Inhibition of Cholesterol Synthesis and Cell Growth by 24(R,S),25-
Inimolanosterol and Triparanol in Cultured Rat Hepatoma Cells*

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24(R,S),25-Inimolanosterol (IL) and triparanol added to cultures of rat hepatoma cells, H4-II-C3 (H4), interrupt the conversion of lanosterol to cholesterol and, depending on their concentrations, cause the accumulation in the cells of intermediates in the lanosterol to cholesterol conversion. At 45 μM, both substances cause the accumulation of 5α-cholesta-8(9),24-dien-3β-ol (zymosterol), and at the low concentration of 4.5 μM, they cause the accumulation of cholesterol 5,24-dien-3β-ol (desmosterol). The effect of intermediate concentrations of 9 or 22.5 μM of either substance is to cause the accumulation in the cells of three sterols: cholesta 5,7,24-trien-3β-ol, zymosterol, and desmosterol. The synthesis of these intermediary sterols, not found normally in H4 cells, is particularly pronounced in cultures kept in lipid-depleted media that contain the inhibitors and proceeds by the use of endogenous substrates at the expense of cholesterol. The synthesis of cholesterol from [14C]acetate or [2-14C]mevalonate is completely blocked by either inhibitor even at 4.5 μM. IL or triparanol inhibits the growth of H4 cells. Cells seeded into either full growth or lipid-depleted medium containing 22.5 μM IL will not grow unless the media are supplemented with low density lipoproteins (60 μg/ml). Supplementation of the media with 4.6 μM mevalonate does not counteract the inhibitory effect of IL on cell growth.

We have reported previously that 2,3-iminosqualene inhibits irreversibly the squalene-2,3-oxide lanosterol synthase (EC 5.4.29.7) in cultured rat hepatoma (H4) and Chinese hamster ovary cells (1-3). It seemed possible that ISq (I) could be epoxidized at its terminal double bond, giving 2,3-epoxyiminosqualene (II) which, by virtue of the symmetry around the two central carbon atoms, is equivalent to 2,3-epoxy-22,23-iminosqualene (IIa). This epoxyiminosqualene could be cyclized to 24,25-iminolanosterol (IL; III, Scheme 1) which conceivably might have been responsible for the effects seen with ISq. This possibility seemed the more plausible as it was reported that ISq was converted in Gibberella fujikuroi to 24,25-iminolanosterol (4, 5). However, the results obtained with IL were totally different from those obtained with ISq. IL did not inhibit the squalene-oxide cyclase and did not cause accumulation of squalene-2,3-oxide in cultured cells but rather blocked the lanosterol to cholesterol conversion. The effects of IL are remarkably similar to those of triparanol, a well known inhibitor of cholesterol biosynthesis. Depending on their concentrations in the culture media, both substances can interrupt the conversion of lanosterol to cholesterol at more than one stage.

EXPERIMENTAL PROCEDURES

Cell Culture—Rat hepatoma cells (H4-II-E-C3, to be referred to as H4 cells) were grown in 25- or 75-cm² Falcon Labware plastic flasks in a modified Swinn S-77 medium supplemented with 5% fetal bovine and 10% horse serum as described (6, 7). This medium will be referred to as full growth medium (FGM). The preparation and composition of a lipid-depleted medium (LDM), almost free of cholesterol and triacylglycerols, have also been described (7).

Cultures grown to confluence in FGM were given on day 0 either fresh FGM or LDM containing 20 μg (45 μM) of 24,25-iminolanosterol per ml or 22.5 μg (45 μM) of triparanol succinate per ml. The two substances were added to the medium as ethanolic solutions. The concentrations of either substance were needed, stocks of media containing the inhibitor at 45 μM were diluted with media without the inhibitor. After 24 h (or longer) incubation, the cells were washed with warm (37 °C) serumless (basal) S-77 medium. The 25-cm² cultures were given 3 ml and the 75-cm² cultures 5 ml of basal S-77 medium containing either 5 μCi of [1-14C]acetate (specific activity, 56 Ci/mol) or 1.74-5 μCi of (R)-[2-14C]mevalonate. The [14C]mevalonate, as bought, had a specific activity of 56.7 Ci/mol and was diluted with unlabeled (R,S)-mevalonate to a specific activity of 0.25 or 10 Ci/mol. The cultures with the labeled acetate were incubated at 37 °C for 2 h and those with mevalonate for 4 h. When cultures were given [14C]mevalonate for 24 h, the labeled substrate was contained in FGM or LDM (not serumless medium). After the incubations, the cells were assayed with 0.1 N HCl and MgO-free phosphate-buffered saline, harvested, and processed for protein determination and extraction of unsaponifiable lipids as described (7). The unsaponifiable lipids were extracted with redistilled light petroleum (b.p., 40-60 °C) containing 0.05% butylated hydroxytoluene.
The synthesis of 24(R,S),25-iminolanosterol from lanosterol by the introduction of the aziridine function over the 24,25-double bond with iodine and LiAlH₄ has been described by Parah and Nes (10). The method is essentially the same as that described by Avrch and Oehlschlenger (11) for the synthesis of 2,3-iminosqualene.

