Chinese hamster ovary-215 (CHO-215) mutant cells are auxotrophic for cholesterol. Berry and Chang (Berry, D. J., and Chang, T. Y. (1982) *Biochemistry* 21, 573–580) suggested that the metabolic lesion was at the level of 4-methyl sterol oxidation. However, the observed cellular accumulation of lanosterol was not consistent with a defect at this metabolic site. With the use of a novel Silica Sep Pak sterol separation procedure, we demonstrated that 60–80% of the acetone-soluble lipid radioactivity in [5-3H]mevalonolactone-labeled CHO-215 cells was incorporated into acidic sterols. 7(8)-Cholest-7-en-4&alpha;-methyl,4a-carboxy,3&beta;-ol was the dominant end product. In addition to this acidic sterol, 7(8),24-cholestadien,4&alpha;-methyl,4a-carboxy,3&beta;-ol and 7(8),24-cholestadien,4a-carboxy,3&beta;-ol were also isolated. Incubation of cell-free extracts with [14C]7(8)-cholest-7-en-4&alpha;-methyl,4a-carboxy,3&beta;-ol and pyridine nucleotides confirmed that CHO-215 4-carboxysterol decarboxylase activity was less than 1% of that for wild type cells. Thus, a correspondence between decreased 4-carboxysterol decarboxylase activity and the spectrum of accumulated sterol products by intact CHO-215 cells was demonstrated. No detectable cholesterol was synthesized by CHO-215 cells. 14C-Product accumulation studies demonstrated that 7(8),24-cholestadien,4&alpha;-methyl,4a-carboxy,3&beta;-ol and pyridine nucleotides confirmed that CHO-215 4-carboxysterol decarboxylase activity was less than 1% of that for wild type cells. Therefore, the steady state ratio for 424-saturated acidic sterols/unsaturated acidic sterols was dependent on media cholesterol source and amount. Finally, the accumulated acidic sterol(s) were not regulatory signal molecules for the modulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity in response to cholesterol availability.

Chinese hamster ovary (CHO) cell lines are suitable experimental model systems for the study of non-parenchymal cell regulation of cholesterol biosynthesis (1). Numerous mutant CHO cell lines with altered cholesterol metabolism have been isolated (2–8). Among these is a cholesterol auxotroph called "CHO-215"; these cells were isolated by Chang et al. (2). Initially, the CHO-215 cell primary metabolic lesion was thought to be localized at the 14&alpha;-methyl demethylation of lanosterol (2). Upon further characterization, Berry and Chang (3) demonstrated that the metabolic lesion was distal to lanosterol 14&alpha;-methyl demethylation; their results supported diminished 4-methyl sterol oxidase activity. However, catalytic defect(s) in 4-methyl sterol oxidation would have been expected to result in 4,4-dimethyl and/or 4-methyl sterol accumulation rather than lanosterol. Recently, Panini et al. (9) have reported that the major sterol product synthesized by CHO-215 cells was lanosterol and possibly 14-desmethyl lanosterol.

In this laboratory we wanted a cholesterol auxotroph which could be used to test the oxysterol hypothesis (10–13). That is, to determine directly (without the contribution of endogenously synthesized cholesterol) whether down-regulation of HMG-CoA reductase activity by regulatory levels of exogenously added highly purified cholesterol correlated with the formation of biologically synthesized oxysterol(s). CHO-215 cells were chosen as our model. However, in view of the apparent uncertainty about the CHO-215 cell metabolic lesion(s), it was important to (i) firmly define the sterologenic defect and (ii) to determine whether the cell did/did not synthesize C-27 sterols in general or cholesterol specifically.

In contrast to the conclusions published by Chang et al. (2) and Berry and Chang (3), we demonstrated that the CHO-215 cell primary metabolic defect was at the level of 4-carboxysterol decarboxylation and not 4-methylsterol oxidation. 4-Carboxysterol accumulation was demonstrated and three acidic sterol derivatives were identified by mass spectral analyses. Furthermore, we demonstrated that this class of oxysterols did not serve as regulatory signal molecules to modulate mevalonic acid synthesis in response to cholesterol availability.

