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# Isolation and partial characterization of a cholesterol-requiring mutant of Chinese hamster ovary cells

(membrane lipid biogenesis/sterol biosynthesis/mutagenesis/gas-liquid chromatography/sterol identification)

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ABSTRACT **A sterol-requiring mutant has been isolated from mutagenized Chinese hamster ovary cells. This mutant grows normally only when cholesterol is present in the medium. Cell lysis occurs within 3 days in the absence of cholesterol. The frequency of reversion of this mutant to prototrophic growth is low ($\leq 10^{-6}$). Whole cell pulse experiments with [$^{14}$C]acetate or [$^{3}$H]mevalonate indicate that the rate of synthesis of digitonin-precipitable material is greatly diminished in the mutant cells as compared to that in normal Chinese hamster ovary cells. Enzyme assays *in vitro* with crude cell extracts show that the biosynthetic conversion of mevalonate to squalene and the conversion of squalene to lanosterol are *not* impaired in the mutant cells. Gas-liquid chromatographic analyses of radioactive sterol composition after whole cell pulse experiments with [$^{3}$H]squalene and with [$^{3}$H]lanosterol suggest that the fundamental enzymatic defect of the mutant is at the stage of lanosterol demethylation. When cells were grown in serum-free medium, lanosterol and dihydrolanosterol accumulated intracellularly in the mutant cells before cell lysis occurred; neither of these two intermediary sterols was detected in the wild-type cells grown under the same condition.**

Specific lipid requiring mutants isolated from microorganisms or animal cells (1, 2) can serve as unique biological tools for studies in lipid biosynthesis and for membrane investigations. In this report, we describe the isolation of a cholesterol-requiring mutant from Chinese hamster ovary (CHO) cells. Preliminary biochemical characterization of this mutant suggests that the fundamental enzymatic defect is at the stage of lanosterol demethylation.

## MATERIALS AND METHODS

Chinese hamster ovary (CHO) cells, media, serum, trypsin, sodium [*1*-$^{14}$C]acetate, and ethyl methanesulfonate were as described previously (2). DL-[2-$^{3}$H]Mevalonolactone (382 mCi/mmol) was from Amersham-Searle. DL-[5-$^{3}$H]Mevalonate (DBED salt, 5 mCi/0.2 mg) was from New England Nuclear. Creatine kinase (42 units/mg) and glucose-6-phosphate dehydrogenase (Brewer's yeast, 350 units/mg) were from PL Biochemicals. Aquasol-2 liquid scintillation counter cocktail was from New England Nuclear. ATP (disodium salt), Fraction V bovine serum albumin, creatinine phosphate, and $NADP^+$ were from Sigma. Cholesterol, desmosterol, and lanosterol were from Steraloids. Egg lecithin was from Analabs. Digitonin and DL-mevalonolactone were from ICN Pharmaceuticals. Squalene was from Eastman. Tween-80 was from Sargent-Welch. Higuchi medium, modified by addition of 0.3 mM L-proline and 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate (Hepes) buffer, pH. 7.4, was prepared as described (3).

**Cell Homogenization and Enzyme Assays.** Cells ($10^9$ or more) grown in suspension culture in $\alpha$-minimal essential medium + 10% fetal calf serum were centrifuged at room temperature (2000 rpm for 5 min), washed with phosphate-buffered saline twice, and recentrifuged. After suspension for 10 min in 10 ml of 20 mM ice-cold potassium phosphate buffer, pH 7.4, containing 1 mM EDTA, the cells were homogenized in a Potter-Elvehjem glass homogenizer equipped with a Teflon pestle at 1000 rpm (15 strokes) and centrifuged at 10,000 rpm for 20 min at 0°. The supernatant was used immediately as the enzyme source.