Desmosterol (cholesta-5,24-diene-3beta-ol) was obtained by mild alkaline hydrolysis of zymosterol acetate (12), a gift from Dr. G. J. Schroepfer, Jr., of Rice University. Lanosterol was a specimen purified by HPLC from a commercial sample, 4,4-dimethylcholesta-8,14-diene-3-ol, isolated from Sacccharomyces cerevisiae, and cholesta-5,24-diene-3-ol was a specimen isolated by Dr. W. R. Nes from Tetrahymena pyriformis incubated with cholesterol. Triparanol succinate was a gift from Dr. Glenn Patterson of the University of Maryland. Human low density lipoprotein (LDL, d < 1.063), obtained from the plasma of one donor by standard differential centrifugation methods, was available from the Core Laboratory of the Atherosclerosis Research Group at UCLA School of Medicine.

Solvents were HPLC grade or were redistilled. The use of redistilled light petroleum is essential in extraction of unsaponifiable lipids when samples are to be analyzed by HPLC, as even analytical grade (AR) "petroleum ether" leaves several nonvolatile residues that interfere with HPLC analysis.

RESULTS

At first we used [14C]acetate as substrate in cultures incubated for 24 h with IL to see whether this substance had effects similar to those of ISo. Fig. 1 shows a typical radiochromatogram of the unsaponifiable lipid extract from a control culture incubated with [14C]acetate for 2 h. In such control chromatograms, the large radioactive peak lies precisely over the cholesterol marker spot which also coincides with the band of endogenously derived cholesterol shown in the lower lane. The two marker spots near the solvent front are squalene-2,3-oxide and squalene. In the chromatogram from the IL-treated culture (Fig. 2), the large radioactive peak does not coincide with the dark cholesterol band (RF 0.40) but with a fainter band touching the band of cholesterol. This fainter band, with an RF value of 0.35, has never been seen in chromatograms of the unsaponifiable lipids from normal cultures, but it invariably appeared in the chromatograms of the unsaponifiable lipids from cultures treated with IL for 24 h or longer and became heavily labeled either from [14C]acetate or [14C]mevalonate. We will show further on that the band at RF 0.35 is zymosterol. When [14C]-labeled acetate or mevalonate is added to cultures that were exposed to IL for 24 h, two or three labeled products in addition to zymosterol are also detected on TLC plates (cf. Fig. 2). Two of these, commonly found at RF values of 0.48 and 0.55, migrate faster than cholesterol (RF = 0.40-0.41); a third moves near lanosterol (RF = 0.61-0.62). There was no mass detectable by the phosphomolybdic acid spray associated with these; they are probably methylsterols (to be discussed later). The small radioactive peak at RF 0.30 (cf. Fig. 2) seen on the ascending limb of the zymosterol peak was not a constant feature on the chromatograms of the unsaponifiable lipids whether [14C]acetate or propan-2-01 for injection onto the HPLC column. When radioactive samples were analyzed, each fraction or a portion of it was added to 10 ml of RPI 3a70B scintillation fluid and counted in a Beckman LS 2800 scintillation spectrometer. Ultraviolet absorption spectra of HPLC fractions were taken in a Unicam SP 1800 scanning spectrophotometer against methanol-water (96:4) at a flow rate of 1 ml/min. The effluent was monitored at 210 nm. Fractions were collected at 0.5- or 1-min intervals. When radioactive samples were analyzed, each fraction or a portion of it was added to 10 ml of RPI 3a70B scintillation fluid and counted in a Beckman LS 2800 scintillation spectrometer. Ultraviolet absorption spectra of HPLC fractions were taken in a Unicam SP 1800 scanning spectrophotometer against methanol-water (96:4) as a reference.

In two large scale experiments (see "Results") in which identical labeled fractions from 16 HPLC runs were pooled for mass spectral analysis, the pooled fractions were concentrated with a stream of N₂ at about 40 °C and then diluted with water. The solutes were extracted with light petroleum and the concentrated extracts run on TLC plates from which the radioactive bands were eluted with ethyl acetate or propan-2-01.

Reversed phase HPLC was carried out with a Beckman model 100 instrument fitted with a Beckman Controller, an Altex variable wavelength detector, and C-18A recorder/integrator. The column, Bio-Rad ODS 5 μ (25 cm × 4 mm), fitted with a Bio-Sil ODS-10 Micro-Guard cartridge, was developed isocratically, as described by Nes et al. (8) with methanol-water (96:4) at a flow rate of 1 ml/min. The effluent was monitored at 210 nm. Fractions were collected at 0.5- or 1-min intervals. When radioactive samples were analyzed, each fraction or a portion of it was added to 10 ml of RPI 3a70B scintillation fluid and counted in a Beckman LS 2800 scintillation spectrometer. Ultraviolet absorption spectra of HPLC fractions were taken in a Unicam SP 1800 scanning spectrophotometer against methanol-water (96:4) as a reference.

