Inhibitors of sterol synthesis. Submicromolar 14α-ethyl-5α-cholest-7-ene-3β,15α-diol causes a major modification of the sterol composition of CHO-K1 cells and a marked change in cell morphology

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Abstract Incubation of Chinese hamster ovary cells (CHO-K1) with 14α-ethyl-5α-cholest-7-ene-3β,15α-diol (0.1 μM) in lipid-deficient medium led to a major change in cellular sterol composition, which was characterized by a very marked accumulation of C_{30} sterols (lanosterol and 24,25-dihydrolanosterol). The accumulation of C_{30} sterols was associated with a striking change in cell morphology. The change in cell shape (elongation) was similar to that described previously (A. W. Hsie and T. T. Puck, 1971. *Proc. Natl. Acad. Sci. USA.* **68:** 358–361; and confirmed herein) for CHO-K1 cells incubated in the presence of dibutyryl cAMP (1 mM). This change in morphology, induced by dibutyryl cAMP, was not accompanied by a change in cellular sterol composition. The cell elongation and accumulation of C_{30} sterols, induced by the 14α-ethyl diol, were prevented by the addition of cholesterol (10 μM or 100 μM) and were reversed by removal of the 14α-ethyl diol from the incubation medium. Incubation of the cells with the 14α-ethyl diol had no effect on the levels of cAMP under the conditions studied. Incubation of the cells with miconazole (10 μM) or with lanosterol (10 μM) was also associated with the accumulation of C_{30} sterols and an elongation of the cells. 24,25-Dihydrolanosterol (10 μM) also induced similar changes in cellular morphology. The results presented herein demonstrate that marked changes in the sterol composition of CHO-K1 cells can be effected by incubation of the cells with 14α-ethyl-5α-cholest-7-ene-3β,15α-diol, miconazole, or lanosterol. In addition, the findings reported herein indicate an important role of sterols in the control of the shape of these cells.—**Izumi, A., F. D. Pinkerton, S. O. Nelson, J. S. Pyrek, P. J. G. Neill, J. H. Smith, and G. J. Schroepfer, Jr.** Inhibitors of sterol synthesis. Submicromolar 14α-ethyl-5α-cholest-7-ene-3β,15α-diol causes a major modification of the sterol composition of CHO-K1 cells and a marked change in cell morphology. *J. Lipid Res.* 1994. **35:** 1251–1266.

Supplementary key words cholesterol • lanosterol • miconazole • inhibitors of sterol synthesis

14α-Ethyl-5α-cholest-7-ene-3β,15α-diol (hereafter referred to as "ethyl diol") is a potent inhibitor of cholesterol biosynthesis. This synthetic 15-oxygenated sterol (1, 2), for which unequivocal assignment of structure was made by X-ray crystal analysis of an appropriate derivative (3), has been shown to be highly active in the inhibition of the incorporation of labeled acetate into digitonin-precipitable sterols in mouse L cells (50% inhibition at 50 nM) and in primary cultures of fetal mouse liver cells (50% inhibition at 60 nM). The ethyl diol also lowered the levels of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase activity in the same cells (1, 4). A second site of action, other than HMG-CoA reductase, was indicated by the observation that the concentration of the sterol required to cause a 50% inhibition of the synthesis of digitonin-precipitable sterols was considerably less than that required to cause a comparable lowering of HMG-CoA reductase activity (4) and the demonstration that the ethyl diol, at a concentration of 1.0 μM, caused an almost complete inhibition of the incorporation of [^{3}H]acetate into C_{27} sterols in the 10,000 *g* supernatant fraction of a rat liver homogenate. This inhibition was associated with

Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; TLC, thin-layer chromatography; dibutyryl cAMP, N^6,O^2-dibutyryl adenosine 3':5'-cyclic monophosphate; G-6-P, glucose-6-phosphate; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis-[2-(5-phenyloxazolyl)]-benzene; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; NCS, newborn calf serum; GC, gas chromatography; TMS, trimethylsilyl; NSL, nonsaponifiable lipid(s); GC–MS, gas chromatography–mass spectrometry; MPLC, medium pressure liquid chromatography.

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a striking accumulation of labeled lanosterol and 24,25-dihydrolanosterol (5). Moreover, preincubation of rat liver microsomes with the ethyl diol (100 μM) had no effect on HMG-CoA reductase activity (5). The ethyl diol (0.5 μM) also caused an inhibition of the synthesis of C_{27} sterols in CHO-K1 cells, which was also accompanied by a striking accumulation of labeled lanosterol and 24,25-dihydrolanosterol (6). The inhibition of the metabolism of these two C_{30} sterols occurred after a very short time (15 min) of exposure of the cells to the 15-oxygenated sterol. A considerably longer exposure of the cells to ethyl diol was required for significant inhibition of the synthesis of nonsaponifiable lipids (NSL) from labeled acetate and for the reduction of the levels of HMG-CoA reductase activity in the cells (6).

The present study was initiated to determine whether the ethyl diol could be used to modify the sterol composition of CHO-K1 cells and, if successful, to explore the effects of this modification on various aspects of cellular function. The results of the studies presented herein demonstrate that incubation of CHO-K1 cells with the ethyl diol, miconazole, or lanosterol leads to the accumulation of lanosterol and/or 24,25-dihydrolanosterol. In addition, our studies indicate an important role of sterols in the control of the morphology of these cells. A preliminary communication of a portion of these results has been presented (7).

EXPERIMENTAL PROCEDURES

Materials

14α-Ethyl-5α-cholest-7-ene-3β,15α-diol, prepared as described previously (1, 2), showed a single component on thin-layer chromatography (TLC) (solvent system, 35% ethyl acetate in $CHCl_3$). Cholesterol was purified by way of its dibromide derivative (8) and recrystallization, and showed a single component on TLC (solvent system, 20% ethyl acetate in toluene). 3β-Hydroxy-5α-cholest-8(14)-en-15-one was prepared as described previously (9, 10), and showed a single component on TLC (solvent system, 33% ethyl acetate in hexane). Lanosterol and 24,25-dihydrolanosterol were prepared as described previously (6) and showed single components on TLC (solvent system, 20% ether in toluene). Authentic samples of 3β-acetoxy-5α-cholest-8(14)-ene (11), 3β-acetoxy-5α-cholest-8-ene (11), 3β-acetoxy-5α-cholesta-8,14-diene (12), 3β-acetoxy-cholesta-5,7-diene (13), cholesteryl acetate (14), 3β-acetoxy-cholesta-5,24-diene (14), 3β-acetoxy-5α-cholesta-8,24-diene (15), lanosteryl acetate (6), and 24,25-dihydrolanosteryl acetate (6) were prepared as described previously. N^6,O^2-dibutyryl adenosine 3',5'-cyclic monophosphate (monosodium salt) (cAMP), glucose-6-phosphate (G-6-P), NAD, NADP, ATP, and bovine serum albumin, were purchased from Sigma Chemical Company (St. Louis, MO). Miconazole ([1-(2,4-dichloro-β-(2,4-dichlorobenzyloxy)-phenylethyl)imidazole), also obtained from Sigma, in the form of its nitrate salt, melted at 185–186°C (lit., 184–185°C (16)) and showed a single component on TLC (solvent system, n-butanol–acetic acid–water 12:1:1; R_f 0.41). [^{3}H]acetic anhydride (0.5 mCi per mg) was purchased from New England Nuclear (Boston, MA). (3RS)-[2-^{3}H]mevalonolactone (176 mCi per mmol) was obtained from the Amersham Corporation (Arlington Heights, IL). Silicic acid (100 mesh) and 2,5-diphenyloxazole (PPO) were obtained from Mallinckrodt, Inc. (St. Louis, MO). 1,4-Bis-[2-(5-phenyloxazolyl)]-benzene (POPOP) was purchased from Packard Instrument Co. (LaGrange, IL). Aluminum oxide (50–200 microns; Woelm neutral), silica gel (32–63 microns; Woelm) were obtained from Universal Scientific, Inc. (Atlanta, GA). Neutral alumina AG-7 (2–44 microns, without binder) was obtained from Bio-Rad Laboratories (Richmond, CA). Hyflo Super Cel was obtained from Johns-Manville Products Corporation (Lompoc, CA). Hexamethyldisilazane and trimethylchlorosilane were obtained from Applied Science Laboratories, Inc. (State College, PA). Powdered Ham's F12 medium (17), Dulbecco's modified phosphate-buffered saline (PBS; KCl, 2.7 mM; KH_2PO_4, 1.2 mM; NaCl, 137 mM; and NaH_2PO_4, 8.1 mM), and newborn calf serum (NCS) were obtained from M.A. Bioproducts (Walkersville, MD) or from Irvine Scientific (Santa Ana, CA). Chinese hamster ovary cells (CHO-K1) were purchased from the American Type Culture Collection (Rockville, MD) and they were subcloned to obtain populations of morphological homogeneity. Male Sprague-Dawley rats were purchased from Sprague-Dawley Farms (Madison, WI) and they were maintained on an alternating light (7:00 AM–6:00 PM)–dark (6:00 PM–7:00 AM) cycle and fed a Purina rat chow diet (Formulab 5008).

