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INHIBITION OF CHOLESTEROL BIOSYNTHESIS IN 3T3 FIBROBLASTS BY 2-AZA-2,3-DIHYDROSQUALENE, A RATIONALLY DESIGNED 2,3-OXIDOSQUALENE CYCLASE INHIBITOR

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Abstract—The effect of 2-aza-2,3-dihydrosqualene, a new compound designed to inhibit the 2,3-oxidosqualene-lanosterol cyclase [A. Duriatti *et al.*, *Biochem. Pharmac.* **34**, 2765 (1985)] was studied as inhibitor of cholesterol biosynthesis in Swiss 3T3 fibroblasts. Treatment with the drug of cells which were grown for 2 days in a delipidated medium resulted in a marked decrease of [^{14}C]acetate incorporation into the C_{27}-sterol fraction. An $IC_{50} = 0.3\ \mu M$ was calculated when the cells were pre-incubated for a period of 4 hr with 2-aza-2,3-dihydrosqualene. This inhibition was correlated with an intracellular accumulation of 2,3-[^{14}C]oxidosqualene and of 2,3:22,23-[^{14}C]dioxidosqualene, indicating that the cyclase was indeed an intracellular target of the drug. A precursor–product relationship of the accumulated [^{14}C]squalene oxide(s) and the [^{14}C]sterols was demonstrated in chase experiments in the absence of drug. Sterols more polar than cholesterol were also detected in treated fibroblasts and in the cells which underwent chase experiments; they were mainly composed of 24,25-epoxycholesterol. The C_{27}-[^{14}C]sterols of [^{14}C]acetate pulse labeled cells consisted in a mixture of desmosterol and cholesterol; treatment of the cells with 2-aza-2,3-dihydrosqualene resulted in a decreased conversion of desmosterol into cholesterol indicating that the Δ^{24}-sterol reductase might be another target of the drug. 2-Aza-2,3-dihydrosqualene at 1 μM affected normal growth of 3T3 fibroblasts, this effect could be prevented by addition of exogeneous cholesterol (50 μM). Growth arrest of the treated cells was correlated with a decrease in cellular sterol content to less than 40% of controls. About 30% of the C_{27}-sterol fraction, of the treated cells, was desmosterol. Our work demonstrates that 2-aza-2,3-dihydrosqualene is a valuable new inhibitor of cholesterol biosynthesis in mammalian cells.

Inhibitors which block specifically enzymes involved in the biosynthesis of cholesterol are of pharmacological interest [1, 2]. They are also tools which have been used to gain information on the regulation of sterol metabolism [3–5], on the role of cholesterol in cell proliferation [4] and in a variety of plasma membrane functions, e.g. transport, activity of membrane-bound enzymes [6–8]. Potent competitive inhibitors of HMG-CoA reductase§, the rate-limiting enzyme in the biosynthesis of cholesterol, have been described [2, 9]. They inhibit the formation of mevalonate which, in addition to being a biosynthetic precursor of cholesterol, plays an essential role, e.g. in DNA replication and in the synthesis of important non-sterol metabolites [10]. Evidence exists that steps occurring after HMG-CoA reductase can also be of regulatory importance [11–13] and only few inhibitors have been rationally designed to block post-mevalonate enzymes in the biosynthesis of cholesterol [14, 15]. We have recently described the synthesis of 2-aza-2,3-dihydrosqualene and related molecules, a new series of compounds which were designed to mimic the first transient carbocationic intermediate occurring in the oxirane ring opening during the cyclization of 2,3-oxidosqualene [16–19]. These high energy intermediate analogues were found to be excellent inhibitors of rat liver microsomal 2,3-oxidosqualene-lanosterol cyclase (EC 5.4.99.7). The present report deals with the effect of 2-aza-2,3-dihydrosqualene on the biosynthesis of cholesterol in 3T3 fibroblasts cultured in a delipidated growth medium. Our aim was to evaluate the specificity of this new drug at a cellular level and its effect on cell growth. 2-Aza-2,3-dihydrosqualene, and its *N*-oxide derivative, proved highly effective in blocking the biosynthesis of cholesterol in 3T3 cells, this inhibition resulting in an arrest of cell growth.