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FIG. 1. Radiochromatogram of unsaponifiable lipids from control confluent culture incubated for 2 h with [1-14C]acetate. TLC on a Whatman LK 5DF plate developed with 10% ethyl acetate in benzene. The markers in the top lane, from left to right, are: cholesterol, squalene-2,3-oxide, and squalene. The lower lane carried the experimental sample; the stained bands are from endogenous material.

or [14C]mevalonol was the labeling substrate. The radioactive peak seen at the origin of the chromatogram in Fig. 2 was not present when [14C]mevalone was the substrate.

To identify the substance with the Rf value of 0.35, the unsaponifiable lipids from four cultures treated with IL for 24 h were pooled, and the unknown substance and cholesterol were eluted together from the chromatograms. GC-MS analysis showed two components (Fig. 3). The first component gave the well known mass spectrum of cholesterol and the second, the spectrum of a cholestadienol with a molecular ion, M+, at m/z 384 which was the base peak (100%) in the spectrum. Further characteristic fragment ions at m/z values (with relative intensities) were: 369, [M - CH3]+ (40%); 366, [M - H2O]+ (8%); 351, [M - (CH3 + H2O)]+ (12%); 300, [M - (CH2 + (CH2)C = CH-CH2)]+ (11%); 271, [M - (side chain + 2H)]+ (13.4%); and 69, [(CH2)3C = CH-CH2]+ (72%). The ions at m/z 300 and 69 made it fairly certain that the cholestadienol had an unsaturated side chain with the double bond between C-24 and C-25, but the position of the second double bond remained uncertain.

Determination of the structure of cholestadienols by mass spectrometry may be ambiguous as they all give the same molecular ion (m/z 384) and also because their fragmentation and the relative intensities of the fragment ions depend not only on the position of the double bonds but also on ion-source pressures and temperatures. The structure of desmosterol for our cholestadienol was excluded as the mass spectrum of desmosterol was reported (13, 14) to have a prominent fragment ion at m/z 271, whereas this fragment ion in our spectrum was rather weak (13.4% of the base peak at m/z 384). Also, the retention time of the dienol relative to that of cholesterol in GC was a little longer than that expected for desmosterol. In Table I are recorded the chromatographic properties of some reference sterols. The data show that to differentiate cholestadienols from one another, reversed phase HPLC is the method of choice.

The identification of the unknown cholestadienol as zymosterol was, indeed, made by reversed phase HPLC. When the unsaponifiable lipids from several cultures treated with IL (45 μM) for 24 h or longer were applied to a C18 reversed phase column, a peak with a retention time αr = 0.686 ± 0.002 (n = 10) relative to that of cholesterol appeared; this peak was absent from the unsaponifiable lipids of control cultures. An
The data are retention times or volumes relative to that of cholesterol. The data of columns a, b, and d are from W. D. Nes and those of column c are from UCLA.

| Sterol                       | Gas-liquid chromatography | Reverse phase HPLC |
|-----------------------------|---------------------------|---------------------|
|                             | a                      | b                    | c                  | d                  |
| Cholesterol                 | 1.00                    | 1.00                 | 1.00               | 1.00               |
| Cholest-7-enol              | 1.06                    | 1.12                 | 0.96               |                   |
| Cholest-8(9)-enol           | 1.01                    | 1.05                 | 0.93               |                   |
| Cholest-5,24-dienol         | 1.04                    | 1.06                 | 0.87               |                   |
| Cholest-8(9),24-dienol      | 1.08                    | 1.09                 | 0.725              | 0.74               |
| Cholest-7,24-dienol         | 1.12                    | 1.22                 | 0.688              | 0.67               |

*DB-5 megabore capillary column operated in the temperature program mode, 180-275 °C at 16 °C/min; He flow rate, 20.6 ml/min.

3% SE-30 packed column run isothermally at 245 °C.

See "Experimental Procedure."

Altex column packed with Ultrasphere ODS 5 μm (4.0 x 250 mm) eluted with methanol/water (96:4) at 1.6 ml/min.

Predicted values.

**TABLE I**

**Chromatographic properties of reference sterols**

**Fig. 4.** Reversed phase HPLC analysis of sterols (A) from a control culture (B) and (C) from a culture treated with 45 μM iminolanosterol and with (R)-[2-14C]mevalonate for 24 h; (C) shows the radioactivity of the fractions (B). There was no detectable radioactivity in fractions corresponding to cholesterol. The authentic specimen of zymosterol from yeast (12) had an α = 0.685 ± 0.002 (n = 6). Zymosterol and desmosterol were well separable from one another, as the α value for the latter was 0.725 ± 0.002 (n = 6).

In Fig. 4, we show segments of the HPLC records, between 25- and 50-min elution times, from the unsaponifiable lipids of a control culture (A) and of one treated with IL (45 μM) and incubated with (R)-[2-14C]mevalonate for 24 h (B and C). In the record from the control culture, the peak at 46.29 min is cholesterol, and the small one at 33.79 min is probably desmosterol. In the specimen from the IL-treated culture, cholesterol was eluted at 46.49 min and contained no radioactivity whatever, but 88% of the applied radioactivity was in the peak eluted at 31.89 min, the elution time being characteristic for zymosterol (31.89/46.49 = 0.686). The observations demonstrate that the action of IL is very rapid; in the experiment shown in Fig. 4, B and C, the IL and the [14C]mevalonate were added simultaneously. Even a few minutes delay in the action of IL would have been sufficient to result in the appearance of detectable amounts of 14C in cholesterol. It needs to be emphasized that zymosterol in the IL-treated cultures is also synthesized from endogenous substrates; addition of precursor substances such as mevalonate is not needed.