Methods

Thin-layer chromatography (TLC) was carried out on plates of silica gel G (Analtech; Newark, DE) unless specified otherwise. Components on the plates were visualized after spraying with molybdic acid (18). Radio-TLC analyses were made as described previously (19). Gas chromatography (GC) was carried out using a Hewlett-Packard Model 402 system or a Hewlett-Packard Model 5730A system equipped with silanized glass columns (6 ft × 4 mm). NSL were analyzed either directly or, after formation of their trimethylsilyl (TMS) ether derivatives, on a glass column (1.8 m × 4 mm) packed with 3% OV-17 on Gas Chrom Q. Nitrogen was used as the carrier gas. The temperatures of the injection port and detector were maintained at 300°C. Column temperature was either held constant at 280°C or programmed to increase from 240°C to 280°C at a rate of 8°C per min unless noted otherwise. On the 3% OV-17 column, under isothermal conditions at 280°C, the following retention times (relative to 5α-cholestane) were observed for authentic standards: cholesterol, 2.17; 5α-

cholest-8-en-3β-ol, 2.28; cholesta-5,7-dien-3β-ol, 2.50; cholesta-5,24-dien-3β-ol, 2.56; 5α-cholesta-8,24-dien-3β-ol, 2.69; 24,25-dihydrolanosterol, 3.00; lanosterol, 3.55; and 14α-ethyl-5α-cholest-7-ene-3β,15α-diol, 5.84. Capillary GC analyses were carried out on a Perkin-Elmer Model Sigma 2000 unit or on a Hewlett-Packard Model 5730 unit, both equipped with a 0.1 μm DB-5 capillary column (30 m × 0.25 mm), using helium as the carrier gas. For the former unit, the temperatures of the injection port and the detector were 290°C and 300°C, respectively. The column temperature was programmed to increase from 50°C to 280°C at a rate of 30°C per min and was maintained at 280°C. For the latter unit, the temperatures of the injection port and the detector were 250°C and 300°C, respectively, and the column temperature was programmed to increase from 220°C to 260°C at a rate of 4°C per min. All sterol compositions are presented in terms of mole percentage. GC of fatty acid methyl esters were carried out on a glass column (1.8 m × 4 mm) packed with 10% SP-2330 on Chromosorb WAW. In these cases, the temperature of the injection port and the detector was 250°C and the column temperature was programmed to increase from 170°C to 210°C at a rate of 2°C per min. A Hewlett-Packard 3090A integrator was used to determine areas under peaks on the chromatograms. GC–mass spectrometry (GC–MS) of the NSL and their TMS derivatives was carried out using a Finnigan 3300 GC–MS unit equipped with a Finnigan 6100 data system. Helium was used as the carrier gas. Analyses were made using either a 3% OV-17 on Gas Chrom Q (60–80 mesh) column (1.2 m × 2 mm) or a 1% OV-17 on Gas Chrom Q (60–80 mesh) column (1.8 m × 2 mm). Column temperatures were as described above. MS were recorded at 70 eV with an emission current of 0.3 mA and an electron multiplier voltage of 1.35 kV. GC–MS analyses of fatty acid methyl esters used GC conditions described above. The identifications of fatty acid methyl esters were based upon retention times on GC (relative to those of authentic standards), the results of GC–MS analyses, and the retention times of the samples on GC after catalytic (5% palladium on carbon) hydrogenation.

Silicic acid–Super Cel (1:1) column (100 cm × 1 cm) chromatography of NSL was carried out as described previously (6, 19–23). Alumina-Super Cel–$AgNO_3$ column chromatography was carried out as described previously (11, 12, 24, 25). Alumina–$AgNO_3$ medium pressure liquid chromatography (MPLC) was carried out using 100 cm × 0.9 cm columns as described previously (6, 14, 15, 26–28).

Radioactivity was measured in a Beckman LS-9000 or a Beckman LS-9800 liquid scintillation spectrometer. Nonaqueous samples were assayed using PPO (0.4%) and POPOP (0.005%) in toluene. Radioactivity in polar solvents (such as $CHCl_3$–methanol solutions) and TLC plate scrapings were assayed using PPO (0.4%) in toluene–ethanol 2:1. Acetylation of sterols was carried out with acetic anhydride and pyridine using a minor modification of the method of Johnston and Bloch (29). Colorimetric assays of sterols and steryl acetates were carried out using a modified Liebermann-Burchard color reagent (30) as described previously (6, 12, 19, 23–28, 31). TMS ether derivatives of sterols were prepared using the reagent of Carter and Gaver (32) (hexamethyldisilazane and trimethylchlorosilane in pyridine) with heating at 80°C for 5 min. Protein was measured according to Lowry et al. (33) or Bradford (34) using bovine serum albumin as standard.

[^{3}H]24,25-dihydrolanosterol was prepared by incubation of a rat liver homogenate preparation with labeled mevalonate in the presence of the ethyl diol (1 μM), a modification of an approach described previously (5). The 10,000 *g* supernatant fraction of a rat liver homogenate, prepared in potassium phosphate buffer (0.1 M; pH 7.4) containing $MgCl_2$ (5 mM) and nicotinamide (30 mM), was incubated with [2-^{3}H]mevalonolactone (176 mCi per mmol) in the presence of added ATP (5 mM), NAD (1 mM), NADP (1 mM), G-6-P (3 mM), and ethyl diol (final concentration, 1 μM; added in propylene glycol) for 1 h at 37°C. After saponification of the incubation mixture (3 h at 90°C with an equal volume of 15% ethanolic KOH), the NSL were subjected to silicic acid–Super Cel 1:1 column (100 cm × 1 cm) chromatography. Using toluene as the eluting solvent, fractions 6.1 ml in volume (45 min per fraction) were collected. The labeled material with chromatographic behavior of lanosterol and 24,25-dihydrolanosterol (fractions 25–38) was acetylated and subjected to alumina–Super Cel–$AgNO_3$ column (100 cm × 1 cm) chromatography using hexane–toluene 85:15 as the eluting solvent (fraction size, 4.2 ml). The material with the chromatographic behavior of the acetate derivative of 24,25-dihydrolanosterol (fractions 12–24) was saponified and further purified by MPLC on a silica gel column (100 cm × 1 cm) using toluene–ether 95:5 as the eluting solvent (fraction size, 5.2 ml). The contents of fractions 50–62 were pooled. Radio-TLC analysis (solvent system, toluene–ether 4:1) showed a single component with the mobility of authentic 24,25-dihydrolanosterol.

CHO-K1 cells were maintained in Ham's F12 medium (17) supplemented with 8% NCS (10 ml) at 37°C in a 5% CO_2–95% air environment. In most experiments, the cells (2.5–3.0 × 10^5 cells) were inoculated into 150-mm plates containing Ham's F12 medium supplemented with 8% NCS (20 ml) and incubated for 48 h. The medium was then aspirated, the plates were rinsed with PBS (30 ml), and fresh Ham's F12 medium supplemented with 8% delipidated (35) NCS was added. The delipidation method used has been found to remove over 99.9% of the cholesterol present in serum (unpublished data). Unless specified otherwise, experimental cells received medium containing the ethyl diol (0.1 μM), while control cells

received medium containing the equivalent amount of ethanol (0.004%). Cell growth was monitored by counting trypsinized cells on a hemocytometer. Cells from three dishes were counted each day. Medium containing the ethyl diol was prepared as described previously (6). In experiments in which cholesterol, lanosterol, [^{3}H]24,25-dihydrolanosterol, and miconazole were added to the cells, ethanol solutions were added to Ham's F12 medium containing 8% delipidized NCS and equilibrated for 24 h prior to use. Control incubations contained the same final concentrations of ethanol as for experimental media. Final concentrations of ethanol in the experiments with the various compounds were as follows: miconazole (0.05%), lanosterol and 24,25-dihydrolanosterol (0.2%), ethyl diol (0.1 μM) plus cholesterol (1 μM and 10 μM) (0.042%), and ethyl diol (0.1 μM) plus cholesterol (100 μM) (0.39%). In each of the experiments, the medium was changed every 2 days. At harvest, the medium was removed by aspiration, the plates were rinsed twice with PBS (10-ml portions), and the cells were collected by scraping with a rubber policeman. The cell suspensions were combined and subjected to centrifugation at 600 *g* for 10 min. The resulting cell pellets were washed twice with PBS. The cell pellets were suspended in water (1 ml) in screw-capped tubes, 15% ethanolic KOH (1 ml) was added, and the resulting mixtures were heated at 80°C for 3 h. After cooling to room temperature, water (2 ml) was added and the NSL were extracted 3 times with petroleum ether (8-ml portions). The combined petroleum ether extracts were washed 3 times with water (8-ml portions). The resulting NSL were then analyzed by GC and GC–MS. Fatty acids were recovered by acidification (pH 1–2) of the residual aqueous phase and extraction 3 times with ethyl ether (8-ml portions). The combined ether extracts were washed 3 times with water (8-ml portions). The resulting fatty acids were methylated with diazomethane (36) and subjected to GC and GC–MS.