MATERIALS AND METHODS

Chemicals. Sodium [2-^{14}C]acetate (48 mCi/mmol) was purchased from CEA (Saclay). Cholesterol, desmosterol and lanosterol were obtained from Sigma (St. Louis, MO) and ergosterol was from Aldrich. 5(6)-Carboxyfluorescein (Eastman Kodak) was purified according to Ralston *et al.* [20]. Phosphatidylcholine was extracted from egg yolk and purified

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§ Abbreviations used: U 18666 A, 3β-[2-diethylamino)ethoxy]androst-5-en-17-one; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; RT, retention time.

as described by Nielsen [21]. The synthesis of 2,3-oxidosqualene, [3-^{3}H]*R,S*-2,3 oxidosqualene, 2-aza-2,3-dihydrosqualene, and its *N*-oxide derivative, and the purification of lanosterol have been reported previously [16, 17]. Enzymatic assay kit for cholesterol determination (cholesterol C-System) was from Boehringer (Mannheim). Cell culture medium and serum were obtained from Gibco and the culture flasks were from Falcon. All other chemicals used were of the highest purity available.

Cell cultures. Swiss 3T3 fibroblasts (obtained from Dr J. P. Beck) were routinely maintained as monolayers in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal calf serum, penicillin (100 units/ml) and streptomycin (100 μg/ml) in plastic culture flasks. The cells were grown in a humidified incubator with a 5% CO_2 atmosphere at 37°. Lipid-depleted serum was prepared from fetal calf serum by treatment with diisopropylether/*n*-butanol (3:1; v/v) according to [22]; more than 95% of cholesterol was removed by this procedure, the residual free and esterified cholesterol amounted to 18 ± 6 μg/ml (N = 6) as determined by an enzymatic method (Boehringer). Cholesterol, 2-aza-2,3-dihydrosqualene and its *N*-oxide derivative were added as solutions in absolute ethanol. Final concentration of ethanol in culture medium did not exceed 0.4% (v/v) and an equivalent volume of solvent was added to the control cultures. No significant effect of ethanol on cell growth could be observed during the time course of the experiments.

Incorporation of radiolabeled precursor into nonsaponifiable sterols. Cells derived from stock cultures were seeded at about 3×10^5 cells in 60 mm dia. plastic Petri dishes in 5 ml medium containing 10% (v/v) delipidated serum. On the second day of culture, the cells were pretreated for indicated times with given amounts of 2-aza-2,3-dihydrosqualene or its *N*-oxide derivative. Labeled acetate (10 μCi, i.e. 0.2 μmol per dish) was then added to the culture medium. After 2 hr of incubation the medium was removed and the dishes washed 3 times with phosphate-buffered saline (pH 7.4). The cells were then treated for 30 min with 2 ml of 0.1 N NaOH and aliquots were removed for protein and DNA determinations [23, 24]. Subsequently the digest was transferred to a glass test tube followed by about 2 ml of the same solution used for rinsing the culture dish. An equivalent volume of 6% potassium hydroxide in methanol (w/v) was then added to the tubes and the saponification performed at 55° for 1 hr. The nonsaponifiable lipids were extracted 3 times with an equivalent volume of hexane. The combined extracts were dried over anhydrous sodium sulfate and evaporated to dryness.

Analytical procedures. The nonsaponifiable lipid extracts were separated by one-dimensional thin-layer chromatography on Silica Gel 60 F_{254} plates (Merck). The following solvent systems were used: system I, double migration in methylene chloride; system II, hexane/ethyl acetate (85:15, v/v); and system III, cyclohexane/toluene (95:5, v/v). The radiolabeled sterols and their precursors were also co-chromatographed with standards: cholesterol, lanosterol, 2,3:22,23-dioxidosqualene, 2,3-oxidosqualene and squalene. The radioactive bands on the TLC chromatograms were located by scanning the plates in a Berthold radiochromatogram reader and were scraped off into scintillation vials and counted. Under our work-up conditions recovery of [^{14}C]cholesterol, added to control experiments, was about 65%. The results are expressed as the means of duplicates which were not found to differ by more than 10%.

