Cholesterol metabolism: use of D$_2$O for determination of synthesis rate in cell culture

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Abstract

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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 $^3$H$_2$O as the most accurate technique (1, 2). Because the amount of $^3$H$_2$O 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$_2$O (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$_2$O (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$_2$O 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$_2$O. For these studies redistilled D$_2$O was purchased. In addition, 25% D$_2$O 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$_2$O 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$_{60}$ + 5 and M$_{60}$ + 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% D₂O.

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 Mo + 6 peak represents 18.3% of the total deuterated species. Therefore the ratio of the area of the Mo + 6 peak × 100/18.3 divided by the area of the Mo 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-cholestan, 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, Mo) 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).

**RESULTS**

Fig. 2 illustrates the complete spectrum of cholesterol isolated from CHO cells grown in 25% D₂O. 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 Mo + 6 peak, not detectable in the spectrum of the protium cholesterol, represents 18.3% of the deuterated spectrum. Conversely, the proportion of protium cholesterol (Mo) that would be expected to occur during cholesterol synthesis in a medium containing 25% D₂O is 0.18%. Although this fraction can be subtracted from the total area of the Mo peak to obtain a corrected Mo/Mo + 6 ratio, under conditions of this study, the relatively large Mo 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 Mo/Mo + 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 Mo/Mo + 6 ratio. The mechanism for the change in ratio was analyzed in detail by comparing the Mo/Mo + 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 H₂O and 25% D₂O. Cells grown in 25% D₂O 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 D₂O 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 D₂O 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% D₂O to theoretical distribution (D/M = 0.25, D + H = 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% D₂O for seven cell population doublings. The most abundant mass peaks are M₀ + 5 and M₀ + 6. The M₀/M₀ + 6 ratio was chosen for quantitation determination of newly synthesized cholesterol.
cholesterols from previous time periods have been subtracted.

"Increase in cell number during each 24-hr incubation period.
1"Raw data M/Z = 368/374 from the mass spectrometer.
2"Ratio obtained from calibration curve (y = 3.65x).
3"Total deuterated cholesterol (net value for 24-hr time period) as determined by gas-liquid chromatography. Total choleroles from previous time periods have been subtracted.
4"Total deuterated cholesterol synthesized in the 24-hr incubation period.
5"Values are pg/106 cells/24 hr.

The proliferation of the CHO cells during the first 24 hr after placing them in D2O-containing media was slower as compared to cells transferred to 26-hydroxycholesterol (7) which is a normal component of human LDL and HDL (11, 15) the mechanism for

As noted (5), the knowledge that D2O 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 D2O 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 vitro and in vivo studies. In retrospect, we have learned (Merck Isotopes, personal communication) that D2O normally made available contains heavy metals and therefore we arbitrarily chose to use glass-distilled D2O. It is of interest that renal failure was reported as one of the toxic effects of D2O (17). In our studies, the initial proliferation of the CHO cells into the medium in 48 hr is a relatively small percent of the total. Under these conditions, changes in the M0/MD + 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 M0/MD + 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

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.

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

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Cell # x 10⁶</th>
<th>M0/MD + 6⁺ Ratio</th>
<th>MD/D⁺ Ratio</th>
<th>Percent D</th>
<th>M0 + D⁺ Cholesterol</th>
<th>D⁺ Cholesterol</th>
<th>Synthesis Rate (pg/10⁶ cells/24 hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>0.35</td>
<td>76.6 3 x 10⁴</td>
<td>21.0 8 x 10³</td>
<td>4.4</td>
<td>2.25 16.5</td>
<td>0.10</td>
<td>0.3</td>
</tr>
<tr>
<td>48</td>
<td>0.94</td>
<td>31.6 600</td>
<td>8.7 165</td>
<td>10.3</td>
<td>5.76 15.3</td>
<td>0.59</td>
<td>0.7</td>
</tr>
<tr>
<td>72</td>
<td>2.00</td>
<td>26.5 200</td>
<td>7.3 55</td>
<td>12.1</td>
<td>10.70 13.6</td>
<td>1.30</td>
<td>0.8</td>
</tr>
</tbody>
</table>

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non-D$_2$O-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$_2$O in metabolic studies has been the difficulty and expense of maintaining mass spectrometers, particularly for biomedical phenomenon is consistent with the observation that, when using D$_2$O, an increase in sensitivity can reduce 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$_2$O, an increase in sensitivity can reduce the proportion of D$_2$O 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