cAMP levels in ethyl diol-treated cells and control cells were measured using a commercial assay kit (Amersham Corporation, TRK.432). Briefly, approximately 1×10^5 cells were inoculated into 100-mm dishes containing Ham's F12 medium supplemented with 5% NCS (10 ml) and incubated for 48 h. The medium was changed to experimental media containing various concentrations of the ethyl diol which were prepared as described above except that 5% delipidated NCS was used. Fresh experimental media were added after 24 h. At the end of 24 or 48 h of incubation, the experimental media were aspirated and the plates were washed three times with ice-cold PBS (10-ml portions). Trichloroacetic acid (10%; 1.25 ml) was added to the plates, and the cells were harvested by scraping with a plastic cell scraper. The cells were incubated on ice for 15 min to insure complete cell lysis. After centrifugation for 10 min at 12,000 *g* in an Eppendorf Microfuge, the resulting supernatants were transferred to glass vials and, after five extractions with ethyl ether (2-ml portions), evaporated to dryness under nitrogen at 45°C. The resulting residues were dissolved in water (150 μl) and the levels of cyclic AMP were determined.

Plasma membranes from CHO-K1 cells were prepared by a minor modification of the method of Brunnette and Till (37). The isolated plasma membranes were suspended in water (1 ml) and the NSL and fatty acids were recovered as described above for the case of whole cells. Sterols of the plasma membranes of control and experimental cells were analyzed by GC and, in the form of their TMS derivatives, by GC–MS as described above. In addition, a portion of the NSL of the plasma membranes obtained from the control cells were acetylated with [^{3}H]acetic anhydride (12 mCi; 25 mg) in dry pyridine (0.5 ml). After standing overnight at room temperature, unlabeled acetic anhydride (0.5 ml) was added and the reaction mixture was heated at 40°C for 3 h. Ice and water were successively added and the resulting mixture was extracted 3 times with petroleum ether (8-ml portions). The combined extracts were washed twice with water (6-ml portions) and evaporated to dryness under nitrogen. A portion of the acetylated material was subjected to MPLC on an alumina–$AgNO_3$ column along with unlabeled samples of the acetate derivatives of 5α-cholest-8(14)-en-3β-ol, 5α-cholest-8-en-3β-ol, cholesterol, 5α-cholesta-8,24-dien-3β-ol, and cholesta-5,7-dien-3β-ol. The column was successively eluted with hexane–toluene 9:1 (fractions 1–80) and hexane–toluene 6:4 (fractions 81–180). Fractions 14 ml in volume were collected. The resulting chromatogram is shown in **Fig. 1.** The contents of fractions 93–100 were pooled and, after the addition of unlabeled samples of the acetate derivatives of cholesta-5,24-dien-3β-ol and 5α-cholesta-8,14-dien-3β-ol, subjected to MPLC on an alumina–$AgNO_3$ column as described above except that hexane–toluene 65:35 was used as the eluting solvent. The resulting chromatogram showed an essentially complete separation of the $\Delta^{5,24}$- and $\Delta^{8,14}$-steryl acetates. Only one peak of ^{3}H was observed which corresponded to that of the authentic standard of the $\Delta^{5,24}$-steryl acetate.

In an attempt to quantitate the changes in morphology (vide infra) the approach introduced by Hsie and Puck (38) was employed. This method involves the determination of the ratio of the longest and the shortest cellular dimensions (L/S ratio). Cell morphology was studied at 100× using a phase contrast microscope and cells were photographed using Kodak Panatomic-X film. The enlarged prints (5 in × 7 in) were further enlarged ($\sim$35%) by photocopying. The photocopy was placed on a Koala Pad (Koala Technologies) which was connected to a Macintosh computer (Apple Computer, Inc.). The coordinates of the longest and shortest cellular axes of the cells were determined using a sharp probe by pointing to the ends of the cell axes on the enlarged photocopy. After measuring the longest and shortest cellular axes of over

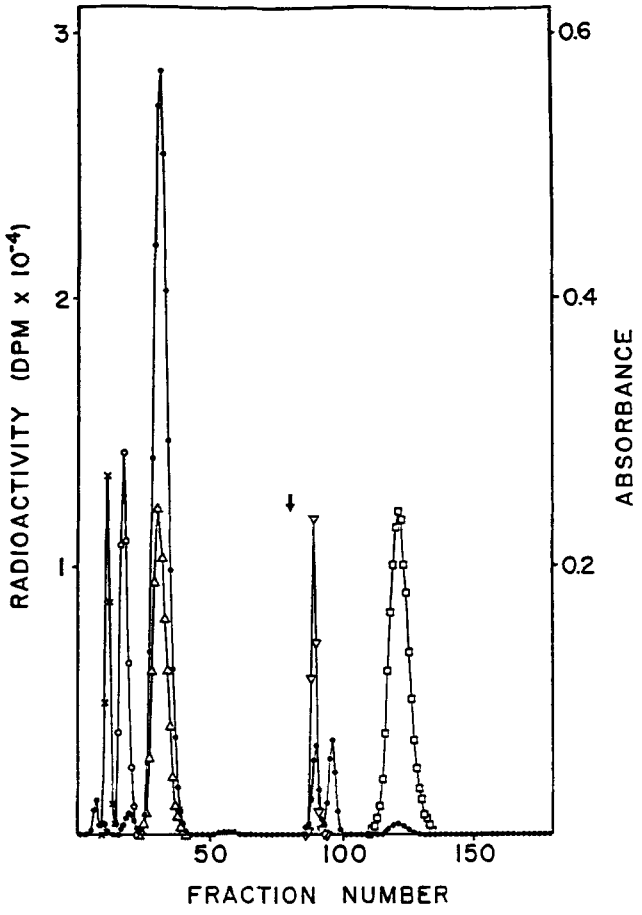

Fig. 1. MPLC analysis on an alumina-$AgNO_3$ column (100 cm × 0.9 cm) of the [^{3}H]acetate derivatives of the nonsaponifiable lipids obtained from plasma membranes of CHO-K1 cells grown for 3 days in lipid-deficient medium. The eluting solvent was hexane–toluene 9:1 which was changed (at arrow) to hexane–toluene 6:4; (●) ^{3}H; (χ) 3β-acetoxy-5α-cholest-8(14)-ene; (○) 3β-acetoxy-5α-cholest-8-ene; (△) cholesteryl acetate; (▽) 3β-acetoxy-5α-cholesta-8,24-diene; and (□) 3β-acetoxy-cholesta-5,7-diene.

100 cells (106 to 336), the mean values of the ratios of the longest to shortest cellular dimensions (L/S ratio) and other statistics were calculated using a computer program that was written in Microsoft Basic (Microsoft Corporation).

For electron microscopic studies, CHO-K1 cells cultured on glass cover slips were incubated in the presence and absence of the ethyl diol (0.1 μM) for 3 days as described above. After washing of the coverslips with saline, the cells were fixed in cold, half-strength Karnovsky's fixative (39) for 1 h at 4°C, rinsed twice (5 min per rinse) in 0.1 M sodium cacodylate buffer, postfixed with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 1 h at 4°C, and then rinsed twice (2 min per rinse) in cold cacodylate buffer. Samples for transmission electron microscopy were en bloc stained with uranyl acetate for 2 h at 4°C, rinsed twice (5 min per rinse) with distilled water, and then dehydrated in graded ethanol. The cells were embedded in Epon 812 (40) and sectioned. Ultrathin sections were stained with uranyl acetate and lead citrate (41) and electron micrographs were obtained using a Phillips EM-420 transmission electron microscope. Samples for scanning electron microscopy were fixed and dehydrated in graded alcohol as described above. The cells were dried in a Denton critical point drier and then coated in a Hummer II vacuum evaporator. Scanning electron microscopy was carried out using an ISI3A scanning electron microscope.

Unless noted otherwise, the variation of mean values is expressed as ± SEM. Differences between mean values were evaluated using Student's *t* test.