The enzymatic assay used for squalene synthesis from mevalonate *in vitro* was essentially that of Popjak (4) in a volume of 2 ml. In all cases the isolated reaction product was verified to be pure [$^{3}$H]squalene by radioactive gas-liquid chromatographic (GLC) analyses with an SE-30 column. The en-

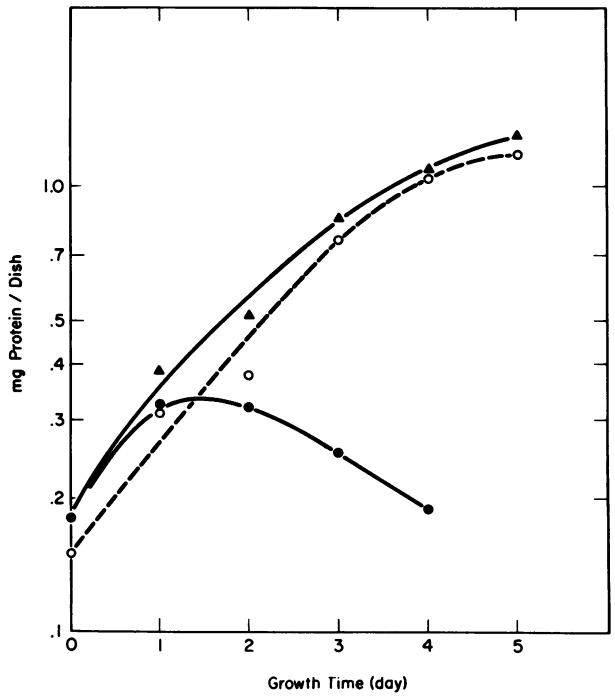


FIG. 1. Growth curves of wild-type and mutant no. 215 cells with or without cholesterol supplement. Cells grown in F-12 + 10% fetal calf serum were trypsinized and replated at $0.5 \times 10^5$ cells per 60-mm dish in the same medium. After 3 days, cells were rinsed once with 4 ml of phosphate-buffered saline and the medium was replaced with 5 ml of F-12 + 10% delipidated bovine serum with or without 30 $\mu$g/ml of cholesterol supplement. Medium was renewed every 2 days. Cell growth was followed in duplicate dishes by measurements of cellular protein content (2). (○ - - - ○) Wild-type cells with or without cholesterol supplement; (● — ●) mutant no. 215 cells without cholesterol supplement; (▲ — ▲) mutant no. 215 cells with cholesterol supplement.

Abbreviations: CHO, Chinese hamster ovary; GLC, gas-liquid chromatography.

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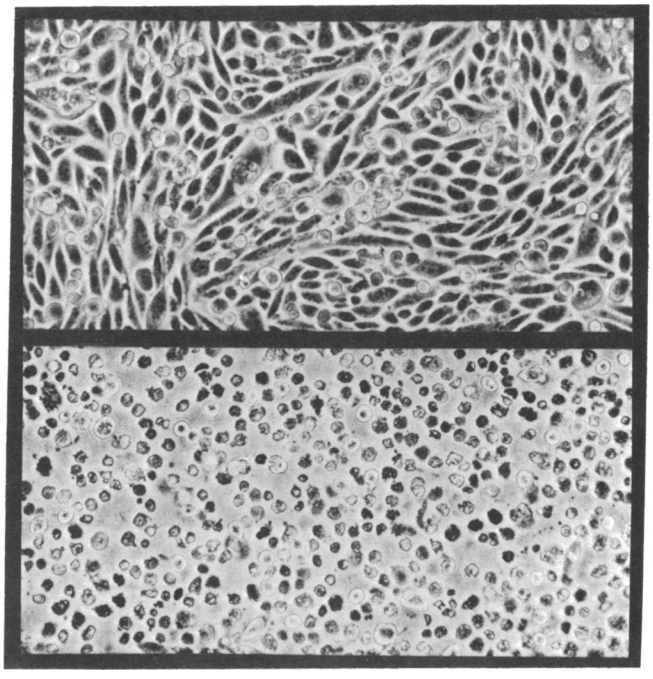

FIG. 2. Morphology of wild-type or mutant no. 215 cells grown with or without cholesterol supplement. Photographs were taken (70-fold magnification with a Nikon phase contrast microscope) on the fourth day after the medium shift. (Top) Mutant no. 215 cells with cholesterol; (bottom) mutant no. 215 cells without cholesterol.

zymatic assay for conversion of squalene to lanosterol *in vitro* was essentially the same as described by Scallen *et al.* (5), using a 10,000 × *g* supernate. The reaction product was isolated and analyzed by thin-layer chromatography on silica gel G using a solvent system of methylene chloride and ethyl acetate (97:3) as described (6).

