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Short Communications

The Stereospecificity of Tritium Distribution in [1-^{3}H]- and [1,2-$^3H_2$]-Cholesterol and -Cholecalciferol

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Recent studies of the metabolism of [1-^{3}H]-cholecalciferol in the chick have demonstrated the existence of a metabolite of cholecalciferol in which at least one of the hydrogen atoms at C-1 of cholecalciferol has been lost (Lawson, Wilson & Kodicek, 1969*a*,*b*), whereas in similar studies with [1,2-$^3H_2$]-cholecalciferol no significant loss of ^{3}H was reported (Haussler, Myrtle & Norman, 1968). It was therefore decided to determine the ^{3}H distribution in the various forms of [1-^{3}H]- and [1,2-$^3H_2$]-cholecalciferol. In view of the difficulty of applying stereochemically unambiguous degradative reactions to cholecalciferol itself, the degradative procedures were performed on the cholesterol used as precursor of cholecalciferol. Results are now presented that permit some speculation as to the nature of the metabolite.

Cholesta-1,4-dien-3-one was reduced with tritium gas to [1,2-$^3H_2$]cholest-4-en-3-one in high yield by using the homogeneous hydrogenation catalyst tristriphenylphosphine–rhodium chloride (Birch & Walker, 1966; Djerassi & Gutzwiller, 1966) in benzene solution. Enol acetylation followed by borohydride reduction gave [1,2-$^3H_2$]cholesterol, of specific radioactivity 59.7Ci/mmol, which was eventually converted into [1,2-$^3H_2$]cholecalciferol of specific radioactivity 508mCi/mmol (B. Pelc, P. A. Bell & E. Kodicek, unpublished work). A sample of the [1,2-$^3H_2$]cholesterol was diluted with carrier to a specific radioactivity of approx. 5μCi/g, and was rigorously purified via the dibromide and by repeated recrystallization.

To determine the location of the ^{3}H label, [1,2-$^3H_2$]cholesterol was reduced to 5α-cholestan-3β-ol (Hershberg, Oliveto, Rubin, Staeudle & Kuhlen, 1951) and oxidized to 5α-cholestan-3-one (Bruce, 1943). To remove labile tritium in the 2-position the 5α-cholestan-3-one was treated with methanolic sodium hydroxide at room temperature for 24h. After purification, the equilibrated material was converted into 5α-cholest-1-en-3-one, both by direct dehydrogenation by using 2,3-dichloro-5,6-dicyanobenzoquinone in refluxing dioxan (Turner & Ringold, 1967) and by dehydrobromination of the intermediate 2α-bromo-5α-cholestan-3-one with calcium carbonate in refluxing dimethylformamide (Zderic, Carpio, Bowers & Djerassi, 1963).

Dibromination of the equilibrated material in the presence of hydrogen bromide gave 2α,4α-dibromo-5α-cholestan-3-one, which was dehydrobrominated with calcium carbonate in dimethylformamide to give cholesta-1,4-dien-3-one; further dehydrogenation with 2,3-dichloro-5,6-dicyanobenzoquinone gave the 1,4,6-trienone, which was subjected to the dienone–phenol rearrangement (Romo, Djerassi & Rosenkranz, 1950) to give 1-methylcholesta-1,3,5(10),6-tetraen-3-ol.

This degradation scheme was also applied to (*a*) a diluted sample of [1,2-$^3H_2$]cholesterol (New England Nuclear Corp., Boston, Mass., U.S.A.), prepared by the heterogeneous catalytic reduction of cholesta-1,4-dien-3-one and used as precursor of [1,2-$^3H_2$]-cholecalciferol (Haussler *et al.* 1968; Neville & DeLuca, 1966), and (*b*) a diluted sample of [1-^{3}H]-cholesterol, the precursor of the [1-^{3}H]cholecalciferol used in this laboratory (Callow, Kodicek & Thompson, 1966), prepared by palladium–charcoal catalytic reduction of 5α-cholest-1-en-3-one. With [1-^{3}H]cholesterol it was not necessary to perform the alkali exchange procedure, which had been carried out during the synthesis. The compounds were purified by column chromatography on alumina and by repeated recrystallization until their physical constants were concordant with those described in the literature for the unlabelled compounds, and their specific radioactivities were constant. Radioactivity was measured by scintillation counting of multiple samples (each 3–8mg) of each compound in a Packard Tri-Carb model 3375 liquid-scintillation spectrometer, quenching being assessed by automatic external standardization by using a standard calibration curve. The molar specific radioactivity of each compound was calculated and expressed relative to that of 5α-cholestan-3-one.

