papers and notes on methodology

Transfer of cholesterol and a fluorescent cholesterol analog, 3'-pyrenylmethyl-23,24-*dinor*-5-cholen-22-oate-3β-ol, between human plasma high density lipoproteins

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Abstract A fluorescent cholesterol analog, 3'-pyrenylmethyl-23,24-*dinor*-5-cholen-22-oate-3β-ol (PMCA), has been synthesized as a spectroscopic probe of cholesterol function. The substrate activity of PMCA, about two-thirds that of cholesterol, with lecithin:cholesterol acyltransferase indicates that PMCA is a reasonable cholesterol analog and that the orientation of the substituted sterol in the phospholipid interface is similar to that of cholesterol. The fluorescence properties of PMCA are similar to those of other pyrene-containing compounds that exhibit concentration-dependent excimer fluorescence. The rate of transfer of [^{3}H]PMCA between HDL is about six times faster than cholesterol. These results indicate that the analog will be useful in studies of cholesterol function. —**Kao, Y. J., M. C. Doody, and L. C. Smith.** Transfer of cholesterol and a fluorescent cholesterol analog, 3'-pyrenylmethyl-23,24-*dinor*-5-cholen-22-oate-3β-ol, between human plasma high density lipoproteins. *J. Lipid Res.* 1986. 27: 781–785.

Supplementary key words cholesterol • lipoproteins • exchange • pyrene • fluorescence • sterol

The singular importance of cholesterol is illustrated dramatically by the pathological deposition of cholesterol and cholesteryl ester in the arterial walls (1, 2). The molecular basis of this phenomenon is unknown. Active hypotheses being studied include *a*) unregulated cellular cholesterol synthesis, *b*) cellular processing of chemically modified cholesteryl ester-rich lipoproteins taken up by specific lipoprotein receptors, and *c*) inadequate egress of cholesterol from various tissue pools (3). Because cholesterol does not possess suitable spectroscopic features, studies (4–10) of its behavior in dilute aqueous solutions of lipid aggregates and in cells have been difficult. The objective of this study was to prepare a fluorescent cholesterol analog and to compare several physical and biological properties of the analog with those of the naturally occurring lipid. This comparison is necessary to establish that the introduction of the spectroscopic reporter group does not change the properties of the analog to such an extent that it is not useful in studies of mechanisms of biologically interesting processes involving the parent compound. An abstract of this work has appeared (11).

MATERIALS AND METHODS

Materials

3-Acetoxybisnorcholenic acid was purchased from Steraloids, Inc. and recrystallized from benzene prior to use. Pyrene-3-carboxyaldehyde and $NaBH_4$ were products of Aldrich Chemical Co. Egg phosphatidylcholine was purchased from Avanti, Inc. $NaB[^3H]_4$ was obtained from ICN Radiochemicals. [G-^{3}H]Cholesterol, 1 Ci mmol^{-1}, was purchased from Amersham. All other chemicals were the highest commercially available grade.

Synthesis

PMCA was synthesized by esterification of 3-pyrenylmethanol with 3β-acetoxy-22,23-*dinor*-5-cholen-24-oyl chloride, followed by selective basic hydrolysis of the acetoxy group.

Pyrene-3-carboxyaldehyde, 5 g, was dissolved in 200 ml of dioxane and 1 g of $NaBH_4$ was added with vigorous stirring. During the reaction, the dull green color of the solution changed to yellow. After 2 hr, the solvent was removed under vacuum. The product was washed with

Abbreviations: PMCA, 3'-pyrenylmethyl-23,24-*dinor*-5-cholen-22-oate-3β-ol; PC, phosphatidylcholine; HDL, high density lipoproteins; VLDL, very low density lipoproteins; LDL, low density lipoproteins; LCAT, lecithin:cholesterol acyltransferase.

1 N HCl, then several times with water. 3-Pyrenylmethanol was obtained from ethanol–H_2O as yellow crystals. Yield 4.4 g; mp 123–125°C (12).

3β-Acetoxybisnorcholenic acid was purified and converted to the acyl chloride as published (13). A mixture of 4 g of the acyl chloride, 1 g of 4-dimethylaminopyridine and 2 g of 3-pyrenylmethanol in 100 ml of benzene was stirred on a steam bath until the solvent had evaporated. The residue, dissolved in 200 ml of benzene, was extracted with 200 ml of 2 N NaOH. After the insoluble material was filtered off, the organic layer was washed with 1 N HCl, then with H_2O until the aqueous solution was neutral. After removal of benzene under reduced pressure, the ester and minor impurities weighed 5.5 g. The crude acetoxy ester was dissolved in 100 ml of methanolic 0.1 N NaOH and refluxed for 30 min. The extent of hydrolysis was monitored by thin-layer chromatography in hexane–diethyl ether–acetic acid–water 90:10:1:1 (v/v). Final purification was carried out with a Merck Lobar G-60 prepacked silica gel column, 3.7 × 44 cm. PMCA was eluted with a linear gradient of hexane–chloroform 4:1 (v/v) and chloroform at 5 ml min^{-1} in 3 hr. The total yield of PMCA was 4.1 g. The purity of PMCA was established by recycling through a Waters C-18 Bondapack column, 4 × 25 mm, with 3000 theoretical plates. After recycling five times, no impurities were observed; mp 225°C; mass spectrum (m/e, relative abundance) 560, M^+ (39%); 542 (10%); 469 (5%); 366 (8%); 329 (10%); 315 (6%); 230 (6%); 215 (100%); 216 (68%); 201 (11%); 207 (12%).