**MATERIALS AND METHODS**

*Chemicals—(3S)-3-Hydroxy-3-methyl-[2-14C]glutaryl coenzyme A was synthesized in this laboratory (14, 15), (R,S)-[5-3H]Mevalonolactone was prepared according to the procedure of Keller (16) and purified by TLC. Prior to use the [3H]mevalonolactone was converted to its sodium salt as described in Ref. 16. The mevalonate solution pH was adjusted to 7.4 prior to each incorporation study. NADP, NAD, glucose 6-phosphate and glucose-6-phosphate dehydrogenase were purchased from Sigma. [1,2-3H](Cholesterol was purchased from Du Pont-New England Nuclear. All other reagents were purchased at the highest grade available from standard suppliers.

*Maintenance of Cells—CHO-215 (1), (obtained from Dr. Roy Vagelos, Merck Research Institute, Rahway, NJ) and CHO-K1, wild type cells (obtained from U. C. S. F. Cell Culture Facility) were maintained in McCoy’s 5A medium which contained 5% fetal calf serum (FCS), 0.05% Tricine, pH 7.4, and 0.5 g of NaHCO3/liter. On day 0 of each experiment, the cells were plated in McCoy’s medium which contained 5% FCS. After 24 h they were refed media supplemented with either 5% FCS, 5% fractionated FCS (lipoproteins with buoyant densities

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Radioactivity flow monitor (United Technologies-Packard, Downers Grove, IL). For neutral sterols a 5% water, 95% methanol elution solvent was used (column was maintained at 33 °C); acidic sterols were resolved with 0.1% acetic acid in 99.9% methanol. Solvent flow rate was always 1 ml/min. Neutral and acidic sterol apparent mass was monitored continuously at 210 nm with a Gilson model 116 UV detector (Gilson Inc., Madison, WI).

Mass Spectrometry—Mass fragmentation analyses were done by the U. C. S. F. Mass Spectrometry Facility (A. L. Burlingame, Director, supported by NIH DRR RR04112). Liquid secondary ionization mass spectrometry in the negative ion mode was used.

HMG-CoA Reductase Assay—Washed, frozen cell pellets were suspended in buffer B (25 mM imidazole-HCl, pH 7.4, 10 mM KEDTA, 5 mM dithiothreitol, and 200 mM KCl) which contained 0.25% (v/v) of the detergent KYRO EOB (Proctor & Gamble, Cincinnati), incubated (37 °C) for 5 min, and centrifuged at 12,000 × g (room temperature). Aliquots (0–25 µl) of the solubilized cell extract were added to 25 µl of a NADPH regenerating system (10 mg of NADP+, 10 mg of dithiothreitol, 100 µl of 0.5 M glucose 6-phosphate, and 25 µl of 350 units) of glucose-6-phosphate dehydrogenase in 1.0 ml of 0.05 M K-phosphate buffer, pH 7.4). [3H]Mevalonate synthesis was initiated by the addition of 5 µl of (S)-[5-3H]HMG-CoA (24 µCi/µmol, 1.32 mCi). The reaction was terminated with 10 µl of concentrated HCl and 10 µl of 10% (w/v) [2,5-diphenyl oxazole in toluene. Non-radioactive standards were added to the reaction mixture before and after extraction to determine the recovery of radioactivity. The reaction mixture was extracted with 50 µl of hexane/ethyl acetate (1:1) and 100 µl of a NADPH regenerating system (10 mg of NADP+, 10 mg of dithiothreitol, 100 µl of 0.5 M glucose 6-phosphate, and 25 µl of 350 units) of glucose-6-phosphate dehydrogenase in 1.0 ml of 0.05 M K-phosphate buffer, pH 7.4). The reaction mixture was extracted with 50 µl of acidified hexane and 100 µl of a NADPH regenerating system (10 mg of NADP+, 10 mg of dithiothreitol, 100 µl of 0.5 M glucose 6-phosphate, and 25 µl of 350 units) of glucose-6-phosphate dehydrogenase in 1.0 ml of 0.05 M K-phosphate buffer, pH 7.4). The reaction mixture was extracted with 50 µl of hexane/ethyl acetate (1:1) and 100 µl of a NADPH regenerating system (10 mg of NADP+, 10 mg of dithiothreitol, 100 µl of 0.5 M glucose 6-phosphate, and 25 µl of 350 units) of glucose-6-phosphate dehydrogenase in 1.0 ml of 0.05 M K-phosphate buffer, pH 7.4). The reaction mixture was extracted with 50 µl of acidified hexane and 100 µl of a NADPH regenerating system (10 mg of NADP+, 10 mg of dithiothreitol, 100 µl of 0.5 M glucose 6-phosphate, and 25 µl of 350 units) of glucose-6-phosphate dehydrogenase in 1.0 ml of 0.05 M K-phosphate buffer, pH 7.4).