The ratio of the area under the cholesterol peak to that under the zymosterol peak in the unsaponifiable lipids from cultures exposed to IL in FGM for 24 h varied between 3.7 (Fig. 4B) and 4.5 with a mean value of 3.5 ± 0.8 (S.D., n = 10).

**Comparison of the Effects of Iminolanosterol and Triparanol**—The effects of IL on the lanosterol to cholesterol conversion reminded us of the action of triparanol (TRP) (1-[p-(2-diethylaminooxy)ethyl]-1-(p-tolyl)-2-(p-chlorophenyl)ethanol) on the same process, except that IL appeared to stop the conversion at a step earlier than TRP. TRP has been known for a long time to inhibit cholesterol biosynthesis and, when given to animals, to cause the accumulation of desmosterol (cholesta-5,24-dien-3β-ol) in the tissues (15-20). In spite of this well attested effect of the drug, we thought it worthwhile to examine the action of TRP on sterol synthesis in the H4 cells. In the first experiments, we used 45 μM TRP in the medium of confluent cultures in FGM, the same concentration as that in the experiments recorded so far with IL. After 24 h with the TRP, the cells were labeled for 2 h with [1-14C]acetate. The unsaponifiable lipids were examined first by TLC and then by HPLC. The scan of the TLC plate gave an image almost identical with that shown in Fig. 2. The HPLC analysis gave a pattern identical with that shown in Fig. 4, B and C. The cells treated with TRP accumulated zymosterol (α = 0.685), not desmosterol, and 96% of the 14C was associated with that sterol, none with cholesterol.

Subsequent experiments showed, however, that the effects of TRP and IL on sterol biosynthesis depended on the concentrations of these drugs in the media.

Therefore, we made a systematic study of the effects of lower concentrations of TRP on cultures kept in FGM and those transferred to LDM. We found that in the presence of TRP or IL in the culture media, the new sterols, not found normally in H4 cells, accumulated faster and were more prominent in cultures transferred to LDM than in those kept in FGM. Further, although both TRP and IL, even at the lowest concentration tested (4.5 μM), completely stopped the incorporation of 14C into cholesterol, they did not prevent the incorporation into squalene and intermediates of the lanosterol to cholesterol conversion. However, the accumulation of the intermediary sterols could be studied without isotopic labeling by analysis of the sterols by HPLC particularly as the accumulation of the sterols became more pronounced in cultures surviving for several days.

**Fig. 5** shows the HPLC pattern of sterols from cultures exposed to 4.5 and 9 μM TRP, respectively, for 5 days. At the lower concentration the fraction with a retention time characteristic for desmosterol (α = 0.725) is dominant, whereas at 9 μM the areas under the zymosterol and desmosterol peak (α = 0.685) are almost equal. There is a small peak in both chromatograms with a relative retention time of 0.65 and another with an α = 0.93. We will show further on that the substance at α = 0.65 is cholesta-5,7,24-trienol; the fraction eluted just before cholesterol is probably lanosterol. The cholesterol eluted at 47-48 min is, of course, a remnant from the initial culturing period.

The ratio of the area of the cholesterol peak to the sum of the zymosterol and desmosterol peaks in cultures kept in FGM ranged from 3.5 to 4.6, but the same ratio for cultures transferred to LDM was close to unity, 0.7-0.9, indicating an
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Minutes

FIG. 5. Reversed phase HPLC analysis of sterols (A) from culture exposed for 5 days in FGM to 4.5 μM TRP and (B) from one exposed for the same time to 9 μM TRP. The numbers above the peaks are the elution times in minutes.

Minutes

FIG. 6. Reversed phase HPLC profiles of sterols from cultures of H4 cells that were exposed to 4.5 (A) and 9 μM (B) iminolanosterol for 3 days.

enhanced synthesis of the intermediary sterols in LDM.

Having seen that TRP at a high concentration caused accumulation of zymosterol in the cells but at low concentrations the accumulation of both zymosterol and desmosterol, it seemed prudent to examine IL also at concentrations lower than used before. Cultures were therefore set up in FGM and LDM with 4.5, 9, and 22.5 μM IL in the media and the unsaponifiable lipids of the cells were analyzed by HPLC after 3- and 5-day incubations.

Fig. 6 shows HPLC profiles of sterols from H4 cells that were exposed to 4.5 and 9 μM IL, respectively, in LDM for 3 days. After exposure to 4.5 μM IL, the cells accumulated only desmosterol (not zymosterol), and after treatment with 9 μM IL in addition to desmosterol (α = 0.725) and a small zymosterol fraction (α = 0.685), there appeared a pronounced peak with a relative retention volume of α = 0.646 similar to the small component noted in the experiments with TRP (cf. Fig. 5).