RESULTS

Effects of 14α-ethyl-5α-cholest-7-ene-3β,15α-diol (0.1 μM) on the sterol composition, cell growth, and other parameters in CHO-K1 cells

CHO-K1 cells were incubated in lipid-deficient medium in the presence and absence of the ethyl diol (0.1 μM) for varying periods of time. After 3 days of incubation in the presence of the ethyl diol, a marked change in the sterol composition of the cells was observed (**Table 1**). Whereas cholesterol was the major sterol in control cells, the cells incubated with the ethyl diol showed a marked accumulation of C_{30} sterols, with lanosterol and 24,25-dihydrolanosterol constituting 70% of total cellular sterols. Lanosterol was characterized by its retention time on GC and by GC–MS in the form of its TMS ether derivative. Its mass spectrum, which was essentially the same as that of the TMS derivative of an authentic sample of lanosterol, showed a molecular ion at *m/z* 498 and other ions in the high mass region at *m/z* 483 (M–CH_3) and at *m/z* 393 (M–CH_3-TMSOH). 24,25-Dihydrolanosterol was characterized by its retention time on GC and by GC–MS in the form of its TMS derivative. Its MS, which was essentially the same as that of the TMS derivative of an authentic sample of 24,25-dihydrolanosterol, showed a molecular ion at *m/z* 500 and other ions in the high mass region at *m/z* 485 (M–CH_3) and *m/z* 395 (M–CH_3-TMSOH). Cholesterol from the experimental and the control cells was similarly characterized by its retention time on GC and by GC–MS. GC analysis of the sterols of control cells also indicated the presence of at least two minor components which were not identified. GC analysis of the sterols from plasma membranes obtained from cells incubated for 3 days in the presence of the ethyl diol also showed a similar accumulation of C_{30} sterols, with lanosterol and 24,25-dihydrolanosterol constituting 71% of total sterols. Lanosterol and 24,25-dihydrolanosterol were characterized by GC and, in the form of their TMS derivatives, by GC–MS. Plasma membrane cholesterol from experimental and control cells was also characterized by GC and GC–MS. GC analysis of the plasma membranes obtained

TABLE 1. Effect of 14α-ethyl-5α-cholest-7-ene-3β,15α-diol (0.1 μM) on the sterol composition of CHO-K1 cells and of plasma membranes derived therefrom after 3 days of incubation in the presence of the ethyl diol

	Sterol Composition			
	Cells		Plasma Membranes	
Sterol	Control[a]	Ethyl Diol[a]	Control[b]	Ethyl Diol[b]
	%			
Cholesterol	87	30	84	31
Other	13	70	16	69
Lanosterol		60		58
24,25-Dihydrolanosterol		10		11
Cholesta-5,24-dien-3β-ol			5	
5α-Cholesta-8,24-dien-3β-ol			5	
5α-Cholesta-7-en-3β-ol			2	
Cholesta-5,7-dien-3β-ol			2	
Unknown			2	

[a] From GC and GC–MS analyses.
[b] From chromatographic mobilities of acetate derivatives upon alumina–$AgNO_3$ MPLC.

from control cells showed the presence of minor sterols other than cholesterol. The NSL of the plasma membranes from the control cells were acetylated with [^{3}H]acetic anhydride and the steryl acetates were subjected to MPLC on an alumina–$AgNO_3$ column. The resulting chromatogram showed the presence of labeled components with the mobilities of the acetate derivatives of cholesterol (84%), 5α-cholest-7-en-3β-ol (2%), cholesta-5,7-dien-3β-ol (2%), and 5α-cholesta-8,24-dien-3β-ol (5%), and cholesta-5,24-dien-3β-ol (5%).

To extend these observations, CHO-K1 cells were incubated in the presence and absence of the ethyl diol (0.1 μM) for varying periods of time up to 10 days. The sterol composition of the cells was studied by GC and GC–MS as described above. The results presented in **Table 2** show that ethyl diol induced a marked change in the sterol composition of the cells which was apparent as early as 2 days, at which time lanosterol and 24,25-dihydrolanosterol constituted 64% of cellular sterols. At 4, 6, 8, and 10 days of incubation in the presence of the ethyl diol, the combination of lanosterol and 24,25-dihydrolanosterol accounted for 75–79% of cellular sterols with cholesterol constituting the remainder (21–25%) of the sterols. The percentage of 24,25-dihydrolanosterol increased throughout the period of study from 14% at 2 days to 34% at 10 days. The ethyl diol was not observed in the cells (limit of detection ~0.1%). Cholesterol represented the major sterol (94–99% of sterols) from the control cells at the various times studied.

The percentage distribution of major fatty acids from control cells was essentially the same as for ethyl diol-

TABLE 2. Effect of 14α-ethyl-5α-cholest-7-ene-3β,15α-diol (0.1 μM) on the sterol composition of CHO-K1 cells

		Sterol Composition[a]			
Day	Sample	Cholesterol	24,25-Dihydrolanosterol	Lanosterol	Other
		%			
0	Control	97	0	0	3 (3.85)[b]
2	Control	94	0	0	6 (2.59)
	Ethyl diol	35	14	50	0
4	Control	96	0	0	4 (2.60)
	Ethyl diol	25	25	50	0
6	Control	98	0	0	2 (2.59)
	Ethyl diol	23	28	49	0
8	Control	>99	0	0	<1 (2.58)
	Ethyl diol	21	31	48	0
10	Control	99	0	0	1 (2.55)
	Ethyl diol	21	34	45	0

[a] From GC and GC–MS analyses.
[b] Values in parentheses are retention times (relative to 5α-cholestane) of the minor components.

treated cells on days 2, 4, 6, 8, and 10 (data not shown). The percentage distribution of major fatty acids in the plasma membranes from control cells and ethyl diol-treated cells did not differ after 3 days of incubation (data not shown) and was similar to the fatty acid distribution in whole cells after 2 and 4 days of incubation in the presence and absence of the ethyl diol. Thus, in contrast to the marked changes in sterol composition, the ethyl diol had no effect on the fatty acid composition of CHO-K1 cells (or of plasma membranes derived therefrom).

No effect of the ethyl diol (0.1 μM) on cell growth was observed (**Fig. 2**). The doubling times for control and ethyl diol-treated cells were 22.6 h and 23.3 h, respectively. Higher concentrations (0.25 μM and 0.5 μM) of the oxysterol significantly inhibited cell growth (data not shown). It should be noted that CHO-K1 cells grown in Ham's F12 medium supplemented with 8% newborn calf serum have a considerably shorter doubling time (13–14 h). The precise reason(s) for the large increase in the doubling time of the cells grown in lipid-deficient medium was not established. However, our observations are consistent with those from another laboratory (42). The ethyl diol (0.1 μM) also had no effect on two other parameters of cell growth, the incorporation of [^{3}H]thymidine and cell protein per dish (data not shown).

Effects of 14α-ethyl-5α-cholest-7-ene-3β,15α-diol (0.1 μM) on the morphology of CHO-K1 cells

The ethyl diol (0.1 μM) induced marked changes in the morphology of CHO-K1 cells incubated in lipid-deficient medium. After 48 h in the presence of the ethyl diol, the cells showed clear evidence of elongation and this change was more pronounced after 4 and 5 days of incubation.

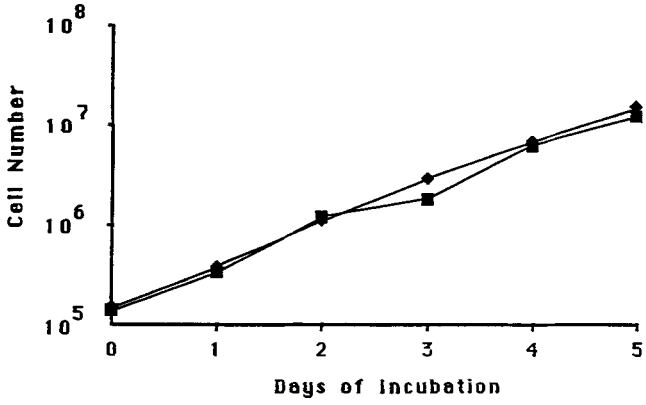

Fig. 2. Growth of CHO-K1 cells in lipid-deficient medium with (♦) or without (■) 14α-ethyl-5α-cholest-7-ene-3β,15α-diol (0.1 μM). Approximately 2.5 × 10^4 cells were inoculated into 100-mm dishes containing lipid-rich medium and incubated for 48 h as described in the text. On day 0, the experimental media were added. Cells were harvested every 24 h and counted on a hemocytometer. Data represent the average from duplicate plates.

Figure 3 shows scanning and transmission electron micrographs of CHO-K1 cells incubated for 3 days in the presence (Fig. 3B and 3D) and absence (Fig. 3A and 3C) of the ethyl diol. Untreated cells were round to ovoid with numerous short and blunt pseudopods and occasional filopodia. Nuclei were irregularly shaped with large nucleoli and comprised nearly half the volume of the cell. Cells treated with the ethyl diol were elongated, much larger than untreated cells, and their surfaces were covered by widely dispersed, uniform microvilli. Many treated cells were multinucleated or with marked nuclear lobation and contained very large nucleoli. Mitochondria were more numerous in treated cells but other cytoplasmic components were similar to control cells.