RESULTS

Incorporation of [5-3H]Mevalonate into Acetone-soluble Lipids—Although Chang et al. (2) and other investigators (3, 9) had labeled CHO-215 cells with either radioactive acetate or mevalonate, their extraction protocols were inadequate for a total assessment of all the potential postsqualene products synthesized. Therefore, we developed the Silica Sep-Pak protocol outlined in Fig. 1. This fractionation scheme facilitated the analytical chromatography of low abundance polar sterols by a prior reduction of cellular cholesterol mass. Acetone was
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used to extract all of the non-phosphorylated isopentenoid lipids (e.g. dolichol phosphate) of potential interest. Although samples were not saponified, the Silica Sep-Pak procedure, coupled with HPLC, captured all of the esterified and unesterified isopentenoid lipid precursors/end products. Radioactive mevalonate was used to label the isopentenoid lipid precursors/end products because of its greater specificity relative to acetate.

CHO-215 cells incorporated (48 h) [3H]mevalonate primarily into compounds which eluted in fraction IV (Table I). This observation came as a surprise because acidic isopentenoid derivatives eluted in this fraction. Chang et al. (2) and others (3, 9) had focused on radioactive precursor incorporation into only the non-acidic lipids such as those eluted in Sep-Pak fractions I–III. For comparison, the radioactivity distribution obtained with [3H]mevalonate labeled wild type CHO K1 cells is also shown in Table I. Whereas 94.8% of the [3H]mevalonate incorporated by CHO-K1 cells eluted in Sep-Pak fraction II, only 11–30% of the CHO-215 cell radioactive lipids were in this fraction.

Since most (60–90%) of the total [3H]mevalonate incorporated into CHO-215 cell acetone-soluble lipids (ASL) eluted in Sep-Pak fraction IV, we set a course to identify its dominant component(s). Reverse-phase HPLC radiochromatograms (Fig. 2) of Sep-Pak fraction IV yielded three distinct peaks with retention times of 6.0, 6.8, and 7.6 min. Each radioactive peak was isolated by repetitive HPLC, checked for purity by TLC, and analyzed by high and low resolution mass spectrometry.

Rs values from the TLC analysis of each purified acidic sterol are summarized in Table II. The Rs values obtained were similar to those reported (3, 24) for the unnatural reference 4-methyl,4-carboxy- and 4-carboxy-cholestan-3β-ols. Mass spectral analysis of the compound with a retention time of 7.8 min gave a parent ion (m+) at m/z 444 which corresponded to a chemical formula of C29H48O3. Characteristic peaks for losses of [M-H2O] at m/z 426, [M-CH3 at m/z 424, [M-(CH3 + H2O)] at m/z 429, [M-(CH2 + H2O)] at m/z 411 and [M-CO2H] at m/z 398 were detected. These data and the Rs values (Table II) for TLC of the 7.8-min peak compound in solvent systems used by others (3, 24) to resolve acidic sterols allowed us to tentatively identify it as 7(8)-cholesten-4β-methyl,4α-carboxy,3β-ol (Fig. 3).