**Radioactive Pulse Experiments.** The cells were processed for saponification and for measurements of cellular protein content and radioactivity incorporation as described (2), except the wash step with 5% trichloroacetic acid was omitted.

**Radioactive GLC Analyses.** A Varian model 2100 gas chromatograph equipped with a factory-installed 1:6 microsample metal splitter was used in these experiments. A U-tube glass column was used (1.8 m × 6.4 mm × 4 mm) packed with either 3% SE-30 or 3% OV-17 and operated at 260°. The carrier gas ($N_2$) flow rate was kept at 9 ml/min through the detector and 45 ml/min through the metal sample collector. After each injection, sample fractions were collected at 0.5- to 2-min intervals with 0.4 m long glass tubes off the metal collector and eluted from the tubes with 6 ml of ether. The recovery of radioactivity from the splitter was about 90% of the expected radioactivity in the split sample.

Table 1. Enzymatic synthesis of squalene from mevalonate

| Source of crude extract | Amount (mg) | Squalene cpm | Squalene cpm/mg P per min |
|---|---|---|---|
| Wild-type | 10 | 2,400 | 80 |
| Wild-type | 20 | 3,800 | 63 |
| Mutant no. 215 | 6 | 1,000 | 56 |
| Mutant no. 215 | 12 | 2,400 | 67 |
| Rat liver | 18.6 | 172,000 | 3,082 |
| Rat liver | 37.2 | 320,000 | 2,842 |

Cells were grown in α-minimal essential medium + 10% fetal calf serum as suspension culture. Cell density was maintained at $1 \times 10^6$ cells per ml. About 1 liter of both wild-type and mutant cells was used. Fresh medium was added to the spinner bottle to double the culture volume the day before cells were harvested. Cell homogenization and enzyme assay were described under *Materials and Methods*.

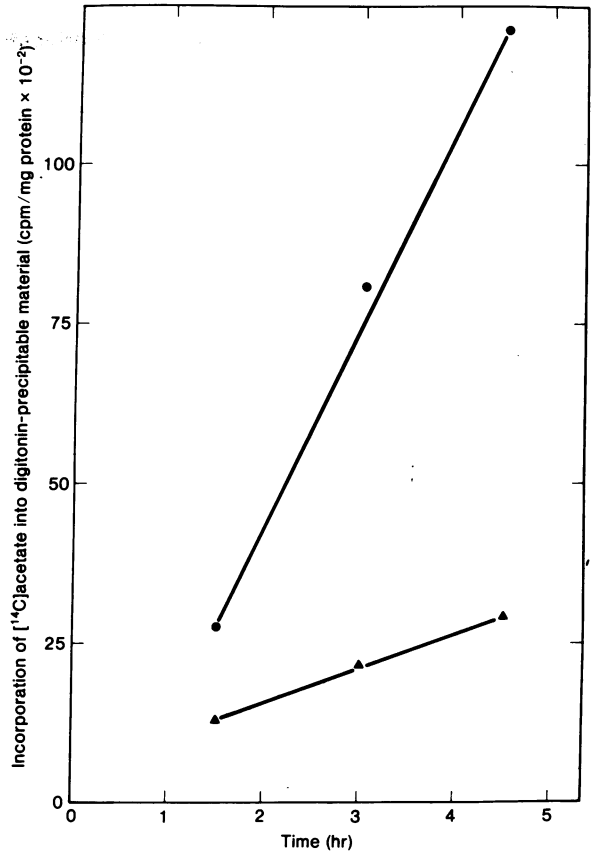


FIG. 3. Incorporation of [$^{14}$C]acetate into digitonin-precipitable material after [$^{14}$C]acetate pulse. Wild-type or mutant no. 215 cells grown in F-12 + 10% fetal calf serum were trypsinized and replated with 4 ml of F-12 + 10% fetal calf serum medium in 60-mm dishes at a cell density of $5 \times 10^5$ cells per dish. On the second day, the medium was renewed twice, each time with 4 ml of fresh F-12 + 10% fetal calf serum. On the third day, the medium was renewed once; 3 hr later, medium was removed, and cells were rinsed once with 4 ml of warm saline buffer. Three milliliters of F-12 + 10% delipidated fetal calf serum containing 15 μCi/ml of sodium [*1*-$^{14}$C]acetate (60 mCi/mmol) were added, and the cells were incubated at 37° for various time intervals. Digitonin-precipitable material was determined as described by Sperry and Webb (8) and protein as described under *Materials and Methods*. (●) Wild-type cells; (▲) mutant no. 215 cells.

