Cholesterol metabolism: use of D_2O for determination of synthesis rate in cell culture

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Abstract Cholesterol synthesis in cell culture in the presence of D_2O yields a spectrum of enriched molecules having a relative abundance that indicates random substitution of deuterium for hydrogen. Quantitation of the absolute rate of cholesterol synthesis is obtained by isotope ratio mass spectrometry. Mevinolin and 26-hydroxycholesterol both decrease cholesterol synthesis rate but have a discordant effect on HMG-CoA reductase activity. —**Esterman, A. L., B. I. Cohen, and N. B. Javitt.** Cholesterol metabolism: use of D_2O for determination of synthesis rate in cell culture. *J. Lipid Res.* 1985. **26:** 950–954.

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Although a variety of radioactive precursors has been utilized for the estimation of cholesterol synthesis rate, uncertainty in regard to the specific activity of the endogenous pool has led to the utilization of ^3H_2O as the most accurate technique (1, 2). Because the amount of ^3H_2O required is a potential biohazard and requires special laboratory facilities, many investigators quantitate the activity of the rate-limiting enzyme, HMG-CoA reductase (EC 1.1.1.34), which under most circumstances correlates with the cholesterol synthesis rate. However, certain instances of a lack of correlation have been reported (3, 4) and these are likely to increase rapidly as new compounds that may regulate cholesterol synthesis are studied. We have validated the use of D_2O (5) for the quantitation of cholesterol synthesis and developed incubation conditions for the evaluation of the effects of exogenous and endogenous compounds such as mevinolin and 26-hydroxycholesterol on synthesis rate.

METHODS AND MATERIALS

Chinese hamster ovary cells (CHO-K1) were maintained in F-12 media (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FCS, Flow Laboratories, McLean, VA) at 37°C in a 5% CO_2 humidified environment. The same lot number of FCS which was found to have a total cholesterol concentration of 360 μg/ml was used for all studies. Prior to each study, the cells were subcultured by trypsinization and seeded into either 60-mm culture dishes or 25-cm^2 culture flasks (Falcon) precoated with fibronectin (Pierce Chemical, Rockford, IL).

D_2O (MSD Isotopes, Montreal, Canada) was added to concentrated stock solutions of F-12 to achieve the desired concentration. Preliminary studies were done with CHO cells grown for 72 hr in media containing 10, 20, and 40% D_2O without any gross inhibition of cell growth and with a progressive increase in the mass of the predominant deuterated species of cholesterol as predicted from the D/H ratio of the media. Based on these findings all further studies were done with media containing 25% D_2O. For these studies redistilled D_2O was purchased. In addition, 25% D_2O was recovered from the expended media by distillation using standard laboratory glassware.

For the determination of the proportion of deuterated cholesterol synthesized by the CHO cells, it was necessary to prepare a cholesterol standard that contained a high proportion of the deuterated species. This was done by growing CHO cells in F-12 media supplemented with 10% delipidated FCS (6, 7) and 25% D_2O until an approximate sevenfold increase in cell count occurred. Following lysis and saponification of the cells, the sterol fraction was extracted into hexane–ether 1:1 (v/v) and cholesterol obtained by elution after thin-layer chromatography using silica gel H and a solvent system of hexane–ethyl acetate 70:30. Analysis of the relative abundance of the enriched cholesterol molecules (see Fig. 3) indicated that the major peaks occurred at $M_0 + 5$ and $M_0 + 6$. No change in the distribution of masses occurred following thin-layer chromatography of the acetate derivative using silica gel plates impregnated with 10% $AgNO_3$ (Analtech, Newark, DE) indicating the absence of significant amounts of dienes (8). A UV absorption spectrum of the cholesterol (2.8 μg/ml in ethanol) from 340 to 200 nm did not detect peaks in the 260–270 nm range characteristic of conjugated

Abbreviations: GLC-MS, gas-liquid chromatography-mass spectrometry; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A.

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dienes of cholesterol. By these techniques there does not appear to be any major change in the cholesterol obtained from the CHO cells grown in the presence of 25% D_2O.