The compound with a 6.8-min retention time gave a m+ at m/z 442. Characteristic fragmentation consistent with the

TABLE I

| Solvent system | I* | II* | III* | IV* |
|----------------|----|-----|------|-----|
| Sterol         | 100| 100 | 100  | 100 |
| C27 (cholesterol) | 100| 100 | 100  | 100 |
| Unidentified compound | 100| 100 | 100  | 100 |
| Cholesteryl-3β-acetate | 100| 100 | 100  | 100 |
| Acetylated unidentified compound | 100| 100 | 100  | 100 |
| 7(8) Cholesterol,4β-methyl,4α-carboxy,3β-ol | 100| 100 | 100  | 100 |
| 7(8),24-Cholestadien,4β-methyl,4α-carboxy,3β-ol | 100| 100 | 100  | 100 |
| 7(8),24-Cholestadien,4α-carboxy,3β-ol | 100| 100 | 100  | 100 |

*Precoted TLC Silica Gel-60 plates.
HPTLC precoated Silica Gel-60 plates impregnated with AgNO3.
HPTLC precoated Silica Gel-60 plates.
RS values in parentheses are for the unnatural cholestan-3β-ol derivatives (21).

Following were detected: [M-CH3] at m/z 427, [M-H2O] at m/z 424, [M-(CH2 + H2O)] at m/z 409 and [M-CO2H] at m/z 396. An additional fragment of m/z 69, characteristic of [CH3CO2C = CHCH3] was also detected. This fragment supported the existence of a double bond between carbon 24 and 25 of the side chain. These data coupled with TLC migration results summarized in Table II were consistent with the compound with a 6.8-min retention time being 7(8),24-cholestadien,4β-methyl,4α-carboxy,3β-ol (Fig. 3).
did not yield a parent ion (m+) but a major ion [M-CO,H] at m/z 382. Furthermore, m/z fragments characteristics for losses of -CH₃, H₂O, -(CH₂ + H₂O) were seen along with a m/z 69 ion. This fragmentation data plus a major ion [M-CO,H] at m/z 382 were consistent with the 6.0-min peak (Fig. 3). Thus, the dominant acidic CHO-215 sterols were 7(8),24-cholestadien,4α-methyl,4α-carboxy,3β-ol, and 3, 7(8),24-cholestadien,4α-carboxy,3β-ol.

The ratio between side chain Δ24 unsaturated (6.8 min peak) and saturated (7.8 min peak) 4α-methyl,4α-carboxysterol derivatives was influenced by media cholesterol source.

Finally, the acidic product with a retention time of 6.0 min did not yield a parent ion (m+) but a major ion [M-CO,H] at m/z 382. Furthermore, m/z fragments characteristics for losses of -CH₂O, H₂O, -(CH₃ + H₂O) were seen along with a m/z 69 ion. This fragmentation data plus a major ion [M-CO,H] at m/z 382 were consistent with the 6.0-min peak compound being 7(8),24-cholestadien,4α-carboxy,3β-ol (Fig. 3). Thus, the dominant acidic CHO-215 sterols were 7(8),24-cholestadien,4α-methyl,4α-carboxy,3β-ol and 7(8)-cholesten,4β-methyl,4α-carboxy,3β-ol (retention times 6.8 and 7.8 min, Fig. 2).

Steady State Concentration of Acidic Sterols—In order to determine the steady state concentration of the acidic sterols, we isolated [3H]7(8)-cholesten-4β-methyl,4α-carboxy,3β-ol by preparative HPLC and determined its mass by acetylation with [1-'Clacetic anhydride of known specific activity (18.7 mCi/mmol). Different aliquot sizes of an isopropyl alcohol solution of the pure unacytlated [3H]-4-methyl,4α-carboxysterol were resolved by reverse-phase C₁₈ HPLC and their peak heights at 210 nm measured. A 4-methyl,4α-carboxysterol peak height versus mass standard curve was generated and used to obtain this sterol's steady state concentration level in CHO-215 cells grown for 48 h in medium which contained 10 μg of cholesterol/ml of the CH-PC dispersion. Total steady state acidic sterol content was determined to be 3–4 μg/mg protein. Cellular cholesterol's concentration was 18–25 μg/mg protein.