**Preparation of [$^3$H]Squalene and [$^3$H]Lanosterol.** [$^3$H]Squalene was prepared from [5-$^3$H]mevalonate by the method of Popjak (4). The radiopurity of [$^3$H]squalene, as assessed by radioactive GLC and by thin-layer chromatography with various solvent systems, was 99%. [$^3$H]Lanosterol was prepared from DL-[2-$^3$H]mevalonate as described (7), and was purified twice on thin-layer silica gel G plates [developed with methylene chloride and ethyl acetate (97:3)]. The purified [$^3$H]lanosterol ($10^4$ cpm/μg) consisted of a 70:30 mix of lanosterol and dihydrolanosterol, as assessed by the radioactive GLC described above.

## RESULTS

**Growth Properties of Mutant No. 215 Cells.** A second isolate obtained from mutagenized CHO cells was selected by its inability to grow on delipidated serum in a similar manner as the

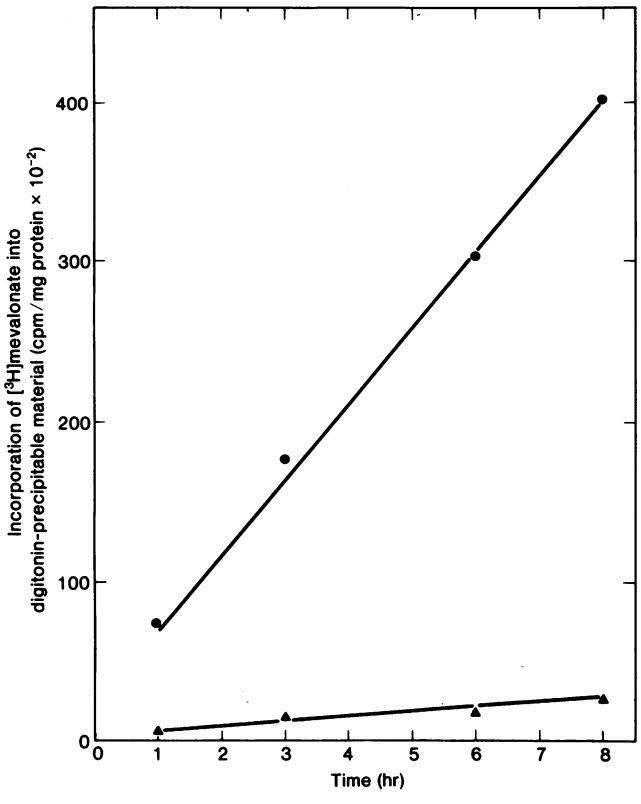


FIG. 4. Incorporation of [$^3$H]mevalonate into digitonin-precipitable materials after [$^3$H]mevalonate pulse. The experimental condition was essentially the same as used in Fig. 3 except cells were plated initially at a cell density of $1.7 \times 10^5$ cells per dish and dialyzed fetal calf serum was used. The medium used for pulse experiment (2 ml per dish) was prepared as follows. DL-[2-$^3$H]Mevalonic acid lactone, 1.5 ml (382 mCi/mmol) in benzene, was dried with a stream of nitrogen in a 100-ml sterile glass bottle. Nonradioactive DL-mevalonate (0.18 ml of a 2 M solution) in sterile phosphate-buffered saline (pH of the solution, 7.4) was added. Then 36 ml of F-12 + 10% dialyzed fetal calf serum medium was added to the bottle and the sample was mixed. After the pulse, cells were processed the same way as described in the legend of Fig. 3. (●) Wild-type cells; (▲) mutant no. 215 cells.