Assuming random distribution in regard to incorporation of D or H and incorporation of 22 atoms (9), it can be calculated (10) for D = 0.25 that the M_0 + 6 peak represents 18.3% of the total deuterated species. Therefore the ratio of the area of the M_0 + 6 peak × 100/18.3 divided by the area of the M_0 peak represents the proportion of the cell cholesterol that was deuterated. The total amount of cholesterol in solution was determined by gas-liquid chromatography (Perkin-Elmer 900) using a 3-ft glass column packed with 3% SP2250 (Supelco, Bellefonte, PA) and an internal standard of 5α-cholestane, 3β-ol. This deuterated standard contains some unlabeled cholesterol from the initial mass of native cholesterol in the cells at the time of plating. This amount was quantitated by GLC-MS and found to be 15% of the total mass of cholesterol (deuterated = 85%, native = 15%). Using this solution of deuterated cholesterol (5.5 μg/ml), different amounts were admixed with a standard solution of protium cholesterol (11.0 μg/ml, M_0) to give protium/deuterium ratios ranging from 1.8 to 115 (**Fig. 1**). Aliquots containing 3 μg were silylated and analyzed by an isotope ratio program using a Hewlett-Packard 5992B GLC-MS instrument using methods identical to those described previously in this Journal (11). The retention time for cholesterol trimethylsilyl ether was 2.3 min.

The effect of cholesterol content in the media on de novo cholesterol synthesis was determined by addition of various amounts of fetal bovine serum. In addition, the effect of 26-hydroxycholesterol, prepared as described previously (12) and mevinolin (Merck Chemical, Rahway, NJ) on cholesterol synthesis rate were determined. HMG-CoA reductase activities were determined as previously described (13).

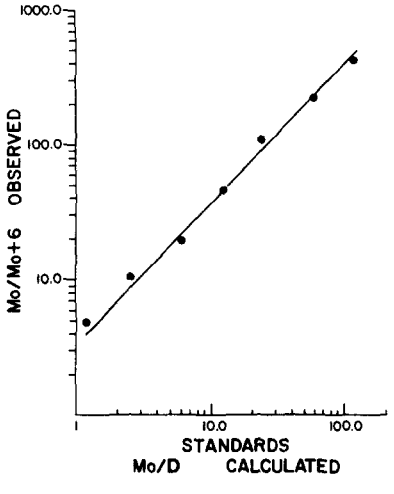

Fig. 1. Calibration curve. Deuterated cholesterol (M_0 + D = 5.5 μg/ml; D = 85%), extracted from CHO cells subcultured for approximately seven doublings in media containing 25% D_2O, was mixed with different amounts of protium (M_0) cholesterol to yield standards ranging in mass ratios (M_0/D) from 1 to 200. The observed M_0/M_0 + 6 ratio (mass spectrum analysis at M/Z = 368/374) is proportional to the M_0/D ratio which permits quantitation of the proportion of newly synthesized cholesterol (D). For any M_0/M_0 + 6 ratio, the M_0/D ratio can be determined by the equation y = 3.65x, where y = M_0/M_0 + 6, x = M_0/D, 3.65 = y/x.

RESULTS

Fig. 2 illustrates the complete spectrum of cholesterol isolated from CHO cells grown in 25% D_2O. Compared to the spectrum of the protium cholesterol, it is apparent that a series of peaks representing masses greater than M/Z 368 and 458 (trimethylsilyl ether) and their respective naturally occurring +1 and +2 peaks are present. In addition to these molecular ions, there are a number of fragments that do not appear in the spectrum of the protium compound.

It was found (**Fig. 3**) that the relative abundance of the deuterium-enriched species of cholesterol corresponded to that predicted by random distribution assuming incorporation of 22 atoms of either hydrogen or deuterium at a D/H ratio of 0.25. The M_0 + 6 peak, not detectable in the spectrum of the protium cholesterol, represents 18.3% of the deuterated spectrum. Conversely, the proportion of protium cholesterol (M_0) that would be expected to occur during cholesterol synthesis in a medium containing 25% D_2O is 0.18%. Although this fraction can be subtracted from the total area of the M_0 peak to obtain a corrected M_0/M_0 + 6 ratio, under conditions of this study, the relatively large M_0 peak, attributable to preformed cholesterol in the cells and/or the medium, was not further corrected in calculation of the amount of newly synthesized cholesterol.

The effect of growing CHO cells in media containing different amounts of fetal calf serum on the M_0/M_0 + 6 ratio of extracted cholesterol is shown in **Fig. 4**. As the lipoprotein content of the media was increased, there was an increase in the M_0/M_0 + 6 ratio. The mechanism for the change in ratio was analyzed in detail by comparing the M_0/M_0 + 6 ratio in the CHO cells and the medium at 24-hr intervals (**Table 1**). At the end of 72 hr, 86% of the newly synthesized cholesterol was in the cells and 14% in the media. Of the total amount of cholesterol in the cells harvested at 72 hr, 2.33 μg was newly synthesized and the remainder, except for the small amount present in the cells initially (0.5 μg), was utilized from the media.

