Agents Affecting Lipid Metabolism. XVIII. A 7-Dehydrocholesterol Δ^7-Reductase Inhibitor (AY-9944) as Tool in Studies of Δ^7-Sterol Metabolism*

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ABSTRACT: A useful method of elucidating intermediates in complex metabolic processes is based on interference with an enzyme by a specific inhibitor resulting in accumulation of its substrate which, in turn, can be isolated and identified. This technique was used with *trans*-1,4-bis(2-chlorobenzylaminomethyl)cyclohexane dihydrochloride (AY-9944), a specific inhibitor of 7-dehydrocholesterol Δ^7-reductase, to determine the biogenetic relationship of Δ^7-sterols. [4-^{14}C]Δ^7-Cholestenol was incubated with rat liver homogenates in the presence of AY-9944, used as a barrier to prevent the enzymatic conversion of the biosynthesized 7-dehydrocholesterol to cholesterol.

Accumulation of ^{14}C-labeled 7-dehydrocholesterol with a radioactivity content virtually identical with that found in cholesterol of control incubations provides additional conclusive evidence for the role of 7-dehydrocholesterol as obligatory intermediate in the enzymatic transformation of Δ^7-cholestenol to cholesterol.

A useful technique of uncovering intermediates in complex metabolic processes is based on the inhibition of one of the enzymes, resulting in accumulation of its substrate. It may then be possible to isolate and to characterize an intermediate even though normally it may be present in too minute a quantity to allow detection (Dixon and Webb, 1964).[1] We have used this approach and AY-9944,[2] a rather specific inhibitor of 7-dehydrocholesterol Δ^7-reductase[3] (Dvornik *et al.*, 1963; Chappel *et al.*, 1964; Kraml *et al.*, 1964; Niemiro and Fumagalli, 1965), to further elucidate the pathway of hepatic cholesterogenesis.[4] The finding of 7-dehydrocholesterol in tissues of laboratory animals treated with AY-9944 (Chappel *et al.*, 1963; Dvornik *et al.*, 1964b; Hill and Dvornik, 1964; Horlick, 1964; Givner and Dvornik, 1965; Rodney *et al.*, 1965) established the earlier suggested role of 7-dehydrocholesterol as intermediate in the pathway of endogenous formation of cholesterol.

Since AY-9944 prevents the enzymatic conversion of 7-dehydrocholesterol to cholesterol, the agent can be used as a tool to demonstrate the role of Δ^7-cholestenol as precursor of Δ^7-dehydrocholesterol. Such experiments with rat liver homogenates are reported herewith. [4-C^{14}]Δ^7-Cholestenol was incubated in the presence of AY-9944 and the 7-dehydrocholesterol accumulated by the inhibitory action of AY-9944 was isolated as its stable transannular 5α,8α-peroxide (Dvornik *et al.*, 1964b). The finding in this peroxide of radioactivity levels which were virtually identical with those found in cholesterol from controlin cubations corroborated the role of 7-dehydrocholesterol as an intermediate in the biosynthesis of cholesterol from Δ^7-cholestenol.

Experimental Section

Cholesterol used as carrier was purified *via* its 5,6-dibromo derivative (Schwenk and Werthessen, 1952).

7-Dehydrocholesterol. Due to the susceptibility of the homoannular $\Delta^{5,7}$-diene system to transannular Diels–Alder-like addition of oxygen (Wieland and Prelog, 1947; Kandutsch, 1962), 7-dehydrocholesterol is usually contaminated with its 5α,8α-peroxide.[5,6] Like 7-dehydrocholesterol (Moore and Bauman, 1952), its peroxide is also fast acting (Dvornik *et al.*, 1963) when treated with the Liebermann–Burchard reagent.[7]

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[1] For limitations of this method, see, *e.g.*, Webb (1963).

[2] *trans*-1,4-Bis(2-chlorobenzylaminomethyl)cyclohexane dihydrochloride (Humber, 1964).

[3] The following trivial names have been used in the text: 7-dehydrocholesterol = cholesta-5,7-dien-3β-ol; Δ^7-cholestenol = cholest-7-en-3β-ol; $\Delta^{5,7,24}$-cholestatrienol = cholesta-5,7,24-trien-3β-ol; 24-dehydrocholesterol (desmosterol) = cholesta-5,24-dien-3β-ol; peroxide of 7-dehydrocholesterol = 5,8-epidioxy-5α,8α-cholest-6-en-3β-ol.