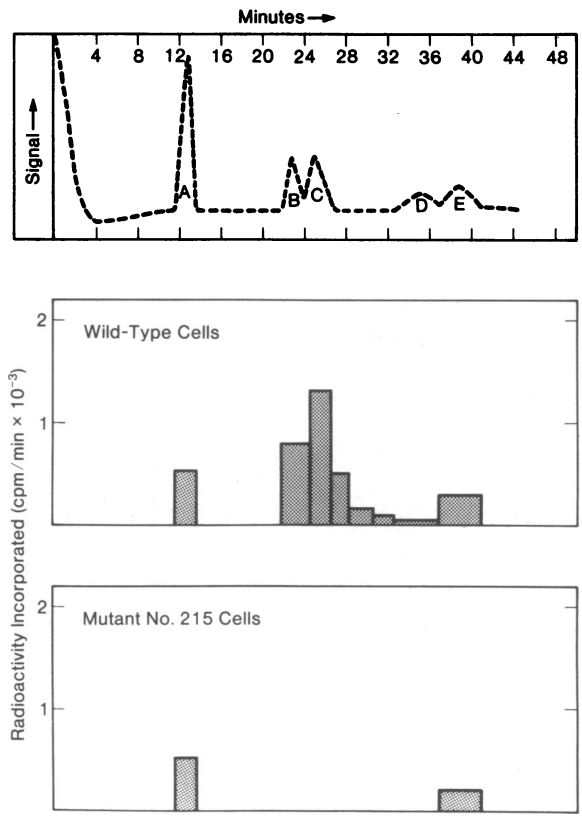


FIG. 5. Incorporation of [$^3$H]squalene into sterol fractions in whole cells. Cells were plated and grown as described in the legend of Fig. 3. The medium used for pulse experiment (4 ml per dish) consisted of [$^3$H]squalene ($10^7$ cpm, $\leq 8$ μg), 0.48 ml of Tween-80 (10 mg/ml), and 6 ml of F-12 + 10% delipidated fetal calf serum. The mixture was sonicated briefly before use. The pulse time was 4 hr at 37°. After the pulse, cells were washed with 4 ml of F-12 + 10% delipidated fetal calf serum medium three times, and were then processed for protein determination and radioactive sterol analysis (*Materials and Methods*). The top section of this figure is a GLC chromatogram with five authentic standards: (A) squalene, (B) cholesterol, (C) desmosterol, (D) dihydrolanosterol, and (E) lanosterol.

mutant that requires unsaturated fatty acids (2). This line, designated as mutant no. 215, did not maintain sustained growth in the absence of cholesterol (Fig. 1). Similar growth curves were obtained for wild-type CHO cells on delipidated fetal-calf serum in the presence or absence of cholesterol, whereas growth of mutant no. 215 leveled off after 1 day in cholesterol-deficient medium. This was followed by a decrease in total cell protein over the next few days. As seen in Fig. 2, mutant no. 215 appeared normal when cultured in medium containing cholesterol (Fig. 2 *top*), whereas the cells appeared to be lysed when the mutant was cultured in the absence of cholesterol (Fig. 2 *bottom*). The morphology of wild-type cells was similar to that seen in Fig. 2 (*top*) when cells were grown in either medium.

**Rates of Sterol Biosynthesis in Whole Cells.** Fig. 3 demonstrates the rate of incorporation of [$^{14}$C]acetate into digitonin-precipitable material of wild-type and mutant no. 215 cells. The precipitation of 3β-OH-sterols by digitonin is known to be a highly specific method for isolation and determination of these sterols (8). This experiment indicates that, using acetate as the substrate, the overall sterol biosynthetic capacity of the mutant cells was defective in comparison with that of the wild-type cells. The incorporation rates of [$^{14}$C]acetate into saponifiable lipid, not shown in this figure, were virtually the same in both types of cells. Because mevalonate has been demonstrated to be one of the obligatory precursors for sterol formation (9), a [$^3$H]mevalonate pulse with whole cells was carried out. Fig. 4 shows that the rate of incorporation of [$^3$H]mevalonate into digitonin-precipitable material in mutant no. 215 cells is grossly deficient when compared to wild-type cells. Preliminary experiments (not shown) indicated that neither labeled acetate nor mevalonate was converted to cholesterol by the mutant cells. The above experiments demonstrate that sterol biosynthesis in mutant no. 215 cells *in vivo* is defective; it also suggests that the biosynthetic lesion of the mutant cells is beyond the stage of mevalonate formation.