The effect of 26-hydroxycholesterol and mevinolin on cholesterol synthesis rate was studied (**Table 2**) using the conditions established above. Both compounds caused a marked inhibition in cholesterol synthesis rate without significantly affecting total cell cholesterol. As has been reported previously, 26-hydroxycholesterol reduces HMG-CoA reductase activity and mevinolin, because of the

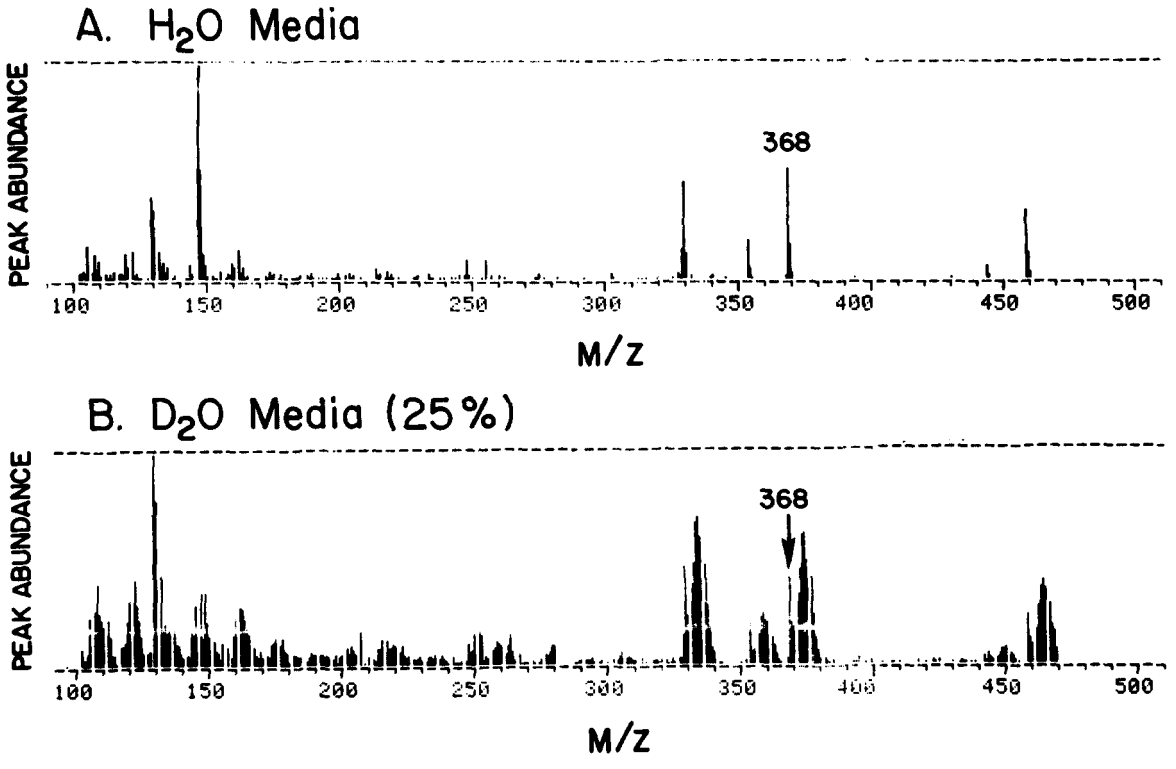

Fig. 2. Complete mass spectrum of cholesterol isolated from CHO cells grown in H_2O and 25% D_2O. Cells grown in 25% D_2O synthesize a spectrum of enriched cholesterol molecules seen as molecular ions greater than M/Z 368 and 458 (trimethylsilyl ether). Fragments of these enriched molecules are also detected.

competitive nature of the inhibition, induces an increase in enzyme activity assayed in vitro.

DISCUSSION

The pioneering studies of Schoenheimer and Rittenberg (5) established that D_2O is incorporated into a molecule of cholesterol during its synthesis and not by exchange. The number of atoms of hydrogen or its isotopes incorporated has generally been stated to be 22 (1, 5), although other values have been proposed (9). Under the conditions of our study, the best fit of the data is to a random distribution using 22 atoms. However, uncertainty can arise when synthesis rate is determined at shorter time intervals when complete mixing of the hydrogen isotope with the subcellular pools contributing hydrogen may not occur. Under these circumstances, the use of D_2O can provide a relative abundance spectrum that can define more closely the precursor/product relationship since it is now apparent that after complete mixing of deuterium, there is random substitution.