3'-Pyrenyl-[^{3}H]-methyl-23,24-*dinor*-5-cholen-22-oate-3β-ol was synthesized by the reduction of pyrene-3-carboxyaldehyde with NaB[^{3}H]$_4$, sp act 249 mCi $mmol^{-1}$. The resultant 3-pyrenyl-[^{3}H]methanol was utilized for the synthesis of PMCA as described above.

For experiments of [^{3}H]cholesterol and [^{3}H]PMCA transfer between HDL and VLDL, 1 μCi of [^{3}H]cholesterol or [^{3}H]PMCA in 0.1 ml of ethanol was injected with continuous vortexing into 1 mg of HDL protein in 1 ml of buffer. The resultant solutions were incubated at 37°C for 2 hr for equilibration of HDL with the labeled sterol and then passed through a 0.22-μm Millipore filter. Ten μg of [^{3}H]sterol-labeled HDL in 1 ml was mixed with 1 ml of 1 mg VLDL at 37°C. At specific time points, 0.1-ml aliquots of the reaction mixture were removed, chilled in ice, and applied to 0.5 × 10 cm A-5m agarose columns at 4°C. Within 30 sec after introduction of the mixture on the column, the separation of HDL and VLDL was essentially complete. Fractions containing VLDL and HDL were collected to quantify radioactivity by scintillation counting. Identical results were obtained when VLDL were precipitated by addition of 75 μl 1 M $MnCl_2$ and separated from HDL by centrifugation at 1500 *g* for 2 min (14). The relatively poor solubility of PMCA in ethanol required the use of relatively high proportions of solvent, which may have changed HDL structure. For comparison of the two sterols, identical conditions were used for labeling of HDL with [^{3}H]cholesterol.

RESULTS AND DISCUSSION

To demonstrate that PMCA was functional as a substrate analog of cholesterol, the two compounds were compared as acyl acceptors in egg PC vesicles in the enzymic reaction catalyzed by lecithin:cholesterol acyltransferase (15). With a partially purified enzyme preparation under experimental conditions previously described (16), 20 and 14 pmols of cholesteryl ester and PMCA ester, respectively, were formed μg^{-1} protein min^{-1}, in a reaction mixture that contained 40 μg of protein and 0.1 mM egg PC containing 10 mol % of either cholesterol or PMCA. The analog was about two-thirds as active as cholesterol, as shown in **Fig. 1.**

The space-filling models of PMCA and cholesterol, shown in **Fig. 2A**, illustrate the extent of differences between the molecules in the side chain region. PMCA contains the pyrene nucleus as the bulky component of the 17β substituent of cholesterol instead of the usual nonpolar aliphatic hydrocarbon region (Fig. 2B). Both the 3β-hydroxy group and an intact steroid core are essential for the interaction between cholesterol and phospholipid, but steric requirements of the hydrophobic alkyl chain are much less stringent (17). Evaluation of several cholesterol analogs indicates that the size of this substituent is less

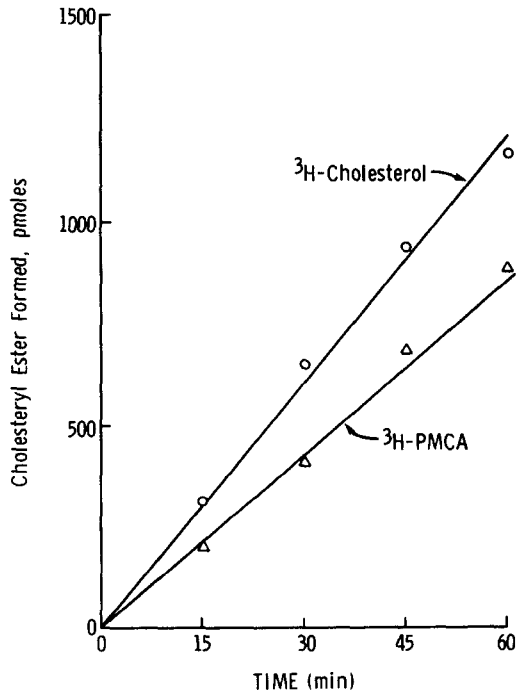

Fig. 1. Comparison of cholesterol and PMCA as substrates for LCAT.