**Squalene and Lanosterol Metabolism in Whole Cells.** We next carried out the whole cell pulse experiments using either [$^3$H]squalene (Fig. 5) or [$^3$H]lanosterol (Fig. 6) as sterol precursors, both of which are obligatory intermediates leading ultimately to the formation of cholesterol in animal systems (10). These two experiments demonstrate that, at least in whole cells, mutant no. 215 cells are capable of synthesizing lanosterol from squalene, but unlike the wild-type cells, they are unable to convert lanosterol to cholesterol. The [$^3$H]lanosterol used as the substrate in Fig. 6 consisted of 70% lanosterol and 30% dihydrolanosterol, yet the ratio of recovered radioactivities under

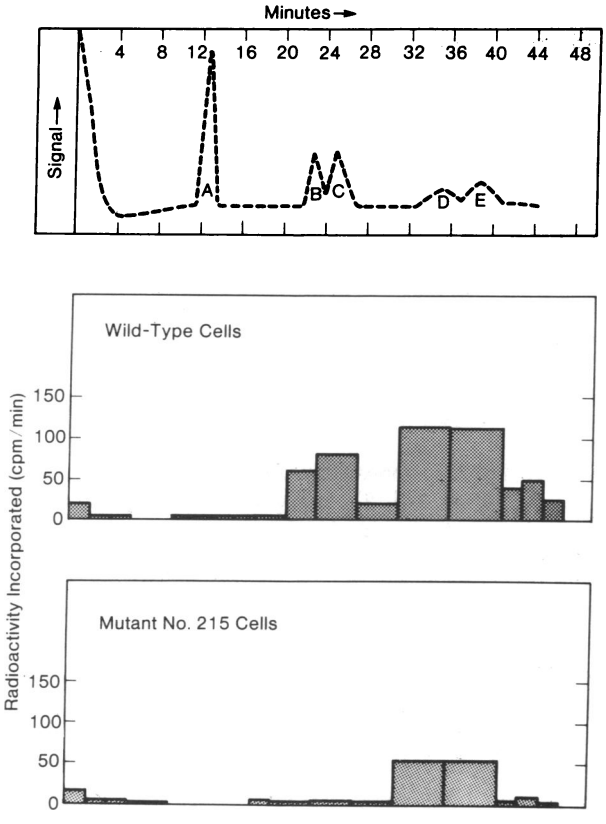


FIG. 6. Incorporation of [$^3$H]lanosterol into sterol fractions in whole cells. Cells were plated and grown as described in the legend of Fig. 4. The medium used for pulse experiment (8.5 ml per dish) consisted of [$^3$H]lanosterol ($2.9 \times 10^5$ cpm, $\leq 24$ $\mu$g), 50 $\mu$l of ethanolic solution of egg lecithin (10 mg/ml), and 8.5 ml of F-12 + 10% delipidated bovine serum. The mixture was sonicated briefly before use. The pulse time was 24 hr at 37°. After the pulse, the cells were processed as described in the legend of Fig. 5. The top section of this figure is a GLC chromatogram with five different authentic standards: (A) squalene, (B) cholesterol, (C) desmosterol, (D) dihydrolanosterol, and (E) lanosterol.