Quantitation of the absolute rate of cholesterol synthesis requires determination of the deuterated cholesterol both in the cells and the medium. We have defined conditions using CHO cells and 1% fetal calf serum in which the amount of newly synthesized cholesterol transported

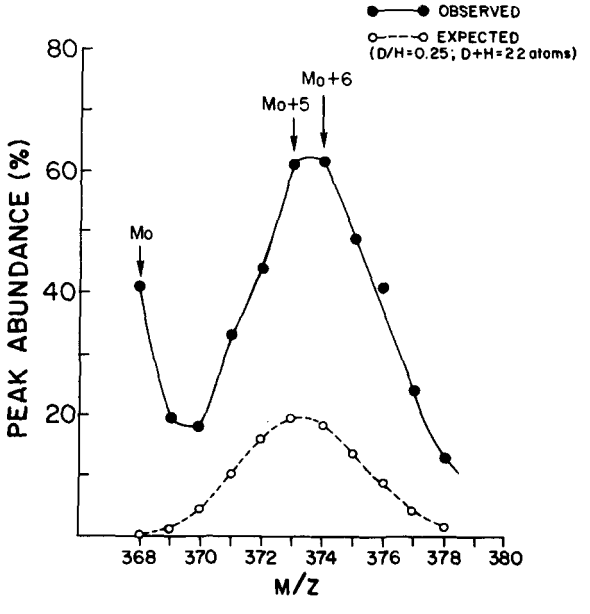

Fig. 3. Comparison of the relative abundance of deuterium-enriched cholesterol extracted from cells grown in 25% D_2O to theoretical distribution (D/M = 0.25, D + M = 22 atoms). The open circles represent the theoretical distribution of peak abundances assuming random distribution of D or H (D/H = 0.25) at 22 positions in the cholesterol molecule. The closed circles represent the actual peak abundance observed with cholesterol extracted from CHO cells grown in media containing 25% D_2O for seven cell population doublings. The most abundant mass peaks are M_0 + 5 and M_0 + 6. The M_0/M_0 + 6 ratio was chosen for quantitation determination of newly synthesized cholesterol.

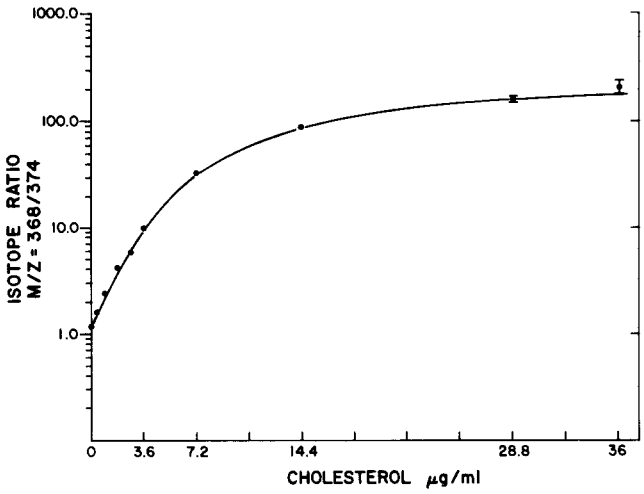

Fig. 4. Effect of lipoproteins on cholesterol synthesis rate in cell culture. CHO cells were grown in media supplemented with different concentrations of fetal calf serum containing high and low density lipoproteins (total cholesterol = 360 μg/ml) for 72 hr. The increase in the M/Z 368/374 ratio (protium cholesterol/deuterated cholesterol) of cholesterol extracted from the cells, together with the finding that relatively little deuterated cholesterol is in the medium (see Table 1), indicates an increase in cholesterol utilization and a decrease in cholesterol synthesis.

into the medium in 48 hr is a relatively small percent of the total. Under these conditions, changes in the M_0/M_0 + 6 ratio of cell cholesterol reflect, for the most part, the change in synthesis rate, since total cell cholesterol did not change.