Biosynthetically labeled [^{14}C]2,3-oxidosqualene was identified chemically according to the procedure of Willett *et al.* [25]. The band migrating with authentic 2,3-oxidosqualene was eluted and hydrolyzed in acidic medium. The material obtained after extraction (about 70% of radioactivity) co-migrated on TLC (hexane/ethyl acetate 75:25, v/v) with authentic squalene 2,3-glycol. This compound was in turn converted into 1,1',2-tris-norsqualene aldehyde by treatment with periodic acid; on TLC (system II), the labeled metabolite showed the same R_F as an authentic sample. The [^{14}C]2,3-oxidosqualene, which accumulated in cells treated with the inhibitors, could also be converted into [^{14}C]lanosterol by a rat liver microsomal system according to a procedure described previously [16]. These results establish the identity of [^{14}C]2,3-oxidosqualene.

Biosynthetically labeled 2,3:22,23-[^{14}C]dioxidosqualene co-chromatographed on TLC (systems I, II) with an authentic sample prepared according to [26]. Similarly labeled [^{14}C]squalene was found to co-migrate with a standard as a single band in all the TLC systems used in this study.

Cholesterol and desmosterol present in the C_{27}-sterol fractions were acetylated and separated by TLC on silver nitrate (10% in methanol/water 70:30, v/v) impregnated silica gel plates by double migration in toluene/cyclohexane (40:60, v/v) as developing solvent. 24(*R,S*),25-Epoxycholesterol was prepared by oxidation of desmosterol as described [27] and purified by TLC by a double migration in System II. The biosynthetically labeled polar sterols were isolated by TLC using the same developing system and the band co-migrating with 24,25-epoxycholesterol was analyzed as described below.

Gas chromatography and mass spectroscopy. Cholesterol content of cultured cells, isolated by thin layer chromatography (Systems I, II), was quantified by gas chromatography on a 25 m 0.25 mm i.d. OV-1 coated fused silica capillary column (Spiral) using ergosterol as an internal standard. A Carlo-Erba (Fractovap 4160) chromatograph equipped with a Spectra Physics (SP 4270) integrator was used. The sample was injected directly into the column at 60° and the analysis was performed (H_2, 2 ml/min) with the following temperature program: 30°/min to 240° followed by 2°/min to 290°. In cells treated with the inhibitors, gas chromatography analysis revealed the accumulation of a compound different from cholesterol in the C-27 sterols band ($RT_X/RT_{cholesterol}$ = 1.037); it was identified as desmosterol by gas chromatography coupled with mass spectroscopy on a LKB instrument. The compounds were separated on a 25 m SE-30 fused silica capillary column and the mass spectrometer was operated at 70 ev: m/z (relative intensity): 384 (20), 369 (22), 300 (32), 271 (80), 253 (25), 105 (40), 69 (100), 41 (75). The

fragmentation of the acetate derivative was also consistent with desmosterol acetate. The compounds more polar than cholesterol and co-migrating on TLC with 24,25-epoxycholesterol were analyzed similarly; the major fraction consisted in this epoxide as evidenced by the fragmentation pattern identical with that recently published [28].

Liposome leakage induced by 2-aza-2,3-dihydrosqualene. Large unilamellar liposomes composed of egg phosphatidylcholine and cholesterol (molar ratio 1:1) and encapsulating 5(6)-carboxyfluorescein (40 mM) were prepared by the reverse phase evaporation method [29]. The vesicles were separated from non-encapsulated material by gel filtration on a Sephadex G-75 column eluted with 5 mM HEPES, 100 mM NaCl (pH 7.4). Lipid phosphorus was determined according to [30]. Leakage experiments were performed, at 25°, with vesicles suspended (1.6 or 16 μM phospholipid) in 2 ml phosphate-buffered saline (pH 7.4). The change of fluorescence, after addition of emulsions of 2-aza-2,3-dihydrosqualene, or its *N*-oxide derivative, in the same buffer, was recorded at 520 nm (λ_{ex} = 490 nm) [31]. It was found convenient to plot the fluorescence change obtained 5 min after the azasqualene addition as a function of the azasqualene concentration. The 100% fluorescence setting was obtained with a vesicle preparation containing 0.5% (v/v) Triton X-100.