[4] Simultaneous oral administration to pigs of two specific inhibitors, *viz.*, triparanol (Avigan *et al.*, 1960) and AY-9944, blocked both the 24-dehydrocholesterol Δ^{24}-reductase and the 7-dehydrocholesterol Δ^7-reductase, respectively, and, consequently, caused accumulation of $\Delta^{5,7,24}$-cholestatrienol, another possible intermediate on the cholesterogenic pathway (Dvornik *et al.*, 1964b). A similar concept was used in studies of hepatic cholesterol synthesis *in vitro* (Dempsey, 1964).

TABLE I: Effect of AY-9944 on the Capacity of Rat Liver Homogenates to Synthesize Cholesterol from Its Precursors DL-[2-^{14}C]Mevalonate, [4-^{14}C]7-Dehydrocholesterol, and [4-^{14}C]Δ^7-Cholestenol.[a]

Precursor		Neutral Lipids (dpm)	Carrier Added: Cholesterol[b] (dpm)	Carrier Added: 7-Dehydrocholesterol[c] (dpm)
DL-[2-C^{14}]Mevalonate (1,110,000 dpm/incubation)	Control	411,000	142,115	
	AY-9944[d]	392,200	578	
[4-^{14}C]7-Dehydrocholesterol (151,000 dpm/incubation)	Control	104,961	18,656	
	AY-9944[d]	88,019	572	
[4-^{14}C]Δ^7-Cholestenol (92,940 dpm/incubation)	Control	86,775	9,978	...
		87,750	...	616
	AY-9944[d]	88,625	746	...
		88,450	...	9784

[a] Each flask contained: 4 ml of homogenate, 2 μmoles of NADP, 5 μmoles of adenosine triphosphate (ATP), 4 μmoles of glucose 6-phosphate, and 2.0 μmoles (0.5 μc) of DL-[2-^{14}C]mevalonate, or 0.13 μmole (0.067 μc) of [4-^{14}C]7-dehydrocholesterol, or 0.63 μmole (0.042 μc) of [4-^{14}C]Δ^7-cholestenol. Incubated at 37° for 1 hr. (Values reported are averages from incubations in duplicate.) [b] Cholesterol (200 mg) added as carrier; isolated, purified, and counted as 5,6-dibromocholestan-3β-ol and calculated as dpm/mg of cholesterol. [c] 7-Dehydrocholesterol (200 mg) added as carrier; isolated, purified, and counted as the 5α,8α-peroxide of 7-dehydrocholesterol and calculated as dpm/mg of cholesterol. [d] Final concentration, 1×10^{-5} M.

The peroxide of 7-dehydrocholesterol differs from its parent diene inasmuch as on treatment with 30 N H_2SO_4 it produces a colored product: after 15 min at room temperature its $E^{1\%}_{1cm}$ at 503 mμ was 432.5 (Givner and Dvornik, 1965). No color appears when 7-dehydrocholesterol is treated in the same way. The presence of the peroxide is readily detected by thin layer chromatography (tlc)[8] (ethyl acetate–CCl_4, 1:4). On spraying with concentrated H_2SO_4 both compounds, *i.e.*, 7-dehydrocholesterol (R_F *ca.* 0.5) and its peroxide (R_F *ca.* 0.3), even at room temperature react immediately with bluish-violet color; in contrast, cholesterol and other slow acting sterols are invisible unless heated, when they appear as reddish-blue spots. Before use as carrier, 7-dehydrocholesterol was crystallized twice from ethyl acetate–methanol, had $E^{1\%}_{1cm}$ at 281.5 mμ of 318 and, according to tlc, contained no peroxide.

[4-^{14}C]7-Dehydrocholesterol was prepared from [4-^{14}C]cholesterol according to Kulkarni *et al.* (1963) and had a specific activity of 0.46 μc/μmole.

[4-^{14}C]Δ^7-Cholestenol was prepared from [4-^{14}C]7-dehydrocholesterol by a modification of the procedure of Schenck *et al.* (1936). More specifically, to a refluxing solution of [4-^{14}C]7-dehydrocholesterol (100 mg) in dry 2-propanol (9 ml), metallic sodium (1 g) was gradually added over a period of 2 hr. During the next 22 hr more 2-propanol (15 ml) and sodium (1.8 g) were added whenever necessary to maintain in the mixture some unreacted sodium. The reaction mixture was finally cooled and the remaining sodium dissolved by careful addition of ethanol. Ether was added and the mixture successively extracted with aqueous HCl, aqueous $NaHCO_3$, and water, respectively, dried over anhydrous $MgSO_4$, and the solvent evaporated under reduced pressure. To remove unchanged 7-dehydrocholesterol (about 10%, as indicated by the absorption at 281.5 mμ), the residue (91 mg) was dissolved in ethanol (5 ml), eosin added, and the solution irradiated and oxygenated for 2 hr at 10°. Ethanol was evaporated under reduced pressure, the residue dissolved in hexane, washed with water, dried over anhydrous $MgSO_4$, and the hexane distilled off. The residue (71 mg) was crystallized three times from ether–methanol to give 31 mg of [4-^{14}C]Δ^7-cholestenol, with a specific activity of 0.066 μc/μmole.