It seemed likely that the compound with an α value of 0.646, which appeared particularly abundant in cells incubated with 9 and 22.5 μM IL in LDM, was yet another intermediate in the lanosterol to cholesterol conversion. To generate enough of this compound for analysis, three 25-cm² cultures were first grown to confluence in FGM, and then the media were changed to LDM with 22.5 μM IL and the cells grown for 5 days. For the last 24 h, the cultures were also given (R)-[2-14C]mevalonate (1.74 μCi/flask = 6.96 μmol/3 ml). The cells from the three cultures were pooled and the unsaponifiable lipids fractionated into squalene and sterols. The sterols were analyzed by HPLC and fractions collected at 1-min intervals. Fig. 7 is part of the combined HPLC and radioactivity profile of the sterols from this experiment.

As the fraction with its peak at 30.7 min was suspected to be cholesta-5,7,24-trienol, the UV spectra of fractions collected from 30 min onward were taken against methanol:water (96:4) immediately after elution. Fig. 8 is the UV spectrum of fraction eluted at 32 min; it is characteristic for a sterol with the 5,7-homoannular conjugated double bond system. Fractions 30, 31, and 33 showed similar spectra but of lesser intensity. All other fractions showed only end absorption below 240 nm.

The HPLC profile shown in Fig. 7 differed from earlier patterns in that it showed the presence of four more components than seen before: one small peak eluted at 16.5 min (α,

FIG. 7. HPLC and radioactivity profile of sterols pooled from three cultures of cells in LDM exposed to 22.5 μM iminolanosterol for 5 days. For the last 24 h, the cultures were given (R)-[2-14C]mevalonate (1.74 μCi = 6.96 μmol/3 ml). The record is from ½ of the total sample; 1-min fractions were counted. From the areas under the peaks and calibration data, the approximate amounts of four sterols in this sample were: cholesterol, 3.45 pg; desmosterol (peak at 34.5 min), 7.52 pg; zymosterol (peak at 32.5 min), 5.55 pg; cholesta-5,7,24-trienol (peak at 30.7 min) on the assumption that it has the same absorbance at 210 nm as cholesta-5,7,22-trienol, 4.33 pg.

FIG. 8. UV spectrum of fraction eluted at 32 min from the experiment shown in Fig. 7.
eluted between 41 and 45 min (α = 0.86 and 0.90, code named U-1a and U-1b, respectively); and a fourth eluted between 51 and 55 min (α = 1.09, code named U-2). All these components as well as the triad between 30 and 36 min became labeled from [2-14C]mevalonate, but cholesterol contained no detectable 14C.

The four additional peaks just mentioned appeared only when the culture media were supplemented not only with IL but also with 2.3 mM mevalonate, labeled or unlabeled. The sterol fraction of the unsaponifiable lipids from cultures grown in FGM or grown first in FGM and then transferred to LDM when the culture media were supplemented not only with IL from [2-'4C]mevalonate, but cholesterol contained no detectable 14C.

Cells exposed to IL (22.5 μM) for 24 h and then given (R)-[2-14C]mevalonate (1.74 pCi) or [2-'4C]mevalonate (2.3 mM), only members of the triad become labeled with 14C and there is no detectable incorporation into cholesterol nor appearance of any other fraction. On the other hand, when the biosynthetic capacity of the IL-treated cells is tested with [2-14C]mevalonate (2.3 mM), the four additional peaks always appear. Figs. 9 and 10 illustrate two further experiments. In one, the cells were treated with IL for 24 h and in the other for 72 h and then were given [2-14C]mevalonate for 5 h. The difference between the two experiments shows that with the longer exposure of the cells to IL in LDM, the ratio of the members of the triad to cholesterol is much increased.

To obtain structural information on components of the sterols from the experiments shown in Figs. 7, 9, and 10, we set up two further experiments similar to the one described in Fig. 7 except that each consisted of five 75-cm² cultures and that for the last day of the incubations two cultures in each batch were given 2.8 μCi (11.2 μmol) of (R)-[2-14C] mevalonate per 5 ml of medium. The unsaponifiable lipids from the pooled cells of each batch were divided into squaene and sterols (see "Experimental Procedures"). The sterols from each batch were first fractionated by HPLC and were further purified by TLC, for mass spectral analysis, as described under "Experimental Procedures." Since all the fractions except cholesterol were labeled with 14C, they could be located on the TLC plates by scanning for radioactivity. Both the purported cholestatrienol and the zymosterol fractions gave single radioactive bands with Rf values of 0.35-0.37. The desmosterol fractions gave a large and a small radioactive band with Rf values of 0.35 (D-1) and 0.53 (D-2), respectively. The latter is probably a small overlap from the neighboring U-1a (α = 0.86) HPLC fraction. U-1a and b were pooled together and also gave two active bands, a larger one at Rf 0.54 and a smaller one at Rf 0.62. U-2 (α = 1.09) gave one radioactive band at Rf 0.61. The mass spectra of the fractions eluted with ethyl acetate from the scrapings of the TLC plates were taken on a direct insertion probe with the mass spectrometer of the Dept. of Pharmacology at UCLA.