RESULTS

Sterol synthesis

Swiss 3T3 fibroblasts grow in lipid-depleted medium; when cultured in the presence of 10% (v/v) delipidated fetal calf serum the cells had an average doubling time of about 24 hr and grew to confluency at about 6.10^4 cells/cm^2. This growth pattern is indistinguishable from the one observed in the presence of normal serum. As expected, 3T3 cells grown in delipidated medium actively incorporated [^{14}C]acetate given in the medium, in the sterol fraction. Confirming the correlation between the proliferative state of cells and sterol biosynthesis [4], the accumulation of the label in the C_{27}-sterol fraction closely paralleled the growth curve (not shown). In contrast, cells grown in normal medium incorporated only marginally the label into sterols, e.g. incorporation of labeled acetate in C_{27}-sterols by cells grown in complete medium was about 4% of that measured in delipidated medium, for cells which have been cultured for 72 hr. When the cells were given [^{14}C]acetate for 2 hr at different times after seeding, the most active incorporation expressed as [^{14}C]cholesterol formed/mg protein was found at 48 hr of growth, i.e. during the logarithmic phase of growth, when the cell number had reached about 35% of saturation density. Consequently, most of our studies on the effect of the inhibitors on cholesterol biosynthesis were carried out with 3T3 cells which have been growing for 2 days in a delipidated medium.

Effect of 2-aza-2,3-dihydrosqualene and 2-aza-2,3-dihydrosqualene N-oxide on sterol synthesis.

Treatment of cells with 2-aza-2,3-dihydrosqualene resulted in an important reduction of [^{14}C]acetate incorporation into the C_{27}-sterols. As shown in Table 1, the extent of inhibition was concentration-dependent and was markedly influenced by the length of the preincubation period of the cells with the drug. A time course experiment was performed to determine how rapidly the drugs exert their inhibition on sterols biosynthesis. The results (Fig. 1) indicate that the effect of 2-aza-2,3-dihydrosqualene is progressive and relatively slow, reaching a maximal effect at about 8 hr. The corresponding *N*-oxide compound provoked its inhibition more rapidly, the maximum being observed within 2 hr of preincubation. At 1 μM, both compounds inhibited maximally the biosynthesis of sterols to a similar extent, i.e. the incorporation of label was about 20% of control. The observations made in Fig. 1 underline

Table 1. Effect of 2-aza-2,3-dihydrosqualene on the incorporation of [^{14}C]acetate into C_{27}-sterols

	Relative rate of C_{27}-sterols synthesis			
	Drug concentration (μM)			
Preincubation time (hr)	0.1	1	10	100
2	ND	85	4	1
4	93	32	3	0.9

3T3 cells grown for 48 hr in a delipidated medium (see Materials and Methods) were preincubated with the indicated concentrations of 2-aza-2,3-dihydrosqualene for 2 or 4 hr. The cells were then given [^{14}C]acetate (10 μCi) and after 2 hr were harvested and the incorporation of label into the C_{27}-sterol fraction determined. The values given are percentages of incorporation of cells grown in the absence of drug, and represent an average of two experiments. Label associated with the C_{27}-sterols in control cells was 1.2×10^4 dpm/mg of cell protein. ND, not determined.

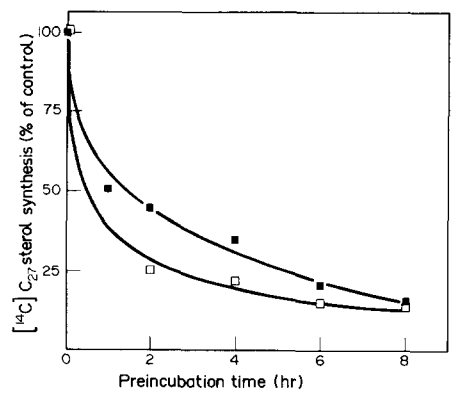

Fig. 1. Influence of drug preincubation time on C_{27}-sterols synthesis. 3T3 cells were cultured in delipidated growth medium for given times with 1 μM 2-aza-2,3-dihydrosqualene (—■—) or 2-aza-2,3-dihydrosqualene *N*-oxide (—□—). After this preincubation, [^{14}C]acetate (10 μCi) was given to the medium and after 2 hr the incorporation of label into C_{27}-sterols was determined. The results are expressed as percentage of [^{14}C]C_{27}-sterols measured in the absence of drug (100% corresponds to 1.10^4 dpm/mg of cell protein).