Incubation Procedure. Livers taken from male albino rats were homogenized and incubated as reported

[5] By analogy, ergosterol is usually contaminated with its 5α,8α-peroxide in amounts detectable by tlc.

[6] In an earlier communication we have reported the isolation of the peroxide of 7-dehydrocholesterol from livers of rats fed AY-9944 (Dvornik *et al.*, 1963). We consider this peroxide to be an artifact rather than a metabolite of 7-dehydrocholesterol as was interpreted by Blondin *et al.* (1964). We have used the isolation of the peroxide of 7-dehydrocholesterol merely to establish the presence of its parent diene but not to indicate formation of the peroxide *in vivo*.

[7] The affinity of 7-dehydrocholesterol to oxygen and the property of the resulting transannular peroxide to react immediately with the Liebermann–Burchard reagent may cause misleading values when the Δ^7-cholestenol content is determined according to Glover and Green (1957), *i.e.*, by subtracting the 7-dehydrocholesterol content (absorption at 281.5 mμ) from the total fast-acting sterols. If so, any amount of 7-dehydrocholesterol converted to the fast-acting but ultraviolet transparent 5α,8α-peroxide would appear as Δ^7-cholestenol.

[8] Abbreviations used: NADPH, reduced nicotinamide–adenine dinucleotide phosphate; NADP, nicotinamide–adenine dinucleotide phosphate; tlc, thin layer chromatography.

earlier (Dvornik *et al.*, 1964a). The cholesterogenic activity of the homogenate was determined by measuring its capacity to convert DL-[2-^{14}C]mevalonate to cholesterol. [4-^{14}C]Δ^7-Cholestenol and [4-^{14}C]7-dehydrocholesterol, respectively, were used as substrates and were incubated simultaneously with aliquots of the same homogenate. Enzymatic activity was arrested by addition of ethanolic KOH (1.0 ml of about 18 N KOH, 4 ml of ethanol, and 5 ml of water), the appropriate carrier was added, and the suspension heated at 75° for 1 hr. The neutral lipids were extracted with light petroleum ether (bp 30–50°) and, depending on the carrier added, treated as described below.

Isolation of Cholesterol. The extract of the saponified incubation mixture containing the neutral lipids was brominated and the 5,6-dibromocholestan-3β-ol isolated and crystallized to constant radioactivity (Biggs *et al.*, 1954). Ethyl acetate–CCl_4, 1:4, tlc revealed a mixture of the two dibromo derivatives (*cf.* Barton and Miller, 1950) in which the thermodynamically stable 5β,6α isomer predominated.

Isolation of 7-Dehydrocholesterol. Mainly due to its proneness to peroxide formation, isolation of 7-dehydrocholesterol can be troublesome and unrewarding (see, *e.g.*, Glover and Green, 1957; Klein and Szczepanik, 1962). Therefore, to facilitate isolation, as before (Dvornik *et al.*, 1964b)[9] 7-dehydrocholesterol was converted to its peroxide by photochemically induced addition of oxygen across the 5,7-diene with eosin as photosensitizer (Schenck *et al.*, 1936). More specifically, 7-dehydrocholesterol was dissolved in ethanol, irradiated, and oxygenated at 10° for about 2 hr; *i.e.*, until the absorption at 281.5 mμ reached a minimum. Disappearance of 7-dehydrocholesterol and appearance of its peroxide was also followed by tlc. Ethanol was removed under reduced pressure, the residue taken up in hexane, passed through anhydrous sodium sulfate, and the solution evaporated to dryness. The residue was dissolved in hexane, filtered, and chromatographed on florisil (1:35, w/w) to remove the bulk of labeled cholesterol as well as to separate the peroxide of 7-dehydrocholesterol from the more polar by-products of the irradiation and oxygenation reactions. A mixture of chloroform and hexane was used; the ratio and volume of a fraction depended on the composition of the eluate as determined by tlc. The chromatogram was also followed by radioactive assay of aliquots of the fractions. Fractions shown by tlc to contain (fast acting) peroxide were combined and crystallized repeatedly until the specific activity of the mother liquor was of the same order of magnitude as that of the crystalline material. Two solvent systems were alternated, ethyl acetate–methanol and aqueous ethanol, respectively.