The fraction corresponding to HPLC fraction α = 0.65 gave a spectrum characteristic for cholesta-5,7,24-trien-3β-ol with M+ at m/z 382 (97.3%) and fragment ions identical with those reported for this substance by Scallen et al. (21) who also presented the most probable mode of fragmentation of this sterol. The relative intensities of the six most prominent fragment ions in our spectrum at m/z values were: 349 (100%), 251 (30.0%), 211 (40.8%), 143 (57.1%), and 69 (61.4%). Scallen et al. (21) indicated relative intensities of ions only by numbers of crosses. The spectrum also showed the presence of a little cholestadienol (m/z 384), no doubt representing zymosterol which cannot be completely separated from the trienol by either HPLC or TLC.

Although zymosterol and desmosterol were identified by their relative retention volumes in HPLC, their mass spectra were also taken for sake of additional evidence of their identity. The spectra of these two sterols were distinctly different. The characteristic ions in the spectrum of zymosterol at m/z values were: M+, 384 (74.1%); [M – CH3]+, 369 (57.2%); [M – (CH3 + H2O)]+, 351 (13.2%); [M – (CH3H2 + 2H)], 271 (27.6%); ring D cleavage with H-rearrangements, 229 (13.3%); and base peak at m/z 69. The structure of another cholestadienol, e.g. 7-dehydrocholesteryl, can be excluded as in the spectrum of that sterol the [M – (CH3 + H2O)]+ is the base peak. The relative intensities of the ions in this spectrum are somewhat different from those reported in the early part of the paper for zymosterol, but the spectrum was obtained under different conditions.

A mass spectrum of the major radioactive component of the TLC-purified fraction of desmosterol (D-1, Rf 0.35) was identical with that of a reference standard, the base peak being at m/z 271, [M – (side chain + 2H)]; the molecular ion at m/z 384 was relatively small (40.5%). Other characteristic fragment ions at m/z values were: 369 (24.7%), 351 (16.2%), 300 (32.4%), and 69 (38.2%). Our spectrum was similar to that reported before for desmosterol (13, 14). No spectrum could be obtained from D-2.
The fraction corresponding to U-1a ($\alpha_a = 0.86$) gave a spectrum compatible with that of 4,4-dimethylcholesta-8,14-dienol with $M^+$ at $m/z$ 426 (100%) and fragment ions at $m/z$ values of 283, [$M - 15]^+$ (81.9%); 365, [$M - (CH_3 + H_2O)]^+$ (17.9%); 287, [$M - \text{side chain}]^+$ (14.5%); and 285, [$M - \text{side chain + 2H}]^+$ (48.2%). The 4-methylsterol structure is suggested on biosynthetic grounds and also because the [$M - CH_2]^+$ ion is smaller than $M^+$. In sterols with a methyl group at position 14, as in lanosterol, the [$M - CH_2]^+$ ion is about 1.8 times more intense than the molecular ion at $m/z$ 426. An unsaturated side chain in U-1a is suggested by the ions at $m/z$ 287 and 285.

The substance U-1b ($\alpha_a = 0.90$, $R_f = 0.62$) gave a weak mass spectrum of lanosterol with an $M^+$ at $m/z$ 426 and fragment ions [$M - CH_2]^+$, 411 (19.9%); and [$M - (CH_3 + H_2O)]^+$, 393 (64.5%); 391, 379, and 377, [$M - (CH_3 + H_2O)]^+$ (30.9%). These ions in the high mass range are similar to those given, on the same instrument under identical conditions, by a reference standard, 4,4-dimethylcholesta-8,14-dienol. However, in the spectrum of our unknown substance, loss of the side chain gave an ion at $m/z$ 301, [$M - C_6H_13]^+$ (9.0%). The relative intensity of this ion in the spectrum of the reference compound was only 1.1%, and the cleavage of the side chain gave the ion at $m/z$ 299, [$M - C_6H_13]^+$ (13.8%). The nonidentity of U-2 with the dimethylcholesta-8,14-dienol was shown further by the latter's $\alpha_a$ value of 0.97 in our HPLC system. Also, U-2 showed an end absorption in the UV, whereas the 4,4-dimethylcholesta-8,14-dienol had a maximum absorption at 248 nm. Although the best candidate for U-2 might be 4,4-cholesta-8(9),24-dien-3-ol (14-norlanosterol), it is difficult to understand why such a substance should have a greater retention volume in HPLC than lanosterol. 14-Norlanosterol was not available for comparison.

Effects of Inominolanosterol and TRP on Cell Growth— Cultures exposed to 45 $\mu$M IL or TRP in LDM deteriorate after 2–3 days. The cells round up and float off the substratum; they all float off after 4 days. In FGM, the effect is not as drastic; cultures may survive for 7 days but with much loss (~50%) of cells.

To assess the effects of IL and TRP on cell growth, we turned to experiments in which we seeded cells harvested from stock culture into FGM or LDM containing the inhibitors at varying concentrations. At first, such experiments were done in the Falcon multivell plates, but because of irregular growth in the small (2-cm²) wells, we changed to the 25-cm² flasks according to the schedules described under “Experimental Procedures.”