The relationship noted between the increase in the amount of fetal calf serum in the medium and the increase in the M_0/M_0 + 6 ratio of cell cholesterol is attributable for the most part to a decrease in cholesterol synthesis rate. This finding would be expected from the knowledge that CHO cells have receptors for low-density lipoproteins (14) and that both LDL and HDL are present in fetal calf serum. Since it is now known that fetal calf serum contains 26-hydroxycholesterol (7) which is a normal component of human LDL and HDL (11, 15) the mechanism for the regulation of HMG-CoA reductase following internalization of LDL is not defined precisely. Nevertheless, using fetal calf serum and probably other sources of LDL, one can adjust the rate of cholesterol synthesis and utilization from the media in cell culture to obtain M_0/M_0 + 6 ratios in a range that permits the study of agents that may affect synthesis and/or transport.

Our need to quantitate cholesterol synthesis rate stemmed from the desire to study the biological effects of 26-hydroxycholesterol independent of its effect on HMG-CoA reductase activity. Although both synthesis rate and enzyme activity appear to vary together, it is clear that, using compounds such as mevinolin, independent analysis is essential for the study of biological effects. Furthermore, it is possible that various agents may have independent effects on receptor-mediated transport and synthesis rate. Under these circumstances, although enzyme activity and synthesis rate may vary together, analysis of exchange rates of the protium and deuterated species of cholesterol between cells and media would permit assessment of changes in transport rate. Thus, we believe further modification of the technique will permit quantitative assessment of both cholesterol synthesis and transport.

As noted (5), the knowledge that D_2O is incorporated into cholesterol helped in the establishment of the value of tracer methodology in metabolic studies. For reasons that are not entirely clear, when D_2O became available, its metabolic effects were studied using enrichments that for the most part exceeded 50% (16). At very high concentration "toxicity" was noted both in in vitro and in vivo studies. In retrospect, we have learned (Merck Isotopes, personal communication) that D_2O normally made available contains heavy metals and therefore we arbitrarily chose to use glass-distilled D_2O. It is of interest that renal failure was reported as one of the toxic effects of D_2O (17). In our studies, the initial proliferation of the CHO cells during the first 24 hr after placing them in D_2O-containing medium was slower as compared to cells transferred to

TABLE 1. Cholesterol synthesis rate in CHO cells grown in media containing 1% fetal calf serum

		M_0/M_0 + 6[b] Ratio		M_0/D[c] Ratio		Percent D		M_0 + D[d] Cholesterol		D[e] Cholesterol		Synthesis Rate[f]
Time	Cell #[a]	Cells	Media	Cells	Media	Cells	Media	Cells	Media	Cells	Media	
hr	× *10⁶*											
24	0.35	76.6	3×10^4	21.0	8×10^3	4.4		2.25	16.5	0.10		0.3
48	0.94	31.6	600	8.7	165	10.3	0.6	5.76	15.3	0.59	0.09	0.7
72	2.00	26.5	200	7.3	55	12.1	1.8	10.70	13.6	1.30	0.25	0.8

[a] Increase in cell number during each 24-hr incubation period.
[b] Raw data M/Z = 368/374 from the mass spectrometer.
[c] Ratio obtained from calibration curve (y = 3.65x).
[d] Total cholesterol (net value for 24-hr time period) as determined by gas-liquid chromatography. Total cholesterols from previous time periods have been subtracted.
[e] Total deuterated cholesterol synthesized in the 24-hr incubation period.
[f] Values are μg/10^6 cells/24 hr.

TABLE 2. Effect of 26-hydroxycholesterol and mevinolin on cholesterol synthesis rate of CHO cells grown in media containing 1% fetal calf serum

	Cell #	HMG-CoA Reductase Activity[a]	M_0/D	% D	M_0 + D	D-Cholesterol	Synthesis Rate[b]
	× 10^6				μg	μg	
Control	5.78	95	13.0	7.10	17.3	1.23	0.21
26-Hydroxycholesterol (1.25 μM)	5.40	21	56.2	1.70	16.2	0.28	0.05
Mevinolin (0.125 μM)	6.65	328	212.0	0.50	15.3	0.08	0.01

[a] pmol/mg protein per min.
[b] $\mu g/10^6$ cells per 24 hr.

non-D_2O-containing media. However, there were no differences noted in doubling times at 48 and 72 hr. This phenomenon is consistent with the observation that, when growing cells are introduced to a new medium which differs qualitatively from the initial growth medium, the cells must adapt to the new environment and go through a "lag" period. Thus, we have not noted any toxic effects under the conditions of our study.