Radioactivity was measured with a Nuclear-Chicago Liquid Scintillation System, Model 720. Compounds to be counted (2–4 mg) were dissolved in 15 ml of the Liquifluor solution [4 g/l. of 2,5-diphenyloxazole and 50 mg/l. of 1,4-bis(5-phenyloxazolyl)benzene] (Nuclear Chicago Corp.). Counting efficiency for ^{14}C was between 73 and 76%.

Results

Incorporation of DL-[2-^{14}C]*mevalonate into cholesterol* was virtually completely blocked by a final concentration of 1×10^{-5} M of AY-9944. The fact that in the presence of an inhibitor of the 7-dehydrocholesterol Δ^7-reductase system practically no radioactivity was found to be associated with cholesterol rules out the possibility that any significant amount of 7-dehydrocholesterol formed in the liver is derived from cholesterol (*cf.* Dvornik *et al.*, 1963).

Conversion of [4-^{14}C]*7-Dehydrocholesterol to Cholesterol.* About 12% of the added radioactivity was recovered in the 5,6-dibromocholestan-3β-ol. In the presence of AY-9944, at a final concentration of 1×10^{-5} M, practically no radioactivity was found to be associated with cholesterol (*cf.* Kraml *et al.*, 1964), thus indicating that under such conditions AY-9944 indeed acts as barrier to prevent the enzymatic transformation of 7-dehydrocholesterol to cholesterol.

Conversion of [4-^{14}C]Δ^7*-Cholestenol to Cholesterol via 7-Dehydrocholesterol as Intermediate.* Approximately 11% of the radioactivity originally added as [4-^{14}C]Δ^7-cholestenol was associated with cholesterol (isolated as its 5,6-dibromo derivative). The conversion yield of Δ^7-cholestenol to cholesterol was thus of the same order of magnitude as that obtained with [4-^{14}C]7-dehydrocholesterol. Similar yields were reported earlier for unfractionated rat liver homogenates (Frantz *et al.*, 1959). The amounts of radioactivity found to be associated with cholesterol (746 dpm) and with 7-dehydrocholesterol (616 dpm) do not necessarily represent their biosynthetic formation, but may reflect their presence as trace impurities in the original substrate.

Discussion

Uncertainty still exists about the sequence of intermediates constituting the normal biogenetic pathway of the enzymatic conversion of lanosterol to cholesterol (see, *e.g.*, Richards and Hendrickson, 1964). Lack of information about the size and lifetime of an intermediate sterol pool permits only tentative deduction as to its place in the normal pathway of cholesterogenesis.

Until recently the concept was often encountered that in animal tissues Δ^7-cholestenol, 7-dehydrocholesterol, and cholesterol are in equilibrium (Glover *et al.*, 1952; Glover and Stainer, 1959; Glover and Green, 1957); *i.e.*, that Δ^7-cholestenol and 7-dehydrocholesterol are metabolites of cholesterol rather than its precursors, or, alternatively, that they are side products derived from Δ^{24}-intermediates (Johnston and Bloch, 1957). This concept, initially weakened by unexpected findings *in vitro* (Schroepfer and Frantz, 1961) and *in vivo* (Mercer and Glover, 1961), was later rendered untenable (a) by the work on the site of action of the cholesterol biosynthesis inhibitor AY-9944 (Dvornik *et al.*, 1963,

[9] The capacity to form readily a peroxide was also used by Dempsey *et al.* (1964) to establish the presence of 7-dehydrocholesterol in an incubation medium.

1964b); (b) by studies *in vitro* of the intermediary role of 7-dehydrocholesterol in cholesterol biosynthesis (Dempsey *et al.*, 1964), and (c) by experiments designed specifically to detect the operation of the sequence cholesterol → 7-dehydrocholesterol *in vivo* (Frantz *et al.*, 1964).