Non-acidic Sterols—Examination of Sep-Pak fraction II by reverse-phase C₁₈ HPLC (Fig. 5) showed that labeled lanosterol and dihydrolanosterol were the dominant mono-oxygenated sterols. However, a radioactive peak which migrated with a retention time similar to that for cholesterol (peak 1) was also detected; ubiquinone was not eluted under these conditions. Normal-phase radio-HPLC (Si-CN column) analysis of CHO-215 cells' Sep-Pak fraction II showed that 5% of the injected radioactive activity was in lanosterol and dihydrolanosterol, 16% in an unidentified isopentenoid which eluted with cholesterol, and the remaining 32% was in ubiquinone (data not presented).

Although Berry and Chang (3) reported (based on TLC migration) that CHO-215 cells synthesized cholesterol at ≤5% of the wild type rate, it was important to determine, with our analytical system, whether this observation was confirmed. The unknown radioactive peak which migrated as cholesterol began to dislodge from the flasks after 72 h of incubation (11 and results not shown).

To ascertain whether acidic sterols were secreted into the medium, CHO-215 cells were prelabeled with [3H]mevalonate (24 h) washed with McCoy's 5A medium and refed with media which contained no mevalonate and either 5% FCS or 5% B₁₂. Media and cells were assayed separately for radioactive sterols over a subsequent 48-h incubation period. Less than 10% of the initial radiolabeled cellular neutral and acidic sterols appeared in the media. Radioactive cellular neutral sterols decreased approximately 90%, and there was a similar increase in the cellular acidic sterol fraction (data not presented). The acidic sterols were localized primarily (94%) in the 100,000 × g particulate fraction of sonicated cell-free extracts. Thus, CHO-215 acidic sterols were not secreted but accumulated in cellular membranes.
the modulation of HMG-CoA reductase activity. Therefore, these observations were consistent with the primary protein factors. Insight about interactive relationships be-
methyl,4a-carboxysterol derivatives (Fig. 2); only a small amount (~2%) of the 4β-desmethyl,4a-carboxysterol was de-
sterol accumulation.
sterols (Table II). Lastly, CHO-215 cell-free extracts decar-
tectable neutral sterols. The only detectable product (l-2% of the input radioactivity) with CHO-215 extracts migrated as
wild type (CHO-K1) and mutant CHO-215 cell homogenates. 
CHO-215 cell homogenate yielded no detectable neutral sterols. The only detectable product (1-2% of the input radioactivity) with CHO-215 extracts migrated as
cholesten,4β-methyl,4α-carboxy,3β-ol, isolated from CHO-215 cells grown on [3H]mevalonate for 48 h, was used as the substrate.
CHO-K1 cell-free homogenates readily converted, [3H]7(8)-cholesten,4β-methyl,4α-carboxy,3β-ol to neutral sterols (Fig. 6). However, the CHO-215 cell homogenate yielded no detectable neutral sterols. The only detectable product (1-2% of the input radioactivity) with CHO-215 extracts migrated as [3H]7(8)-cholesten,4α-carboxy,3β-ol. Thus, the in vitro assay confirmed the intact cell observations (Table II and Fig. 4). CHO-215 decarboxylation of 4β-methyl,4α-carboxysterols was strongly decreased and decarboxylation of 4α-carboxysterols was not detectable.

HMG-CoA Reductase Activity in CHO-215 Cells—Since CHO-215 cells accumulated primarily acidic oxysterols (Table II) and not lanosterol as reported previously (2, 3), there was none with total 4-carboxysterol level. Therefore, 4-carboxysterols (acidic oxysterols) were unlikely regulatory molecules responsible for the modulation of reductase activity.