Cultures exposed to 45 $\mu$M IL or TRP do not get attached to the substratum and do not divide. Cells exposed for 24 h to such concentrations of the inhibitors do not revive when the medium is changed to one without the inhibitors.

The experiments with TRP in the multivell plates showed that cells seeded into FGM or LDM containing 4.5 or 9 $\mu$M of the inhibitor will divide normally for 2 days but stop growing afterward and are even killed at 9 $\mu$M in LDM after 2 days. Further experiments with TRP were not done.

Most of the experiments on the effect of IL on cell growth were done in the 25-cm² flasks. In FGM, 4.5 and 9 $\mu$M IL caused no inhibition of cell growth. IL at 9 $\mu$M in LDM caused only a modest (30%) inhibition of growth: control cells in LDM grew in 3 days from the initial inoculum equivalent of 580 $\mu$g of protein to 1350 $\mu$g (growth, 770 $\mu$g), and the cells exposed to IL grew to 1120 $\mu$g (growth, 540 $\mu$g). In the medium which contained IL and also LDL, 60 $\mu$g/ml, the growth was 940 $\mu$g.

Fig. 11 illustrates two of four experiments of similar protocol with IL at 22.5 $\mu$M. The important results of these experiments are that IL at this concentration in either LDM or FGM prevented the growth of the cells and that the LDL supplement (60 $\mu$g/ml) to the medium counteracted the effect of IL and allowed the cells to grow normally. In contrast, supplementation with mevalonate had no beneficial effect.

**DISCUSSION**

We have shown that cultured rat hepatoma cells treated with 24,25-inominolanosterol or TRP accumulate sterols not found normally in these cells except perhaps in trace amounts. The nature of these sterols depends on the concentration of the inhibitors in the culture media and can be best explained by supposing that both substances inhibit two enzyme systems involved in the conversion of lanosterol to cholesterol. At the highest concentration tested (45 $\mu$M), both substances prevented the reduction of the 24,25-double bond and the isomerization of the 8(9)-double bond. As a result, the cells accumulated zymosterol. At intermediate concentrations (9 and 22.5 $\mu$M), the inhibition of the reduction of the 24,25-double bond still prevails, but the inhibition of the isomerization of the 8(9)-double bond is incomplete, and the cells
actively synthesizing sterols, accumulate not only zymosterol but also cholesta-5,7,24-trien-3β-ol and desmosterol. At the low concentration of 4.5 μM, inhibition of the isomerization is no longer apparent, and the cells accumulate mostly desmosterol and only traces of zymosterol and the trienol.

The move of the 8(9)-double bond from zymosterol to 5(6) in desmosterol occurs probably in three steps: (i) isomerization to 7(8); (ii) dehydrogenation at positions 5 and 6; and (iii) NADPH-dependent reduction of the 7(8)-double bond (Scheme 2). Our finding simultaneously zymosterol, the cholestatrienol, and desmosterol in IL- and TRP-treated cells makes this sequence highly probable. The possibility of cholesta-5,7,24-trienol being an intermediate in the conversion of lanosterol to cholesterol was first mooted by Johnston and Bloch (22). Frantz et al. (23) were probably the first to identify this substance in the intestine of TRP-treated guinea pigs. It was unambiguously identified by its mass spectrum, UV, infrared, and 1H NMR spectra by Scallen et al. (21) in the lung of weaning pigs treated with AT-9944 [trans-1,4-bis(2-chlorobenzylaminomethyl)cyclohexane dihydrochloride] and 20,25-diazacholesterol. The former is an inhibitor of Δ7-sterol reductase and the latter an inhibitor of Δ5-sterol reductase. Scallen (24) had also demonstrated the conversion of [3α-2H]cholesta-5,7,24-trien-3β-ol into cholesterol in the rat. Johnston and Bloch (22) were the first to show the conversion of [4C]zymosterol into cholesterol in liver homogenates. Schroepfer (25) showed also the conversion of [4C]zymosterol into cholesterol in liver homogenates but found that such conversion did not occur in the TRP-treated rat. There is evidence also from experiments with yeast, S. cerevisiae, that TRP inhibits not only the Δ7-sterol reductase but also the Δ5-Δ7-isomerase (e.g. 26). The status of intermediates in the lanosterol to cholesterol conversion has been extensively reviewed by Schroepfer (27).

The use of tracers to demonstrate the formation of the intermediary sterols was not needed in our experiments as they could be identified by mass in HPLC analysis; the cells used their endogenous substrates. The use of tracers was needed only to ascertain the extent of the interference with the lanosterol to cholesterol conversion. When [1-14C]acetate was used as tracer (60 μM) with cells treated with IL for 24 h or longer, cholesterol remained unlabeled, and the bulk of the labeled was in the three main intermediates accumulated in the cells, the cholestatrienol, zymosterol, and desmosterol and in traces in other substances that became apparent by mass when [2-14C]mevalonate (2.3 mM) was used as substrate. These additional substances had relative retention volumes (αr) in reversed phase HPLC of 0.35, 0.86, 0.90, and 1.09 (cf. Figs. 7–9). The substance αr = 0.35 has not been identified yet, but its retention volume suggests that it might be related to the 32-hydroxylanosterol identified recently by Saucier et al. (28) in mevalonate-treated cell cultures. The substance αr = 0.86 was identified by its mass spectrum as 4-methylcholestadienol with the double bonds probably in positions 8(9). Compound αr = 1.09 by its mass spectrum is a 4,4-dimethylcholestadienol with one of its double bonds being at C-24, the other remaining conjectural.