The findings that Δ^7-cholestenol and 7-dehydrocholesterol occur in nature (Cook *et al.*, 1954) and are readily convertible to cholesterol (Biggs *et al.*, 1954; Frantz *et al.*, 1959; Kandutsch and Russell, 1960; Mercer and Glover, 1961; Schroepfer and Frantz, 1961; Kandutsch, 1962) were suggestive of their possible role as intermediates in hepatic cholesterogenesis (Langdon and Bloch, 1953; Frantz *et al.*, 1959; Schroepfer and Frantz, 1961; Mercer and Glover, 1961; Goodman *et al.*, 1963). Supporting evidence was in the finding that on incubation of rat or mouse skin with labeled acetate more radioactivity was associated with the Δ^7-sterols than with cholesterol (Gaylor and Baumann, 1959). However, the existence of such a pathway was detected only in preputial gland tumors in mice (Kandutsch and Russell, 1960).[10]

Of decisive importance was the study in intact rats of the time course of hepatic cholesterogenesis following intravenous administration of [2-^{14}C]mevalonate (Goodman *et al.*, 1963). The rapid appearance of radioactivity in lanosterol and Δ^7-sterols[11] together with its progressive disappearance from these sterols and reaccumulation in cholesterol, demonstrated that Δ^7-sterols lie on the major biogenetic pathway to cholesterol.

The presence of a cholesterogenic pathway with 7-dehydrocholesterol as intermediate was established by two approaches. In both of them the enzymatic transformation of 7-dehydrocholesterol to cholesterol was arrested, thus creating a pool of 7-dehydrocholesterol sufficiently large to ensure its isolation and characterization.

The approach of the Ayerst group was based on the finding that AY-9944, a potent inhibitor of cholesterol biosynthesis, acts by blocking the enzymatic conversion of 7-dehydrocholesterol to cholesterol, thus causing *in vivo* a readily detectable accumulation of 7-dehydrocholesterol (Dvornik *et al.*, 1963, 1964b; Chappel *et al.*, 1963, 1964; Hill and Dvornik, 1964; Givner and Dvornik, 1965; Dvornik *et al.*, 1965).

Based on their earlier work (Frantz *et al.*, 1959; Schroepfer, 1961; Schroepfer and Frantz, 1961; Dempsey, 1962) and in accordance with the observation of Kandutsch (1962), the Minneapolis group has established *in vitro* that reduced nicotinamide–adenine dinucleotide phosphate is essential for the formation of cholesterol from 7-dehydrocholesterol (Dempsey *et al.*, 1963). Subsequently, incubation of Δ^7-cholestenol with a rat liver homogenate fraction in the absence of NADPH led to the accumulation of 7-dehydrocholesterol and allowed its characterization (Dempsey *et al.*, 1964).[12]

In the present study the capacity of AY-9944 to inhibit the 7-dehydrocholesterol Δ^7-reductase was used as barrier between 7-dehydrocholesterol and cholesterol and thus cause accumulation of 7-dehydrocholesterol biosynthesized from Δ^7-cholestenol *in vitro*. A rat liver homogenate converted [4-^{14}C]Δ^7-cholestenol to cholesterol with a total radioactivity content of 9978 dpm (isolated and purified as its 5,6-dibromo derivative). On the other hand, in the presence of AY-9944, a total radioactivity of 9784 dpm was found to be associated with 7-dehydrocholesterol (isolated and purified as its stable 5α,8α-peroxide). The finding of virtually identical levels of radioactivity in the biosynthesized sterols, *i.e.*, in the cholesterol from control incubations and in the 7-dehydrocholesterol isolated in the presence of AY-9944, coupled with the earlier established role of 7-dehydrocholesterol as precursor (and not metabolite) of cholesterol (Dvornik *et al.*, 1963; Frantz *et al.*, 1964), provides additional conclusive evidence that 7-dehydrocholesterol is an obligatory intermediate in the enzymatic conversion of Δ^7-cholestenol to cholesterol.

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[10] It is tempting to postulate that Δ^7-cholestenol and 7-dehydrocholesterol were recognized in tumor tissues because the uncontrolled rate of cholesterogenesis which appears to be associated with some carcinogenic states (*cf.* Siperstein and Fagan, 1964) was distributed unevenly amongst the enzymatic reactions constituting the pathway of cholesterol biosynthesis, thus creating large, detectable pools of the intermediary Δ^7-cholestenol and 7-dehydrocholesterol.

[11] The technique used for the isolation of Δ^7-sterols did not differentiate Δ^7- and Δ^8-sterols nor Δ^7-cholestenol and 7-dehydrocholesterol, respectively.

[12] It is pertinent to note that, at a final concentration of 1×10^{-3} M, AY-9944 did not affect the generation of NADPH *in vitro* from NADP, glucose 6-phosphate, and glucose 6-phosphate dehydrogenase (*cf.* Lohr and Waller, 1963) (unpublished results of Dr. M. L. Givner).

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