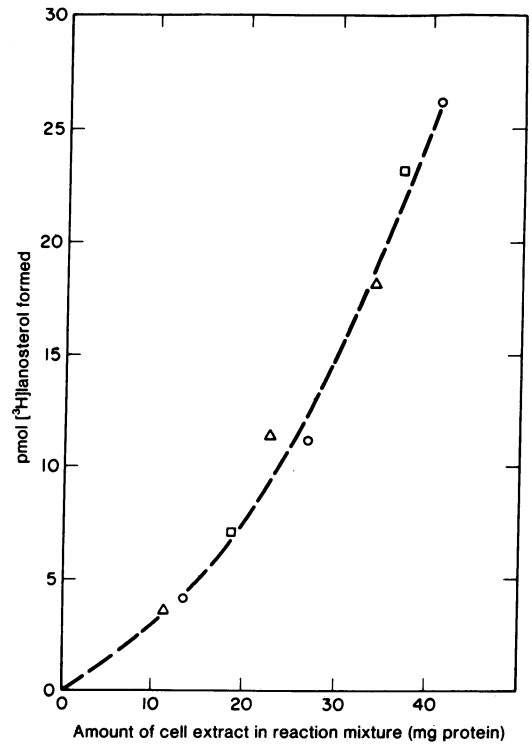


FIG. 7. Enzymatic synthesis of lanosterol from squalene *in vitro*. Cells were grown as described in Table 1. About 4 liters of both wild-type and mutant cells were used. Cell homogenization and enzyme assay were described under *Materials and Methods*. (○) Wild-type cell extracts; (△) mutant no. 215 cell extracts; (□) ½ wild-type cell extracts + ½ mutant no. 215 cell extracts.

lanosterol and dihydrolanosterol peak areas for both wild-type and mutant no. 215 cells approached 50:50. This suggests that part of the incorporated lanosterol was converted to dihydrolanosterol intracellularly, and therefore, the sterol Δ24-reductase activity (11) in the mutant cells is probably intact. This was confirmed by the finding (not shown) that desmosterol was reduced to cholesterol by mutant no. 215 cells.

**Squalene Synthesis and Lanosterol Synthesis *In Vitro*.** Enzymatic synthesis of squalene from mevalonate (4) and of lanosterol from squalene (5) were measured with crude extracts of wild-type cells and mutant no. 215 cells. The data for squalene synthesis are shown in Table 1 and for lanosterol synthesis in Fig. 7. The chemical identity of [$^3$H]squalene formed as product of the first reaction was confirmed by radioactive GLC. This technique was used to demonstrate that with the assay system used for lanosterol synthesis, approximately 80–90% of the radioactive sterols synthesized was lanosterol. The data in Table 1 and Fig. 7 indicate that mutant no. 215 cells possess intact enzymatic activities for lanosterol synthesis from mevalonate, as would be expected from the results of the *in vivo* experiments.

**Cellular Sterol Composition of Cells Grown in Serum-Free Medium.** We have found that wild-type CHO cells can grow from low cell density ($1 \times 10^5$ cells per 60-mm dish) to essential confluency ($\geq 1 \times 10^6$ cells per 60-mm dish) in a serum-free, chemically defined medium (3), provided these cells are plated initially in regular tissue culture medium plus 10% fetal calf serum before the medium switch. As shown in Fig. 8, mutant no. 215 cells grew almost as well as the wild-type cells under the same conditions if 25 $\mu$g/ml of cholesterol was included in the medium; without cholesterol the mutant cells started to die on the third day after the medium was switched from medium supplemented with 10% fetal calf serum to serum-free medium. At various time periods of this growth experiment, cells were harvested and sterol content was analyzed. As can be seen from Table 2, lanosterol and dihydrolanosterol started to accumulate in mutant no. 215 cells on the second day after the medium switch, while in the wild-type cells, no sterols other than cholesterol could be detected throughout the entire time course of this experiment. In this experiment, lanosterol and dihydrolanosterol were identified by both GLC (with SE-30 column and OV-17 column), and by GLC-mass spectrometry (with OV-17 column).

These data strongly suggest that the fundamental biochemical defect of mutant no. 215 is the inability of the cells to convert lanosterol and dihydrolanosterol to cholesterol.

## DISCUSSION

All of the evidence presented in this paper, including growth experiments, whole cell pulse experiments, specific enzyme assays, and cellular sterol composition analysis, indicates that mutant no. 215 is a cholesterol-requiring mutant, defective in its ability to convert lanosterol to cholesterol. The biosynthetic pathway converting lanosterol to cholesterol involves successive demethylation reactions of methyl groups at the 14 $\alpha$-position and the 4$\alpha$- and 4$\beta$-positions of lanosterol. Each of the three