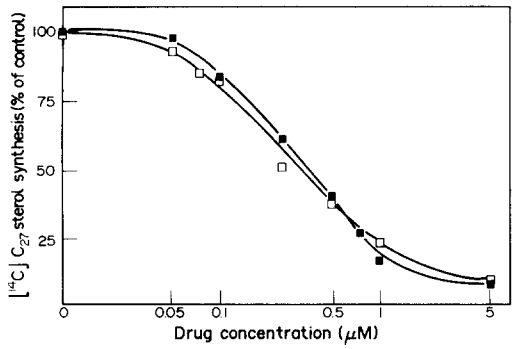

Fig. 2. Influence of 2-aza-2,3-dihydrosqualene (—■—) and its *N*-oxide (—□—) concentration on C_{27}-sterols biosynthesis. 3T3 cells, cultured in delipidated growth medium (see Materials and Methods), for 48 hr, were incubated for 4 hr, in the presence of the indicated concentrations of drugs. [^{14}C]acetate (10 μCi) was then given to the medium and after 2 hr the incorporation into C_{27}-sterols was determined. The results (average of two experiments) are expressed as percentages of [^{14}C]cholesterol measured in the absence of drug (100% corresponds to 1.5 10^4 dpm/mg of cell protein).

that 2-aza-2,3-dihydrosqualene, which is a hydrophobic compound of limited solubility, is most probably slowly taken up and accumulated by the cells. Half-maximal inhibition was determined using a 4 hr preincubation time (Fig. 2); under these conditions both compounds were equally inhibitory and interestingly, the measured IC_{50} (0.3 μM) was largely inferior to the constant obtained with rat liver microsomal, 2,3-oxidosqualene cyclase, e.g. 2-aza,2,3-dihydrosqualene had an IC_{50} of 8.8 μM [16] (IC_{50} corresponds to the inhibitor concentration which reduces the observed biosynthesis rate by 50%).

The specificity of the inhibitors for the sterol biosynthetic pathway is suggested by the fact that the [^{14}C] radioactivity associated with total nonsaponifiable lipids was essentially unchanged after the cells were treated with 1 μM 2-aza-2,3-dihydrosqualene up to 6 hr, i.e. conditions which profoundly affected the incorporation of [^{14}C]acetate into C_{27}-sterols. To identify the step(s) where the drugs exert their inhibitory action on the biosynthesis of cholesterol, the nonsaponifiable lipids were analyzed by thin-layer chromatography. As indicated in Table 2, in the absence of inhibitors, the totality of [^{14}C]acetate-derived nonsaponifiable lipids were sterols, predominantly C_{27}-sterols, a minor fraction co-migrating with 2,3-epoxysqualene. Analysis of the C_{27}-sterol fraction by TLC on silver nitrate impregnated silica plates revealed the presence of both radiolabeled cholesterol and desmosterol; their relative proportion varies from 9:1 to 2:8 depending on the age of the culture, i.e. desmosterol increases at the expense of cholesterol with an increasing number of passages. This was somewhat unexpected since, as we will describe later, analysis of the sterol content in untreated cells by gas chromatography indicated, the only presence of cholesterol. Minor amounts of desmosterol, i.e. 2.5–5% of the C_{27}-sterol fraction becoming detectable only when cells reached confluency. It is possible that in the Swiss 3T3 fibroblasts the Δ^{24}-sterol reduction is a limiting step; cell lines are known in which desmosterol is the end product of the sterol synthetic pathway, e.g. L cells [13]. Treatment of the cells with 0.5 μM 2-aza-2,3-dihydrosqualene reduced the incorporation of [^{14}C]acetate into C_{27}-sterols, this decrease being accompanied by an increase in radioactivity in several other regions of the chromatogram (Table 2). The most pre-eminent peak co-chromatographed with a 2,3-oxidosqualene standard. This compound was identified, as described in Materials and Methods, by its chemical conversion into squalene 2,3-glycol and 1,1',2-tris-norsqualene aldehyde, according to Willett *et al.* [25]. The biosynthetically labeled 2,3-oxidosqualene could also be converted into [^{14}C]lanosterol by a rat liver microsomal fraction, under anaerobic conditions. In addition to 2,3-oxidosqualene, two other minor labeled peaks appeared. The least polar compound co-chromatographed with a squalene standard in all the chromatographic systems tested and the other peak co-migrated with a 2,3:22,23-dioxidosqualene standard. The effect of 1 μM 2-aza-2,3-dihydrosqualene on the sterol distribution in the C_{27}-fraction and on the accumulation of sterols more polar than chol-