Perhaps the major deterrent for the use of D_2O in metabolic studies has been the difficulty and expense of maintaining mass spectrometers, particularly for biomedical use. Recent improvements in design have greatly reduced maintenance problems and also lowered costs and the need for highly specialized laboratory personnel. We expect that further improvements will increase the sensitivity, especially for isotope ratio measurements, which has broad applicability for metabolic studies using known compounds. For estimation of cholesterol synthesis rates using D_2O, an increase in sensitivity can reduce the proportion of D_2O used in the media, and high resolution analysis of fragmentation patterns can give further insights into the regulation of cholesterol synthesis.

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REFERENCES

1. Lakshmanan, M. R., and R. L. Veech. 1977. Measurement of rate of rat liver sterol synthesis in vivo using tritiated water. *J. Biol. Chem.* **252:** 4667–4673.
2. Jeske, D. J., and J. M. Dietschy. 1980. Regulation of rates of cholesterol synthesis in vivo in the liver and carcass of the rat measured using [^{3}H]water. *J. Lipid Res.* **21:** 364–376.
3. Dugan, R. E., L. L. Slakey, A. V. Briedis, and J. W. Porter. 1972. Factors affecting the diurnal variation in the level of 3-hydroxy-3-methylglutaryl coenzyme A reductase and cholesterol-synthesizing activity in rat liver. *Arch. Biochem. Biophys.* **152:** 21–27.
4. Dietschy, J. M., and M. D. Siperstein. 1967. Effect of cholesterol feeding and fasting on sterol synthesis in 17 tissues of the rat. *J. Lipid Res.* **8:** 97–104.
5. Rittenberg, D., and R. Schoenheimer. 1937. Deuterium as an indicator in the study of intermediary metabolism. XI. Further studies on the biological uptake of deuterium into organic substances, with special reference to fat and cholesterol formation. *J. Biol. Chem.* **121:** 235–253.
6. Cham, B. E., and B. R. Knowles. 1976. A solvent system for delipidation of plasma or serum without protein precipitation. *J. Lipid Res.* **17:** 176–181.
7. Esterman, A. L., H. Baum, N. B. Javitt, and G. J. Darlington. 1983. 26-Hydroxycholesterol: regulation of hydroxymethylglutaryl-CoA reductase activity in Chinese hamster ovary cell culture. *J. Lipid Res.* **24:** 1304–1309.
8. Gibbons, G. F., K. A. Mitropoulos, and K. Ramananda. 1973. A method for the rapid qualitative and quantitative analysis of 4,4-dimethyl sterols. *J. Lipid Res.* **14:** 589–592.
9. Fears, R., and B. Morgan. 1976. Studies on the response of cholesterol biogenesis in feeding in rats: evidence against the existence of diurnal rhythms. *Biochem. J.* **158:** 53–60.
10. Olkin, I., L. J. Gleser, and C. Derman. 1978. Probability, Models and Applications. Macmillan Publishing Co., New York. 169–176.
11. Javitt, N. B., E. Kok, S. Burstein, B. I. Cohen, and J. Kutscher. 1981. 26-Hydroxycholesterol: identification and quantitation in human serum. *J. Biol. Chem.* **256:** 12644–12646.
12. Scheer, I., M. J. Thompson, and E. Mosettig. 1956. 5-Cholestene-3β-26-diol. *J. Am. Chem. Soc.* **78:** 4733–4738.
13. Chang, T-Y., and J. S. Limanek. 1980. Regulation of cytosolic acetoacetyl coenzyme A thiolase, 3-hydroxy-3-methylglutaryl coenzyme A synthetase, 3-hydroxy-3-methylglutaryl coenzyme A reductase, and mevalonate kinase by low density lipoprotein and by 25-hydroxycholesterol in Chinese hamster ovary cells. *J. Biol. Chem.* **255:** 7787–7795.
14. Krieger, M., M. S. Brown, and J. L. Goldstein. 1981. Isolation of Chinese hamster cell mutants defective in the receptor-mediated endocytosis of low density lipoproteins. *J. Mol. Biol.* **150:** 167–184.
15. Brooks, C. J. W., R. M. McKenna, W. J. Cole, J. MacLachlan, and T. D. V. Lawrie. 1983. 'Profile' analysis of oxygenated sterols in plasma and serum. *Biochem. Soc. Trans.* **11:** 700–701.
16. Thompson, J. F. 1963. Biological Effects of Deuterium. Macmillan Publishing Co., New York.
17. Barbour, H. G., and J. Trace. 1936. The pharmacological action of deuterium oxide. I. Toxicity and symptoms; metabolic rate; water exchanges. *J. Pharmacol. Exp. Therap.* **58:** 460–482.