preparation. All animals were pretreated with Questran.

DISCUSSION

The results depicted in Fig. 1, Table 2, and Table 3 demonstrate that during the 7α-hydroxylation of exogenous cholesterol, the stoichiometry between the formed 7α-hydroxycholesterol and the release of 7α hydrogen (as H_2O) is maintained for the tritium atom present in that position. The demonstrated absence of an isotope effect involving this in vitro enzymic cleavage of the carbon–tritium covalent

TABLE 5. Incubation of rat liver microsomes with [7α,7β-^{3}H]cholesterol, followed by acetylation of the pooled steroids with [^{14}C]acetic anhydride

No.		Protein	Cholesterol			Fractional Conversion of Exogenous Cholesterol to 7α-Hydroxycholesterol	7α-OH Cholesterol Formed/30 min.		^{3}H/^{14}C Cholesterol Acetate A[a]	^{3}H/^{14}C 7α-Hydroxycholesterol Diacetate B[a]	A/B
			Endogenous	Exogenous	Total						
		mg/incubation	*μmoles/incubation*				*Total n moles*	*n moles/mg protein*			
1	a	10.2	0.86	0.46	1.32	0.0096	12.7	1.24	2.28	0.62	3.7
	a	10.2	0.86	0.46	1.32	0.0084	11.0	1.08			
	b	7.6	0.64	0.46	1.10	0.0084	9.2	1.21	2.89	0.68	4.3
	b	7.6	0.64	0.46	1.10	0.0084	9.2	1.21			
	c	5.1	0.43	0.46	0.89	0.0054	4.8	0.94	3.26	0.69	4.7
	c	5.1	0.43	0.46	0.89	0.0054	4.8	0.94			
							Average	1.10 ± .06 SE		Average	4.23 ± .26 SE
2	a	8.7	0.77	0.94	1.71	0.0089	15.2	1.74	3.08	0.84	3.7
	a	8.7	0.77	0.94	1.71	0.0096	16.4	1.88			
	b	7.2	0.64	0.79	1.43	0.0086	12.3	1.71	3.15	0.76	4.1
	b	7.2	0.64	0.79	1.43	0.0089	12.7	1.76			
	c	5.8	0.52	0.63	1.15	0.0084	9.7	1.67	3.08	0.72	4.3
	c	5.8	0.52	0.63	1.15	0.0085	9.8	1.69			
							Average	1.74 ± .03 SE		Average	4.03 ± .02 SE

[a] Average determination for each pair of duplicate incubations.

In experiment 1, all of the microsomes were prepared from the same source. The letters indicate the dilution of enzyme, and the duplicate incubations. Experiment 2 demonstrates the results from a series of incubations run in duplicate with varying amounts of enzyme. In these instances the total amount of cholesterol (exogenous and endogenous) is proportional to the amount of protein present. The ratios of ^{3}H/^{14}C determined after acetylation of the total cholesterol, relative to the product, indicate that the mass conversions are well approximated from that calculated for the fractional conversion of exogenous [^{3}H]cholesterol to 7α-hydroxycholesterol by the tritium release method.

bond at the 7α position agrees with the in vivo findings of Bjorkhem (23). The overall metabolic pathway that the hydrogen or tritium atom pursues to terminate as H_2O is not known. The stoichiometric data indicate that, under the conditions of our assay, there is no additional isotope effect that alters the rate of incorporation (or equilibration) of the hydrogen atom with water once it is detached from the steroid. The absence of these complicating isotope effects permits a tritium release method for monitoring this enzymic conversion as fractional conversion of added cholesterol substrate. In addition one can use synthetic [7α,7β-^{3}H]cholesterol, after allowing for the fact that 50% of the tritium, i.e., 7β-^{3}H, is not released during the enzymic hydroxylation. The use of [7α,7β-^{3}H]cholesterol for this purpose has additional advantages. It is relatively simpler to synthesize, and one can prepare material with greater specific activity by the method described above. More important is the fact that [7α,7β-^{3}H]cholesterol can be used to test the degree of intermixing between exogenous and endogenous cholesterol. The data in Table 4 show that for every ^{3}H lost as ^3H_2O, one tritium atom is retained on the product, 7α-hydroxycholesterol. Acetylation of such material (product 7α-hydroxycholesterol) with [^{14}C]-acetic anhydride of known specific activity, and then relating its determined ^{3}H/^{14}C ratio to the total ^3H_2O formed will yield the mass of 7α-hydroxycholesterol present at the end of the incubation. If intermixing was complete, then the acetylation of the cholesterol and 7α-hydroxycholesterol present should yield cholesterol acetate with a ^{3}H/^{14}C ratio 4 times greater than that expected in 7α-acetoxycholesterol acetate. The results of such experiments, Table 5, give average ratios of 4.3 in one case and 4.03 in another. In these acetylation experiments the presence of endogenous 7α-hydroxycholesterol was not taken into account. These results demonstrate that, under the conditions employed, intermixing occurred to a sufficient extent to allow for the use of the tritium release method as an approximation of mass conversion.

The method can be scaled downward when lesser amounts of biological material are available for assay. Increased sensitivity can be achieved by using tritiated material of higher specific activity, since the [7α,7β-^{3}H]cholesterol was diluted by a factor of 2.5 with unlabelled material. Additional sensitivity can be achieved with the use of scintillation cocktails that allow for the assay of volumes of water greater than those employed.

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