In an attempt to quantitate these changes in morphology, the approach introduced by Hsie and Puck (38) to follow the changes in morphology of CHO-K1 cells induced by dibutyryl cAMP was used. This method involves the determination of the ratio of the longest to the shortest cellular dimensions. The ethyl diol caused substantial increases in this ratio (**Table 3**). After 2 days of incubation in the presence of the sterol, the mean value of the ratio of the longest to the shortest dimensions (L/S ratio) was 57% higher than that of control cells (4.67 ± 0.24 vs. 2.97 ± 0.12), a difference that was highly significant. After 4 days of incubation in the presence of the sterol, the mean value of the L/S ratio increased to 6.29 ± 0.34, which was very much higher (+ 115%) than the corresponding value for the control cells.

The changes in morphology of the CHO-K1 cells incubated in the presence of the ethyl diol were highly reproducible, both qualitatively and quantitatively (as estimated by determination of changes in the L/S ratio). For example, also shown in Table 3 are the results of two other independent experiments in which the cells were incubated with the sterol for 4 and 4.5 days. Increases in the L/S ratios of 115% and 125% ($P < 0.001$ in each case) were observed on days 4 and 4.5, respectively, relative to control cells on the same days.

Reversal of elongation of CHO-K1 cells and of C_{30} sterol accumulation induced by 14α-ethyl-5α-cholest-7-ene-3β,15α-diol by incubation of cells in medium in the absence of the 14α-ethyl-Δ^7-3β,15α-diol

Table 4 shows the mean values for the L/S ratio for: (A) cells prior to incubation with the ethyl diol, (B) cells treated with the ethyl diol for 4 days, and (C) cells incubated for 4 days in the presence of the sterol followed by 5 days of incubation in the absence of the sterol. Removal of the ethyl diol from the incubation medium was associated with a complete reversal of the elongation of the cells, and with a partial reversal of the accumulation of the C_{30} sterols (Table 4). Removal of the 15-oxygenated sterol was associated with striking changes in the percentage of cells with L/S ratios in excess of 4.0 (12% for cells prior to sterol treat-

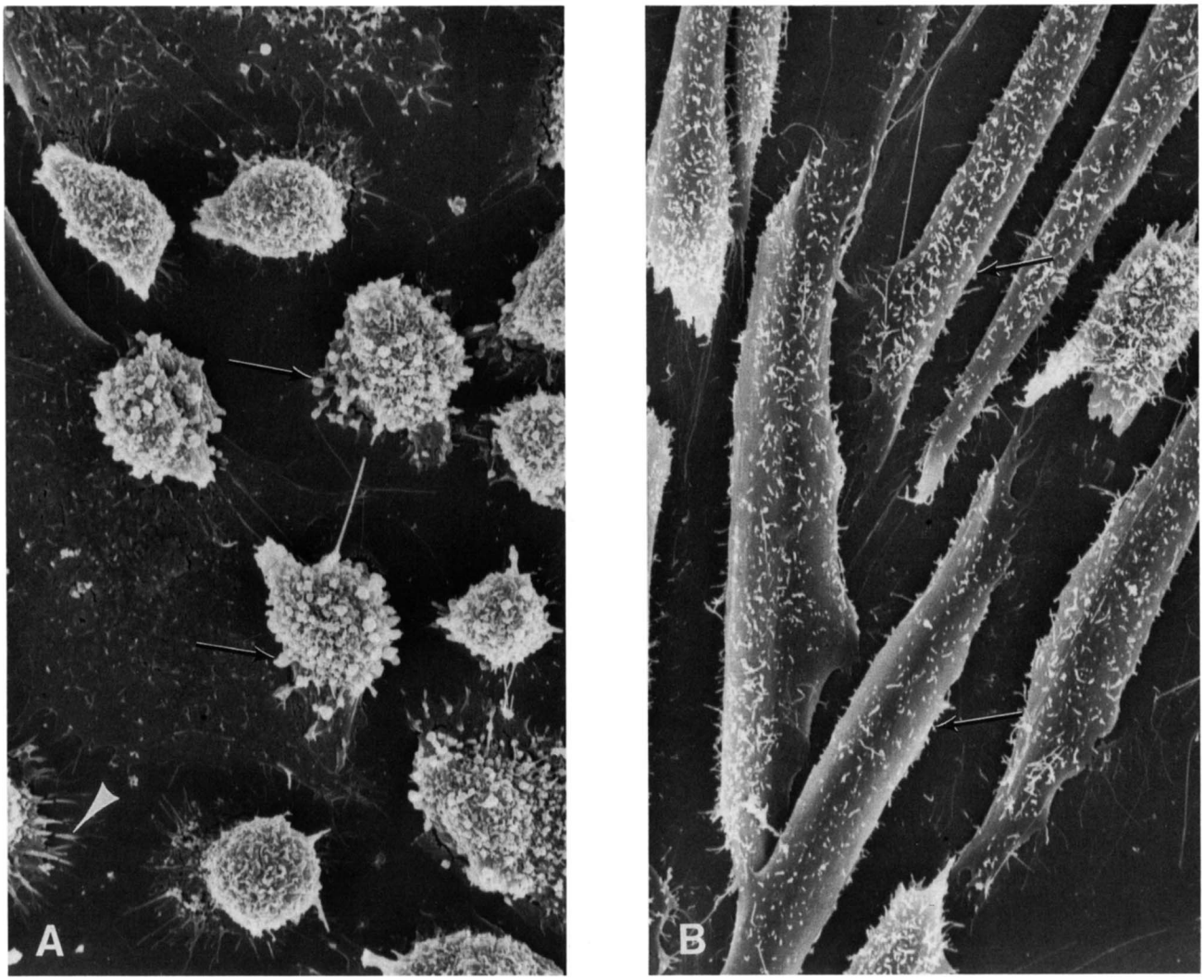

Fig. 3. Scanning (A and B) and transmission (C and D) electron micrographs of CHO-K1 cells after 3 days of incubation in the presence (B and D) and absence (A and C) of 14α-ethyl-5α-cholest-7-ene-3β,15α-diol (0.1 μM). A: Untreated cells are small round to ovoid and covered with short blunt pseudopods (arrows). Occasional clusters of filopodia (arrowhead) are seen. × 2000. B: Ethyl diol-treated cells are elongated and much larger than untreated cells and are covered by widely dispersed short microvilli (arrows). × 2000.

ment, 65% for cells after 4 days of sterol treatment, and 13% for sterol-treated cells after 5 days of incubation in the absence of the sterol). Removal of the 15-oxygenated sterol also led to marked changes in percentage sterol composition. The percentage of cholesterol increased from 21% to 57%. The percentage of lanosterol decreased from 45% to 17%. 24,25-Dihydrolanosterol showed a decrease that was much less than that of lanosterol.

Effect of exogenous cholesterol on the changes in morphology and sterol composition induced by 14α-ethyl-5α-cholest-7-ene-3β,15α-diol (0.1 μM) in CHO-K1 cells

CHO-K1 cells were incubated for 4 and 10 days in lipid-deficient medium containing the ethyl diol (0.1 μM) and varying concentrations of cholesterol (1 μM, 10 μM, and 100 μM). Cholesterol, added at concentrations of 10 μM and 100 μM, but not at 1 μM, prevented the elongation of the cells induced by the ethyl diol (**Table 5**). The results of GC analyses of the sterol composition of the ethyl diol-treated cells incubated in the presence of varying concentrations of exogenous cholesterol for 10 days are also shown in Table 5. The addition of cholesterol (10 μM and 100 μM) resulted in reductions of the percentage of total sterols as C_{30} sterols from 87% to 15% and 12%, respectively.

Effect of miconazole on the sterol composition and morphology of CHO-K1 cells

CHO-K1 cells were incubated in the presence and absence of various concentrations of miconazole for 5 days.