DISCUSSION

We demonstrated that [3H]mevalonate-labeled CHO-215 mutant cells, accumulated 4-carboxysterols (Figs. 2 and 4) and not lanosterol or 14-desmethyl lanosterol as reported by others (2, 3, 9). This is the first report of cellular 4-carboxysterol accumulation.

The dominant CHO-215 acidic sterol products were 4β-methyl,4α-carboxysterol derivatives (Fig. 2); only a small amount (~2%) of the 4β-desmethyl,4α-carboxysterol was detected. Furthermore, CHO-215 cells did not synthesize C27 sterols (Table II). Lastly, CHO-215 cell-free extracts decarboxylated pure [3H]7(8)-cholesten,4β-methyl,4α-carboxy,3β-ol at 1% the rate measured with wild type cell extracts (Fig. 6). These observations were consistent with the primary
cholesterol availability on CHO-215 4-carboxysterol level and HMG-CoA reductase activity

| Cholesterol source | Total 4-carboxysterol | Cholesterol-egg lecithin dispersion | 4-carboxysterol conversion to CZ7 sterols | A24,4-carboxysterol conversion to 24,25-dihydro, 4-carboxysterols | 24,25-dihydro, 4-carboxysterols close to 1.0 | HMG-CoA reductase activity | ρg/ml of protein pmol/min/mg of protein |
|--------------------|-----------------------|-------------------------------------|----------------------------------------|-----------------------------------------------|----------------------------------------|----------------------------------------|----------------------------------------|
| 5% FCS             | 3.2                   | 79                                  |                                        |                                               |                                        |                                        |                                        |
| 1.063 g/ml bottom  | 4.0                   | 705                                 |                                        |                                               |                                        |                                        |                                        |
| dispersion         | 4.3                   | 846                                 |                                        |                                               |                                        |                                        |                                        |

between 4-methyl sterol oxidation and decarboxylation must await purification of these enzyme complexes.

The ratio of Δ24,4-carboxysterols:24,25-dihydro, 4-carboxysterols was dependent on exogenous cholesterol source. Continuous [3H]mevalonate labeling studies with CHO-215 incubated with a cholesterol source (B2) which did not support long term growth resulted in a steady state ratio of 1:4 (Figs. 2 and 4). However, exogenous cholesterol sources (FCS and CH-PC) which supported continuous CHO-215 growth established steady state ratios of Δ24,4-carboxysterols:24,25-dihydro, 4-carboxysterols close to 1:0 (Fig. 9). This distribution did not reflect the steady state lanosterol/dihydrolanosterol ratio of 4:1 reported for CHO-K1 cells grown in the presence of FCS (25). Thus, it appeared that 24,25-dihydro, 4-carboxysterol accumulation was facilitated by the lack of Δ24, 4-carboxysterol conversion to C27 sterols.

Berry and Chang (3) reported that approximately 5% of the radioactivity incorporated into CHO-215 cell neutral sterols (Sep-Pak fractions I-III) was in C27-C27 sterols and/or cholesterol. We demonstrated that the product which migrated with a HPLC (Fig. 5) retention time similar to cholesterol was not this sterol. Unfortunately, insufficient amounts of the unknown material was available for determination of its identity. However, migration characteristics on HPTLC suggested that it might be a 4-methyl sterol.

Since the end products synthesized by CH-215 mutant cells were oxidized at C-4, it was possible that they could act as "oxysterol" regulatory signal molecules (11) to modulate HMG-CoA-reductase activity. However, endogenously synthesized 4-carboxysterols accumulated independent of cholesterol availability (Table III, Fig. 2). Therefore, 4-carboxysterols were unlikely participants in the regulatory cascade which modulated HMG-CoA reductase activity.

The finding that CHO-215 cells did not synthesize detectable levels of C27 sterols was important for future studies designed to test the oxysterol hypothesis without the contribution of endogenous cholesterol synthesis. Preliminary results obtained with CHO-215 cells (26) have validated their use as a model to assess whether 25-hydroxycholesterol was biosynthesized from regulatory levels of exogenous cholesterol (purity >99.3%).

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