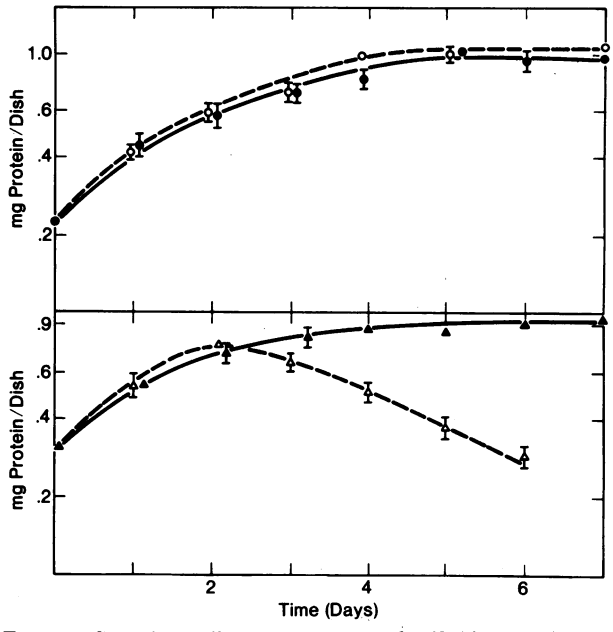


FIG. 8. Growth of cells in serum-free media. Wild-type cells were plated at $1.5 \times 10^5$ cells per 60-mm dish and mutant no. 215 cells at $2 \times 10^5$ cells per 60-mm dish in F-12 + 10% fetal calf serum medium. After 3 days, cells were rinsed twice with 4 ml of saline buffer and the medium was switched to 10 ml per flask of Higuchi + 0.1% Fraction V bovine serum albumin. Medium was renewed once every 2 days. Cellular protein content was determined as described under *Materials and Methods*. (● — ●) Wild-type cells with 25 μg/ml of cholesterol supplement; (○ - - - ○) wild-type cells without cholesterol supplement; (▲ — ▲) mutant no. 215 cells with 25 μg/ml of cholesterol supplement; (△ - - - △) mutant no. 215 cells without cholesterol supplement.

demethylations involves several enzymatic reactions (12–14). Although the evidence presented strongly suggests that demethylation is blocked, the site of the defect in this series of reactions has not yet been delineated.

Table 2. Cholesterol (C), lanosterol (L), and dihydrolanosterol (DL) content of cells grown in serum-free medium

| Time* (days) | C† | L† | DL† |
|---|---|---|---|
| | Wild-type | | |
| 0 | 21.3 | 0 | 0 |
| 6 | 22.0 | 0 | 0 |
| | Mutant no. 215 | | |
| 0 | 21.8 | 0 | 0 |
| 1 | 21.8 | 0 | 0 |
| 2 | 17.1 | 0.14 | 0 |
| 3 | 15.8 | 0.95 | 0.04 |
| 4 | 16.8 | 1.8 | 0.9 |
| 5 | 14.1 | 2.3 | 1.2 |
| 6 | 12.5 | 2.9 | 2.3 |

Cells were grown as described in the legend of Fig. 8. Cellular sterols were analyzed by GLC as described under *Materials and Methods* and by GLC-mass spectrometry. Five micrograms of 4α-methylcholesterol was added to every sample before extraction, and used as an internal standard.

* Time in serum-free medium.

† Sterol content expressed as μg/mg of protein.

The mutant is nonleaky with very low reversion frequency ($\leq 10^{-6}$). In the absence of cholesterol supplementation, these cells die even if they are plated at high cell density ($\geq 2 \times 10^6$ cells per 60-mm dish). Phenotypic expression of this mutant seems to be very stable because cultures of mutant no. 215 cells that have been carried continuously for several months have retained the same cholesterol requirement for growth. Unlike the pleiotropic yeast petite mutants, which require oleate and sterol for growth (15, 16), growth of mutant no. 215 cells in the presence or absence of cholesterol cannot be stimulated by exogenous oleate supplementation. Moreover, rates of saturated and unsaturated fatty acid synthesis in whole cells, measured by the [$^{14}$C]acetate pulse technique (2), were virtually identical in wild-type and mutant no. 215 cells, indicating that mutant no. 215 cells possess an intact fatty acid synthetase and desaturase.

This work and our previous work on a mutant that requires unsaturated fatty acid (2) clearly demonstrate that it is now entirely possible to use standard conventional techniques (17) to select for specific lipid-requiring mutants from cultured mammalian cells.

The availability of such mutants with specific defects in lipid synthetic enzymes should facilitate studies of the biological functions of unsaturated fatty acids and particularly cholesterol in animal cells.

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