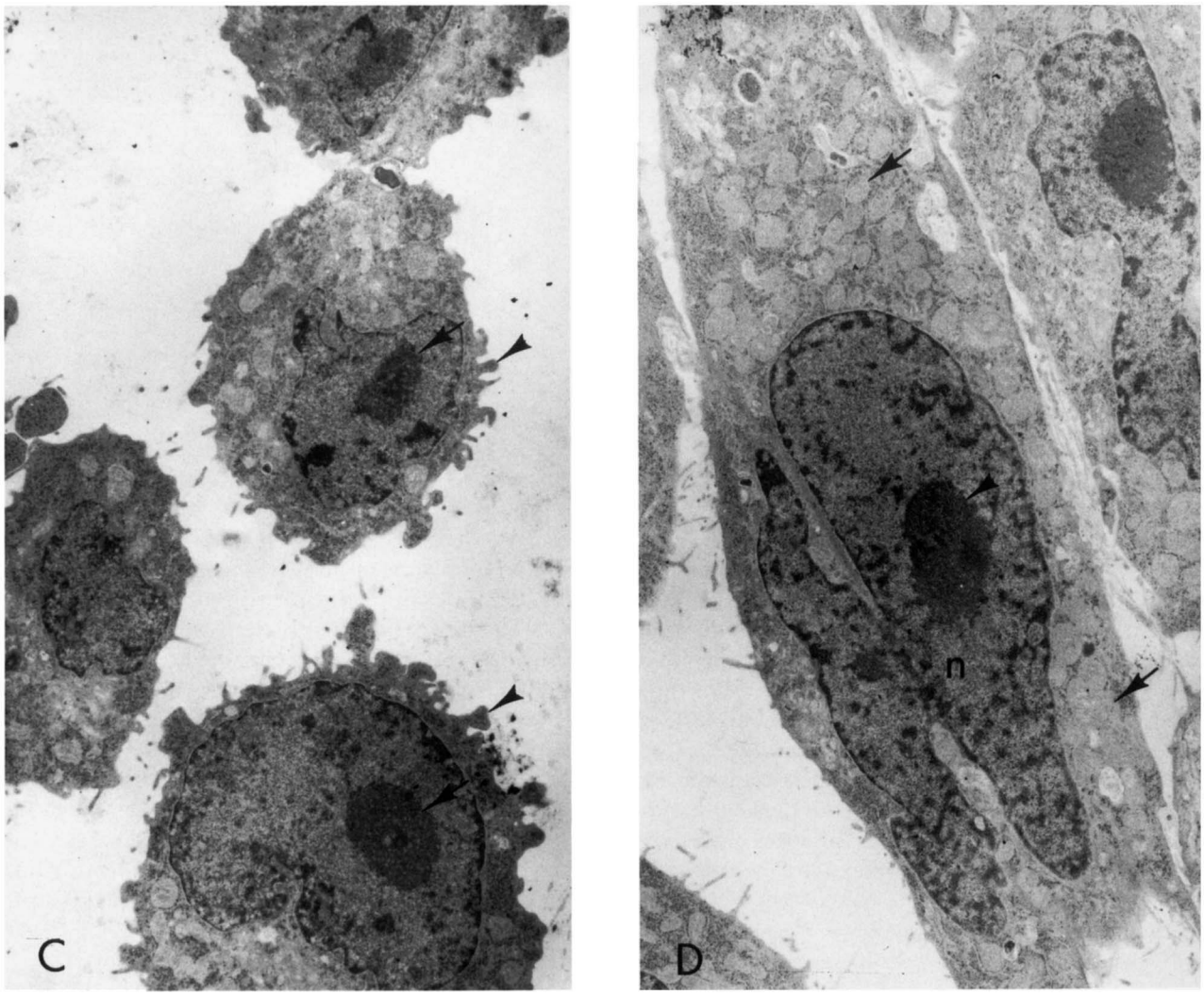

Fig. 3 (cont.) C: Untreated cells have short pseudopods (arrowheads) and large irregular nuclei with large nucleoli (arrows). × 6600. D: Ethyl diol-treated cells contain numerous mitochondria (arrows) and have large multilobate or multiple nuclei (n) with large nucleoli (arrowheads). × 6600.

The results of capillary GC analyses and GC–MS analyses of the NSL from cells incubated with miconazole (10 μM) showed a marked accumulation of lanosterol and, to a lesser extent, of 24,25-dihydrolanosterol that was associated with a decrease in cholesterol (**Table 6**). In contrast, cholesterol was the major sterol from control cells and from cells incubated with miconazole (0.1 μM and 1.0 μM). Miconazole, at the various concentrations studied, had little or no effect on the total sterol concentration of the cells (19.6, 15.3, 20.6, and 18.5 μg sterol per mg protein at concentrations of miconazole of 0.0, 0.1, 1.0, and 10 μM, respectively).

CHO-K1 cells incubated in the presence of miconazole, at a concentration of 10 μM (but not at 0.1 μM or 1.0 μM), showed the same morphological changes as observed with cells incubated with the ethyl diol (0.1 μM). In two separate experiments, incubation of the cells with miconazole (10 μM) was associated with 152% and 166% increases in the mean values for the L/S ratio (Table 6).

Effect of lanosterol on morphology and sterol composition of CHO-K1 cells

Lanosterol, at concentrations of 0.1 μM, 0.5 μM, 1.0 μM, and 5.0 μM, had no effect on the morphology of CHO-K1 cells incubated for 3 days in lipid-deficient medium. However, at a concentration of 10 μM, lanosterol addition led to an elongation of the cells that was evident on day 2 and more pronounced on day 3. Cholesterol (10 μM) addition did not cause elongation of the cells. Incubation of CHO-K1 cells for 3 days in the

TABLE 3. Effect of 14α-ethyl-5α-cholest-7-ene-3β,15α-diol (0.1 μM) on the morphology of CHO-K1 cells as estimated by the determination of the ratio of the longest to shortest dimensions (L/S ratio)

Day	Sample	L/S Ratio (mean ± SEM)	% Change from Control
Experiment 1			
2	Control	2.97 ± 0.12 (n = 171)	
2	Ethyl diol	4.67 ± 0.24 (n = 119)	+57 $P < 0.001$
4	Control	2.92 ± 0.10 (n = 135)	
4	Ethyl diol	6.29 ± 0.34 (n = 134)	+115 $P < 0.001$
5	Control	2.83 ± 0.13 (n = 156)	
5	Ethyl diol	5.98 ± 0.31 (n = 127)	+111 $P < 0.001$
Experiment 2			
4	Control	2.59 ± 0.09 (n = 132)	
4	Ethyl diol	5.56 ± 0.22 (n = 134)	+115 $P < 0.001$
Experiment 3			
4.5	Control	2.64 ± 0.10 (n = 116)	
4.5	Ethyl diol	5.94 ± 0.26 (n = 116)	+125 $P < 0.001$

presence of either lanosterol (10 μM) or [^{3}H]24,25-dihydrolanosterol (10 μM) was associated with markedly elevated mean values of the L/S ratio (5.47 ± 0.21 and 5.81 ± 0.22, respectively) that were clearly different ($P < 0.0001$) from that of cells incubated in the presence of cholesterol (10 μM). The mean values of the L/S ratio of cells treated with lanosterol and 24,25-dihydrolanosterol did not differ significantly ($P > 0.2$).

Capillary GC analyses of sterols from cells incubated for 3 days in the presence of added lanosterol (10 μM) or cholesterol (10 μM) revealed a marked effect on cellular sterol composition. Whereas the total sterol content of the lanosterol-treated and cholesterol-treated cells were essentially the same (18.5 and 17.7 μg per mg protein, respectively), the sterol composition differed markedly. The lanosterol-treated cells showed a marked accumulation of lanosterol (68% of total sterols) along with 9% 24,25-dihydrolanosterol, whereas the cholesterol-treated cells showed mostly cholesterol (89%) accompanied by lower levels of lanosterol (3%) and 24,25-dihydrolanosterol (8%). The sterol composition of the cells incubated with [^{3}H]24,25-dihydrolanosterol was not studied by GC. The NSL recovered after incubation of the cells with the [^{3}H]24,25-dihydrolanosterol were subjected to silicic acid–Super Cel column chromatographic analysis. The resulting chromatogram showed that most (86%) of the ^{3}H corresponded to 24,25-dihydrolanosterol. Approximately 12% of the recovered ^{3}H had the chromatographic mobility of cholesterol and other C_{27} monohydroxysterols. Very little (~ 0.1%) of the recovered ^{3}H was eluted from the column with the mobility of a polar sterol (i.e., after change of the elution solvent from toluene to chloroform–methanol 2:1). Thus, under the conditions of this study, only limited metabolism of the added 24,25-dihydrolanosterol was observed.

Effect of dibutyryl cyclic AMP on morphology and sterol composition of CHO-K1 cells

CHO-K1 cells were incubated for 24 h in lipid-deficient medium in the presence and absence of dibutyryl cAMP

TABLE 4. Effects of 14α-ethyl-5α-cholest-7-ene-3β,15α-diol on cell morphology and sterol composition in cells incubated for 4 days with the ethyl diol (treatment B) followed by incubation for 5 days in the absence of the sterol (treatment C)

Treatment	L/S Ratio (mean ± SEM)	Percent Change from A	Sterol Composition		
			Cholesterol	24,25-Dihydrolanosterol	Lanosterol
				%	
A. Control (no oxysterol)	2.58 ± 0.01 (n = 129)				
B. After 4 days with the ethyl diol	6.29 ± 0.34 (n = 134)	+ 144 $P < 0.001$	21	34	45
C. After 4 days with the ethyl diol followed by 5 days in the absence of the sterol	2.55 ± 0.16 (n = 113)	0 $P > 0.8$[a]	57	25	17

[a]The percentage change from B to C was − 59% ($P < 0.001$).