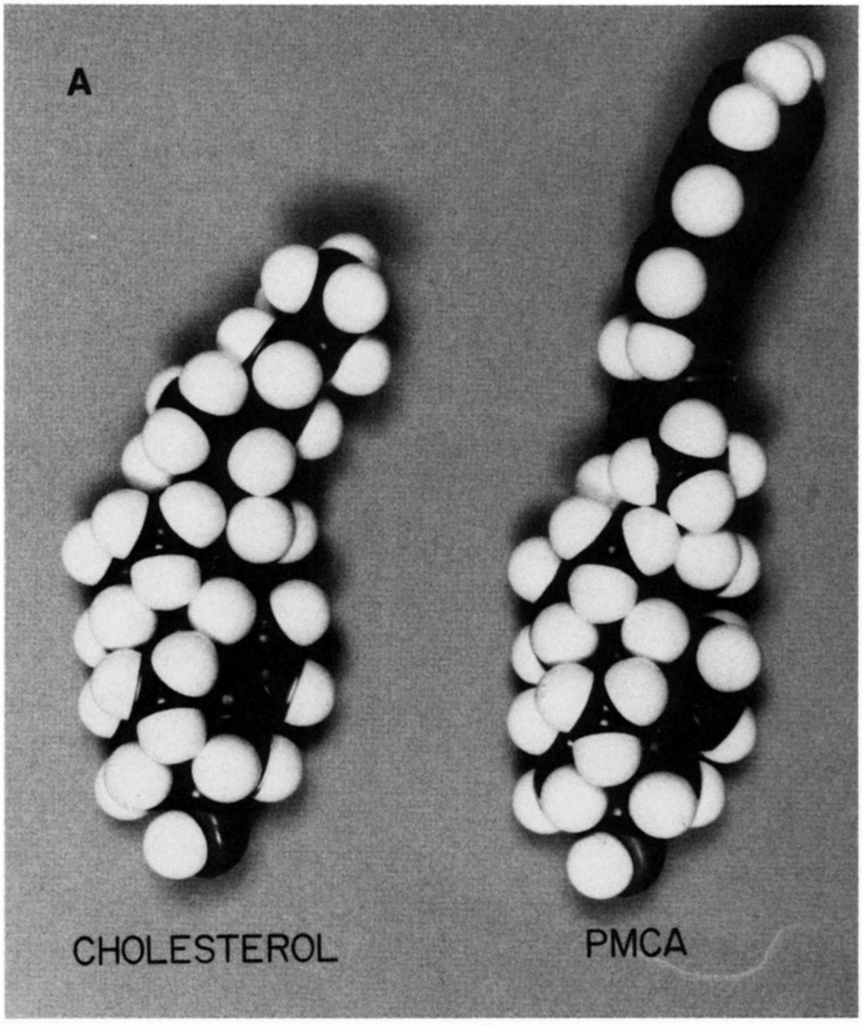

Fig. 2. A, Molecular models of cholesterol and PMCA. B, Molecular formula for PMCA.

important than the hydrophobicity (18–20). Although the bulk of the substituent differs, both cholesterol and PMCA have a hydrophobic side chain region. Previous studies with similar cholesterol analogs (13, 21, 22) demonstrated that modification of this structural region of cholesterol had only minor effects on the physical and enzymatic properties of the sterol.

The spectroscopic properties of PMCA are similar to those reported for other pyrene and pyrene-containing lipids studied in this laboratory (23–27). PMCA in HDL exhibited a linear relationship between I_e/I_m and the amount of PMCA between 3 and 12 molecules per HDL. As expected, when mixed with unlabeled HDL, the excimer fluorescence decreased. The pseudo first order observed rate constant was 0.5 min^{-1} and was independent of the concentration of acceptor and donor HDL and of the amount of PMCA per HDL.

The transfer of [^{3}H]cholesterol between HDL and VLDL was an apparent first order process, with a rate constant of 0.09 min^{-1} under these experimental conditions. This rate, observed with unfractionated HDL, is only about twofold different from the rate of transfer of

cholesterol from HDL_2 and HDL_3 in the absence of organic solvents (8). This comparison suggests that the faster rate that we observed was due to the solvent effect on the solubility of the two sterols (7, 9, 23, 24), and that the solvent effect was minor. Control experiments with [^{3}H]PMCA demonstrated that the rate constants for transfer of PMCA from HDL to VLDL determined spectroscopically and by physical separation of donor and acceptor lipoproteins were identical, 0.5 min^{-1}. The rate of transfer of PMCA is about six times faster than cholesterol.

The oleate ester of PMCA has proven to be highly useful in studies of LDL metabolism. LDL containing PMCA oleate[1] was utilized to visualize LDL receptor by fluorescence microscopy (28, 29) and to produce cell mutants by receptor-dependent photosensitization (30–32). Since little is known about the dynamics of the intracellular distribution of cholesterol and related compounds, this pyrene analog, with other fluorescent sterols and derivatives (33–36), should be valuable in studies of cellular uptake of sterols. ■

[1]Available from Molecular Probes, Inc., 4849 Pitchford Avenue, Eugene, OR 97402.

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