The results are shown in Table 1(*a*). Conversion of cholesterol into 5α-cholestan-3-one occasioned no loss of radioactivity in any of the samples. The losses of 36 and 40% of the initial radioactivity in

Table 1. *Relative molar specific radioactivities of derivatives of* [1-^{3}H]- *and* [1,2-$^3H_2$]-*cholesterol*

Standard deviations were in all cases less than 0.01 (six samples). Abbreviation: DDQ, 2,3-dichloro-5,6-dicyanobenzoquinone.

Derivative	(a) Relative molar specific radioactivity		
	[1-^{3}H]Cholesterol	[1,2-$^3H_2$]Cholesterol (homogeneous catalysis)	[1,2-$^3H_2$]Cholesterol (heterogeneous catalysis)
Cholesterol	1.00	1.01	0.98
5α-Cholestan-3-one	1.00	1.00	1.00
After alkaline exchange:			
5α-Cholestan-3-one	—	0.64	0.60
5α-Cholest-1-en-3-one (DDQ)	0.15	0.15	0.22
2α-Bromo-5α-cholestan-3-one	1.00	0.60	0.60
5α-Cholest-1-en-3-one (from 2α-bromo derivative)	0.17	0.17	0.16
	(b) Derived percentage distribution of total ^{3}H		
Position 1α	83–85	47–49	38–44
Position 1β	15–17	15–17	16–22
Positions 2α and 2β	—	36	40

the two doubly tritiated samples after alkali exchange represent radioactivity at the 2-position, and are in agreement with results obtained with [1,2-$^3H_2$]testosterone (Gut & Hayano, 1963). The difference in incorporation at the 1- and the 2-position is accounted for by incorporation of hydrogen, provided in these carrier-free syntheses at the expense of the solvent (Gut & Hayano, 1963).

1,2-Dehydrogenation of 5α-3-oxo steroids with 2,3-dichloro-5,6-dicyanobenzoquinone has been shown to proceed by specific *trans*-diaxial elimination of the 2β- and 1α-hydrogen atoms (Ringold, Gut, Hayano & Turner, 1962; Turner & Ringold, 1967). The radioactivity lost on treatment of 5α-cholestan-3-one with 2,3-dichloro-5,6-dicyanobenzoquinone therefore represents the amount incorporated at the 1α-position: 85% for the [1-^{3}H]-cholesterol, and 49 and 38% for the [1,2-$^3H_2$]-cholesterol prepared by homogeneous hydrogenation and by heterogeneous hydrogenation respectively. That the remaining radioactivity was located at the 1β-position was demonstrated by rearrangement to 1-methylcholesta-1,3,5(10),6-tetraen-3-ol, in which the 1β-hydrogen atom is displaced by the 19-methyl group; less than 2% of the initial radioactivity remained after this treatment in any of the samples.

5α-Cholest-1-en-3-one was also obtained by dehydrobromination of 2α-bromo-5α-cholestan-3-one. Radioactivity was lost on dehydrobromination, and the loss was similar to that obtained by dehydrogenation. This implies that dehydrobromination is also specific for removal of the 1α-hydrogen atom. Although Schmitz & Johnson (1962) found that dehydrobromination of 2α-bromo[1α-^{2}H]5α-cholestan-3-one caused the loss of only 75% of the initial deuterium content, their assignment of configuration was based only on the measurement of the n.m.r. coupling constant of the remaining (1β) hydrogen. This can be expected to indicate only the predominant configuration.