TABLE 5. Effects of exogenous cholesterol on elongation and the change in sterol composition of CHO-K1 cells induced by 14α-ethyl-5α-cholest-7-ene-3β,15α-diol (0.1 μM) after 4 and 10 days of incubation

Days	Treatment	L/S Ratio (mean ± SEM)	Percent Change		Sterol Composition		
					Cholesterol	24,25-Dihydrolanosterol	Lanosterol
						%	
4	Ethyl diol	5.66 ± 0.27 (n = 129)					
	Ethyl diol + cholesterol (1 μM)	5.98 ± 0.23 (n = 121)	+6	$P > 0.3$			
	Ethyl diol + cholesterol (10 μM)	2.99 ± 0.10 (n = 139)	−47	$P < 0.001$			
	Ethyl diol + cholesterol (100 μM)	2.39 ± 0.07 (n = 149)	−58	$P < 0.001$			
10	Ethyl diol	5.53 ± 0.27 (n = 116)			12.6	41.8	45.6
	Ethyl diol + cholesterol (1 μM)	5.73 ± 0.22 (n = 130)	+4	$P > 0.5$	18.4	39.6	42.0
	Ethyl diol + cholesterol (10 μM)	2.50 ± 0.09 (n = 103)	−55	$P < 0.001$	84.6	7.5	7.8
	Ethyl diol + cholesterol (100 μM)	2.53 ± 0.09 (n = 128)	−54	$P < 0.001$	87.9	3.3	8.8

(1 mM). Cells incubated in the presence of dibutyryl cAMP showed elongation within 6–12 h and, after 24 h of incubation, had a mean L/S ratio of 7.74 ± 0.30 (n = 126) that was +137% higher ($P < 0.001$) than that of control cells incubated for 24 h in the absence of dibutyryl cAMP (3.26 ± 0.12; n = 148). Experimental cells showed a marked difference in the percentage of cells with L/S ratios in excess of 6.0 (experimental, 65.1%; control, 4.1%) and in excess of 8.0 (experimental, 36.5%; control 0.0%). The results of GC analyses of the NSL from experimental and control cells after 24 h of incubation were essentially identical, with cholesterol representing the only detectable cellular sterol. The accumulation of C_{30} sterols, observed in the cases of incubations of the cells with the ethyl diol, miconazole, and lanosterol, all of which induced similar morphological changes, was not found after incubation of the cells with dibutyryl cAMP.

The possibility that the ethyl diol caused an elevation of intracellular levels of cAMP levels, which in turn led to the observed morphological changes, was investigated by incubating the cells in the presence of varying concentrations of the ethyl diol (0.1, 0.25, 0.5, and 1.0 μM) for 24 h or 48 h, and then assaying for cAMP. The results showed that the ethyl diol (at each of the concentrations studied) had no effect on the cellular levels of cAMP at either time point (data not shown). It should be emphasized that the ethyl diol-treated cells demonstrated the expected elongation at all sterol concentrations after 48 h of incubation. 3β-Hydroxy-5α-cholest-8(14)-en-15-one, at the single concentration studied (0.25 μM), had no effect on the levels of cAMP in the cells at 24 h and 48 h (32.5 ± 0.7 and 32.0 ± 1.5 pmol cAMP per mg protein, respectively).

DISCUSSION

The results presented herein demonstrate that incubation of CHO-K1 cells in lipid-deficient medium containing the ethyl diol (0.1 μM) caused a marked modification of the sterol composition of the cells which was characterized by a striking accumulation of lanosterol and 24,25-dihydrolanosterol. The accumulation of the C_{30} sterols was also observed in plasma membranes derived from the ethyl diol-treated cells. In contrast to these marked changes in sterol composition, the ethyl diol had no detectable effect

TABLE 6. Effect of miconazole on the morphology and sterol composition of CHO-K1 cells incubated for 5 days in lipid-deficient medium containing the indicated concentrations of miconazole

Experiment	Miconazole	L/S Ratio (mean ± SEM)	Percent Change	Sterol Composition			
				Cholesterol	24,25-Dihydrolanosterol	Lanosterol	Other
					%		
	μM						
1	0	3.30 ± 0.14 (n = 252)					
	0.1	3.46 ± 0.12 (n = 226)	+5 $P > 0.4$				
	1	3.65 ± 0.19 (n = 203)	+11 $P > 0.1$				
	10	8.79 ± 0.53 (n = 123)	+166 $P < 0.001$				
2	0	3.15 ± 0.12 (n = 145)		79.1	3.4	10.7	6.9
	0.1	3.03 ± 0.12 (n = 180)	−4 $P > 0.4$	87.4	< 0.1	3.9	8.5
	1	3.37 ± 0.12 (n = 139)	+7 $P > 0.1$	75.5	4.7	15.9	3.9
	10	7.95 ± 0.34 (n = 151)	+152 $P < 0.001$	23.2	25.1	48.8	2.9

on fatty acid composition of the cells or of plasma membranes derived therefrom.

The unimpaired growth of the cells in lipid-deficient medium containing the ethyl diol, despite the marked accumulation of C_{30} sterols (70–80% of total sterols) in the cells (and in plasma membranes derived therefrom), is especially worthy of note. The results of early studies suggested that lanosterol alone appeared to be functionally incompetent in eukaryotic cells (43, 44). This postulation was based upon the lack of effectiveness of lanosterol when present as the sole sterol in fulfilling the sterol requirement for the growth of yeast under anaerobic conditions (45–47) and for the pupation of the hide beetle *Dermestes vulpinus* (48). In addition, lanosterol and 24,25-dihydrolanosterol were found to be considerably less effective than cholesterol or 5α-cholestan-3β-ol in supporting the growth of the sterol-requiring *Mycoplasma capricolum* (49, 50). Also, lanosterol and 24,25-dihydrolanosterol did not support the growth of a sterol-requiring strain of *Saccharomyces cerevisiae* under aerobic conditions (51). However, after adaptation of this yeast strain to 24,25-dihydrolanosterol, the cells were found to grow well in the presence of these C_{30} sterols (52). Lanosterol permitted growth of the same strain under anaerobic conditions, albeit not as well as cholesterol (51).

Related studies have also been carried out with mammalian cells in culture. In 1977, Chang et al. (53) isolated a cholesterol-requiring mutant of Chinese hamster ovary cells which was reported to be defective in the metabolism of lanosterol and 24,25-dihydrolanosterol (53, 54). When the mutant cells were grown in serum-free medium in the absence of added cholesterol, the cells ceased growing after 1 day and started to die on the third day. The mutant cells grown under these conditions were found to accumulate lanosterol and 24,25-dihydrolanosterol intracellularly before cell lysis occurred. When wild-type cells were grown under the same conditions, no sterols other than cholesterol were detected. It is important to note that the accumulation of lanosterol and 24,25-dihydrolanosterol in the mutant was very considerably less than that found in the present study. For example, after 4 days of incubation of the mutant cell in serum-free medium, the extents of accumulation of lanosterol and 24,25-dihydrolanosterol were 9.2% and 4.6% of total sterols, respectively. Our results demonstrate that CHO-K1 cells incubated in the presence of the ethyl diol showed a marked accumulation of C_{30} sterols (70–80% of total cellular sterols). Since the growth of these cells was not inhibited, we suggest that the simple accumulation of these sterols in the mutant CHO-K1 cells (which was at a much lower percentage of total cellular sterols (53)) may not represent a unique explanation for the lysis and death of the mutant CHO cells when they were grown in serum-free medium. Recently, Plemitas, Havel, and Watson (55) reported that the mutant cells are deficient in 4-carboxysterol decarboxylase activity and that these cells, incubated in medium containing fetal calf serum, accumulated primarily labeled 4-carboxysterols and not lanosterol, 24,25-dihydrolanosterol, or material believed to be a 14-desmethyl derivative of lanosterol as reported by others (53, 54, 56) in studies in which the cells were incubated in a medium containing delipidized serum or serum-free medium. The acidic sterols were reported to be without effect on the levels of HMG-CoA reductase activity (55).

Chen et al. (57) reported the isolation of another CHO-K1 cell mutant defective in lanosterol metabolism. The defect in this mutant was attributed to an absence of detectable lanosterol 14α-methyl demethylase activity. The mutant cells showed significant accumulation of material with the GC behavior of lanosterol plus 24,25-dihydrolanosterol. After 30 h of incubation of the cells in delipidated medium, the level of total sterols was similar to that of CHO-K1 cells but contained significant amounts of lanosterol plus 24,25-dihydrolanosterol ($\sim$38% of total sterols). In delipidated medium the mutant showed a very severe impairment of growth which could be alleviated by the addition of cholesterol.