The appearance of the additional sterols after use of mevalonate, we believe, is not a direct consequence of the enzyme inhibition by IL (this was not studied in conjunction with TRP) but a consequence of the sterol-synthesizing enzyme system being overburdened by the nonphysiological concentration of mevalonate. In control cells first grown to confluence in FGM and then transferred to LDM, most (80%) of the label from [1-14C]acetate after a 4-h exposure appears in cholesterol and 20% in an HPLC fraction with the retention volume of desmosterol associated with barely detectable mass. When similar cells are given labeled or unlabeled mevalonate, well defined peaks of desmosterol and fractions with αr values of 0.35, 0.86, 0.90, and 1.09 in reversed phase HPLC are found. When [2-14C]mevalonate (2.3 mM) is fed to such control cells, desmosterol and cholesterol become almost equally labeled and contain the bulk of the label (42.6 and 45.6%, respectively, of the total); the rest is about equally distributed among the substances with αr values of 0.35, 0.86–0.90, and 1.09. TLC is inadequate for clear demonstration of the diverse sterols that might accumulate in cells under the influence of inhibitors of sterol biosynthesis; for example, the triad of cholesta-5,7,24-trienol, zymosterol, and desmosterol cannot be resolved by TLC.

The accumulation of intermediates of the lanosterol to cholesterol conversion was particularly pronounced in cultures that were first grown to confluence in FGM and then transferred to LDM. The effect of such medium change in H4 cells (as in many other cultured cells of animal origin) is the induction of 3-hydroxy-3-methylglutaryl-CoA reductase by the enhanced transcription of its gene (7). This induction may have been responsible for the more vigorous use of endogenous substrates for sterol synthesis in the IL-treated cells in LDM as compared with synthesis seen in cells kept in FGM.

Iminolanosterol and TRP share another property beyond the ability to interrupt the conversion of lanosterol to cholesterol: both inhibit cell growth and also prevent the alkylation at C-24 in the side chain of sterols in plants (29), S. cerevisiae (30), and G. fujikuroi (31, 32). Malhotra and Nes (29) drew attention to the analogy between the reduction of the 24,25-doubled bond and the alkylation at C-24 in that in both reactions an electrophilic species, H+ and CH3+, respectively, is being added to C-24.

A plausible explanation for the cell-growth inhibition caused by IL and TRP is that the cells under the influence of these inhibitors are deprived of endogenously synthesized cholesterol and that they also accumulate unusual sterols which may perturb membrane structures. In fully developed cultures, cells can survive for some days even in the presence of high concentrations of inhibitors as cells in such cultures contain a full complement of cholesterol. However, endogenously synthesized cholesterol may be of consequence only when the exogenous supply of cholesterol is limited as in freshly seeded cultures and particularly in cultures initiated in LDM. The data of Fig. 11 showed that supplementation of media with LDL completely counteracted the growth-inhibitory effect of IL, but supplementation with mevalonate did not. Of course, mevalonate, in the presence of IL, cannot be converted into cholesterol. Thus, cultures initiated in LDM in the presence of IL (10 μg/ml) are entirely dependent on LDL for growth and differ in an important way from cells whose growth is inhibited by compactin, the competitive

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**Scheme 2.** Probable steps in the transformation of zymosterol into desmosterol.
inhibitor of 3-hydroxy-3-methylglutaryl-CoA reductase. It has been shown by Brown and Goldstein (33) that compactin inhibited the growth of Chinese hamster ovary cells. The growth inhibition caused by 2 μM compactin could be overcome by LDL in the medium (25 μg/ml) but not by 0.5 mM mevalonate. However, the growth inhibition caused by 40 μM compactin could be overcome only by a combination of LDL (25 μg/ml) and 0.5 mM mevalonate. From this and other experiments, Brown and Goldstein concluded that while LDL provides the sterol requirement of the cells, mevalonate furnishes a nonsteroidal substance essential for growth. Similar conclusions as to the existence of a nonsteroidal factor derived from mevalonate and needed for cell division were reached by Quesney-Huneeus et al. (34) and by Habenicht et al. (35). We have inferred and have preliminary experiments to show that IL in LDM does not suppress 3-hydroxy-3-methylglutaryl-CoA reductase. Thus, enough mevalonate could be available for the synthesis of cholesterol, which seems essential for growth of H4 cells, is completely blocked. LDL provided the sterol requirements of the cells. The total dependence of IL-treated cells on LDL for growth is thus understandable and can be reconciled with all experimental observations. IL, by its peculiar mode of action on sterol biosynthesis, may turn out to be more than a substance of idle curiosity and of value in further studies of the role of sterols and mevalonate in the life of cells.

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