It is concluded from these results that the radioactivity distribution (Table 1*b*) is 83–85% 1α and 15–17% 1β for the [1-^{3}H]cholesterol and consequently for the [1-^{3}H]cholecalciferol; 36% in the 2-position, 47–49% 1α and 15–17% 1β for the [1,2-$^3H_2$]cholesterol obtained by homogeneous catalytic tritiation; and 40% in the 2-position, 38–44% 1α and 16–22% 1β for the [1,2-$^3H_2$]-cholesterol obtained by heterogeneous catalytic tritiation, and hence the [1,2-$^3H_2$]cholecalciferol derived from this material. It should be noted that, as a result of the inversion of ring A during the conversion of 7-dehydrocholesterol into cholecalciferol, the actual configuration at the 1-position is the reverse of that indicated by the nomenclature, which is in accordance with the usual practice of denoting the configuration of groups or atoms attached to ring A of secosteroids by the configuration that they possessed in the parent steroid.

The degree of stereospecificity (83–85%) for the 1α-position exhibited in the palladium-catalysed tritiation of 5α-cholest-1-en-3-one is similar to that observed for palladium-catalysed tritiation of androst-1-ene-3,17-dione (Brodie, Hayano & Gut, 1962). However, these authors found that heterogeneous catalytic tritiation of 17β-hydroxyandrosta-1,4-dien-3-one gave 25% 1α-^{3}H and 75% 1β-^{3}H, in contrast with the present findings of 38–44% 1α-^{3}H and 16–22% 1β-^{3}H for the reduction of cholesta-1,4-dien-3-one. This appears to indicate

that the change of side chain causes a sufficient change in the accessibility of the α-face to account for the different distribution. The observation that homogeneous catalytic tritiation of the 1,4-diene is not appreciably more stereoselective than heterogeneous catalytic tritiation was unexpected in view of the studies of Djerassi & Gutzwiller (1966). They found that homogeneous catalytic deuteration of androsta-1,4-diene-3,17-dione gives entirely [1α,2α-$^{2}H_2$]androst-4-ene-3,17-dione, but their conclusions were based on the evidence of n.m.r. coupling constants. This may be expected only to indicate the predominant configuration, as in fact has been demonstrated by Brodie, Kripalani & Possanza (1969). They showed that [1-^{2}H]androst-4-ene-3,17-dione prepared by this method lost 15% of its deuterium on conversion into oestrone *in vitro*. This indicates that the stereoselectivity was 85% 1α and 15% 1β.

After the administration of [4-^{14}C,1-^{3}H]cholecalciferol to rachitic chicks, Lawson *et al.* (1969*a*,*b*) detected a polar metabolite of vitamin D that possessed a lower $^{3}H/^{14}C$ ratio than did the administered cholecalciferol. This polar metabolite has also been obtained after the administration of 25-hydroxy[4-^{14}C,1-^{3}H]cholecalciferol. The metabolite represents almost all of the ^{14}C in chick intestinal nuclei, where the $^{3}H/^{14}C$ ratio was less than 0.2, compared with 4.7 for the administered [4-^{14}C,1-^{3}H]cholecalciferol. This represents a loss of more than 96% of the ^{3}H label on a molar basis. Since only 83–85% of the tritium is located in the 1α-position in this material, these results imply that both the 1α- and 1β-hydrogen atoms are lost during conversion of 25-hydroxycholecalciferol into this polar metabolite.

As Lawson *et al.* (1969*b*) have suggested, the increased polarity of this metabolite compared with 25-hydroxycholecalciferol is probably due to the incorporation of one or more additional oxygen functions into the molecule. The loss of both hydrogen atoms from C-1 implies that one of these additional groups is located at C-1, either as a ketone or as a hydroxyl group, and that in the latter case at least some rearrangement of double bonds has occurred involving C-1. It is difficult to see how alternative hypotheses involving double-bond shifts and the insertion of an oxygen function other than at C-1 could account for the loss of both the 1α- and 1β-hydrogen atoms.

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