More recently, Buttke and Folks (58) reported the isolation of a human T cell line in which significant accumulation of lanosterol and 24,25-dihydrolanosterol was observed when the cells were grown in a medium containing 1% fetal bovine serum. Repeated subculturing of the cells in serum-free media in the absence of added cholesterol led to an almost complete replacement of cellular cholesterol by lanosterol and 24,25-dihydrolanosterol. This marked change in sterol composition was not accompanied by a change in cell growth. The latter finding with the T cell line is similar to the lack of effect of very substantial modification of the sterol composition of CHO-K1 cells on the growth of the cells. However, in the present study we have not established whether or not a low level of cholesterol is required for the unimpaired growth of the cells in the presence of the ethyl diol (0.1 μM).

In the present study we have shown very substantial accumulation of lanosterol and 24,25-dihydrolanosterol in CHO-K1 cells incubated in delipidated medium containing added ethyl diol (0.1 μM). For example, after incubation of the cells for 4 days under these conditions, the extents of accumulation of lanosterol and 24,25-dihydrolanosterol were 50% and 25% of total sterols, respectively (Table 2). Under these conditions, no effect on cell growth was observed. Thus, our findings and those of Buttke and Folks (58) demonstrate unimpaired growth of two mammalian cell lines despite very substantial replacement of cellular cholesterol by the C_{30} sterols.

In some of the experiments reported here, the presence of sterols other than cholesterol (including lanosterol and 24,25-dihydrolanosterol) was observed in control CHO-K1 cells or in plasma membrane derived therefrom. The sterols detected in plasma membranes of control cells corresponded to known sterol intermediates in the biosynthesis of cholesterol (59), i.e., zymosterol (5α-cholesta-8,24-dien-3β-

ol), lathosterol (5α-cholest-7-en-3β-ol), desmosterol (cholesta-5,24-dien-3β-ol), and 7-dehydrocholesterol (cholesta-5,7-dien-3β-ol) (Table 1).[5] We attribute these findings to a very marked increased synthesis of sterols that was induced by incubation of the cells in the lipid-deficient medium.

Incubation of CHO-K1 cells with the ethyl diol not only resulted in a marked change in cellular sterol composition but also a marked change in morphology. The change in cell shape was similar, if not identical, to that reported by Hsie and Puck (38) for CHO-K1 cells incubated in the presence of dibutyryl cAMP, i.e., a change from the randomly oriented, compact shape characteristic of transformed cells to an elongated fibroblast-type form. The elongation of the cells induced by the ethyl diol could be reversed by removal of the ethyl diol from the medium, a change that also resulted in a decrease in the accumulation of C_{30} sterols induced by the ethyl diol. The change in morphology induced by the ethyl diol was prevented by the addition of cholesterol (10 μM or 100 μM), concentrations at which the marked accumulation of C_{30} sterols induced by the ethyl diol was also prevented.

It is important to note that the morphological changes induced by the ethyl diol in CHO-K1 cells were not observed in comparable studies with 3β-hydroxy-5α-cholest-8(14)-en-15-one. This finding can be correlated with the observation that the 15-ketosterol (100 μM) had no effect on the distribution of radioactivity in NSL derived from labeled acetate in the 10,000 *g* supernatant fraction of a rat liver homogenate (61). This latter observation is in marked contrast to the case of the ethyl diol which, at a concentration of 1 μM, caused a marked accumulation of labeled lanosterol and 24,25-dihydrolanosterol. Similarly, in CHO-K1 cells, the 15-ketosterol (0.5 μM) had no effect on the distribution of ^3H in NSL derived from [^3H]acetate, while the ethyl diol, at the same concentration, caused a marked accumulation of labeled lanosterol and 24,25-dihydrolanosterol (6).

Miconazole is one of a group of imidazole-substituted compounds that have been shown to be potent antimycotic agents. Prominent among the actions of these compounds is their inhibitory effect on the enzymatic removal of the 14α-methyl group in sterol biosynthesis (62–68). Kempen et al. (67) reported a decrease in cholesterol and a slight accumulation of lanosterol and dihydrolanosterol (less than 20% of total sterol) upon incubation of HepG2 cells for 24 h with ketoconazole (20 μM, 40 μM, and 60 μM). In the present study, we have found that incubation of CHO-K1 cells with 10 μM miconazole caused a marked accumulation of C_{30} sterols. Under these conditions, lanosterol and 24,25-dihydrolanosterol constituted 49% and 25% of total cellular sterols, respectively. Miconazole, at a concentration of 10 μM, also caused a marked elongation of the cells which was similar, if not identical to that induced by the ethyl diol (0.1 μM) or dibutyryl cAMP (1 mM). At lower concentrations of miconazole (0.1 μM and 1.0 μM), cholesterol represented the major cellular sterol and, at these concentrations, no significant elongation of the cells was observed.

The observation that both the ethyl diol and miconazole, at concentrations that caused a marked accumulation of lanosterol and 24,25-dihydrolanosterol, induced significant elongation of the cells, prompted the investigation of the effect of the addition of those sterols on cell morphology. Both sterols, at a concentration of 10 μM, induced significant elongation of the cells. Incubation of the cells with lanosterol (10 μM) for 3 days led to a marked accumulation of lanosterol. After 3 days of incubation of the cells with [^3H]24,25-dihydrolanosterol, most of the ^3H recovered in the NSL of the cells corresponded chromatographically to the incubated substrate.

The combined findings reported herein suggest that the elongation of the CHO-K1 cells induced by the ethyl diol (0.1 μM), miconazole (10 μM), lanosterol (10 μM), and 24,25-dihydrolanosterol (10 μM) is related to the accumulation of C_{30} sterols (lanosterol and/or 24,25-dihydrolanosterol). The concentration at which lanosterol and miconazole were effective in causing elongation of the cells was that which induced significant accumulation of the C_{30} sterols. At the same time it should be noted that incubation of the cells with the ethyl diol (0.1 μM), miconazole (10 μM), and lanosterol (10 μM) was not only associated with a marked accumulation of C_{30} sterols but also with a decrease in the cholesterol concentration of the cells. While further experimentation on this matter is clearly indicated, it should be noted we have not observed these changes in morphology upon incubation of the cells with other potent inhibitors of sterol synthesis, which in our experience do not cause the accumulation of C_{30} sterols. Irrespective of these considerations, it should be noted that in the cases of cells incubated with miconazole (10 μM) or lanosterol (10 μM), for which marked changes in morphology were observed, no change in the total cellular sterol concentration was found.

As noted previously, the morphological changes induced by the ethyl diol, miconazole, lanosterol, and 24,25-dihydrolanosterol in CHO-K1 cells appear to be similar, if not identical, to those reported for dibutyryl cAMP by Hsie and Puck (38) in CHO-K1 cells grown in Ham's F12 medium supplemented with the macromolecular fraction of fetal calf serum in an amount equivalent to 10% of whole serum. In the present study, similar observations were made for dibutyryl cAMP (1 mM) in cells grown in Ham's F12 medium supplemented with 8%

[5]Echevarria et al. (60) have reported the presence of small amounts (1% of total sterols) of zymosterol in human fibroblasts incubated in the presence of triparanol and, on the basis of separate isotopic studies, that the zymosterol formed from [^3H]acetate was concentrated in plasma membranes.

delipidated NCS. Hsie and Puck (38) also noted that incubation of the cells with testosterone propionate (45 μM) for 3 days also induced elongation of the cells. The changes in cell shape induced by dibutyryl cAMP occurred much more rapidly ($\sim$6 h) than those induced by the ethyl diol, miconazole, lanosterol, or 24,25-dihydrolanosterol under the conditions described herein (2–3 days). Moreover, the changes in morphology induced by dibutyryl cAMP were not associated with a change in the percentage composition of cellular sterols. Further, the possibility that the ethyl diol might change intracellular cAMP levels such as to induce the observed changes in morphology has been excluded by our observation that the ethyl diol had no effect on the levels of cellular cAMP.

The interaction of cytoskeletal elements with the plasma membrane represents an important matter in the control of cell shape and other processes (69). While a detailed consideration of this subject is beyond the scope of this work, it is important to note that modification of the sterols of the plasma membrane, as demonstrated herein for the case of CHO-K1 cells incubated in the presence of ethyl diol, may represent a promising approach for the exploration of the role(s) of sterols in the interaction of the plasma membrane with those cytoskeletal elements critical to the control of cell morphology. Moreover, Buttke and Folks (58) noted their T cell mutant defective in lanosterol demethylation could prove to be useful in defining the role of cholesterol in the induction of syncitia formation and cytopathic effects by the human immunodeficiency virus. If replacement of cellular cholesterol by lanosterol and 24,25-dihydrolanosterol should prove beneficial, the ethyl diol could represent a potent effector for this modification of sterol composition.

This work was supported by grants (HL-15376 and HL-22532) from the National Institutes of Health and by a grant (C-583) from the Robert A. Welch Foundation. The support of the Ralph and Dorothy Looney Endowment Fund and the American Cyanamid Company is gratefully acknowledged. We thank Martha Glenn for excellent technical assistance.

Manuscript received 4 October 1993 and in revised form 18 February 1994.

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