Table 2. Analysis by thin layer chromatography of the incorporation of [^{14}C]acetate into nonsaponifiable lipids. Effect of 2-aza-2,3-dihydrosqualene

	Lipids			
Supplements	C_{27}-sterols	2,3:22,23-Dioxidosqualene	2,3-Oxidosqualene	Squalene
None	89	0.2	9	1.5
2-Aza-2,3-dihydrosqualene (0.5 μM)	55	7.5	25	7.5

3T3 cells were grown in delipidated growth medium. After 2 days, they were preincubated 4 hr in the presence or absence of 0.5 μM drug; this treatment was followed by a 2 hr labeling period with [^{14}C]acetate (10 μCi). The cells were then harvested and the nonsaponifiable lipids analyzed by thin layer chromatography using system II as developing solvent. The results, which are means of two experiments, are given as percentages of the radioactivity associated with the nonsaponifiable lipid fractions.

Table 3. Analysis by thin layer chromatography of the incorporation of [^{14}C]acetate into nonsaponifiable lipids after pulse and pulse-chase experiments by 3T3 fibroblasts pretreated with 2-aza-2,3-dihydrosqualene

	Lipids				
Conditions	Polar sterols	C_{27}-sterols	2,3:22,23-Dioxido-squalene	2,3-Oxido-squalene	Squalene
Pulse-label	3.7	16.1	14.1	53.8	12.3
Pulse-label and chase	9.4	43.6	2.7	42.2	2.1

Cells were grown in delipidated growth medium. After 2 days, they were preincubated 4 hr in the presence of 1 μM drug; this treatment was followed by a 2 hr labeling period with [^{14}C]acetate (pulse-label experiment). For the chase experiment the cells were then washed and incubated further 4 hr without the drug in the presence of cold acetate (20 mM). The cells were then harvested and the nonsaponifiable lipids analyzed by TLC using system II (double migration) as developing solvent. The results are given as the percentages of total radioactivity associated with the nonsaponifiable lipid extracts. Label recovered in these fractions was 1.23×10^5 dpm/mg of cell protein in the pulse-label experiment and $0.93\ 10^5$ dpm/mg in the pulse-label and chase experiment.

esterol was studied. At this concentration, 4 hr of pretreatment with the drug reduced the incorporation of labeled acetate into the C_{27}-fraction to 22% of control; the fraction was composed of desmosterol (98%) and cholesterol (2%). A small fraction of polar sterols which co-migrated with a 24,25-epoxycholesterol standard, was detectable in the treated cells, it represented about 8% of the label recovered in the nonsaponifiable lipids, i.e. 30% of the label associated with the C_{27}-sterol fraction. Analysis of this fraction by GC-MS revealed that it was mainly composed of 24,25-epoxycholesterol (more than 50%). Further purification of this fraction has not yet been attempted.

In order to demonstrate a precursor–product relationship between the accumulated [^{14}C]2,3-oxidosqualene and the C_{27}-sterols, a chase experiment was performed. A typical experiment is given in Table 3. After the chase a decrease in labeled squalene and 2,3-oxidosqualene was observed which was correlated with a corresponding increased incorporation of the label in the C_{27}-sterol fraction. Similarly, the counts associated with the 2,3:22,23-dioxidosqualene fraction was reduced and more label was associated with polar sterols. These experiments indicate that (i) the effect of 2-aza-2,3-dihydrosqualene is reversible, although not completely, probably because of its retention by the cells, (ii) squalene diepoxide is probably converted into polar sterols when the inhibition of the cyclase is released.

Effect of 2-aza-2,3-dihydrosqualene on cell growth

Incubation of 3T3 fibroblasts in delipidated medium with 2-aza-2,3-dihydrosqualene at concentrations superior or equal to 0.5 μM inhibited cell growth in a dose-dependent manner (not shown). At 1 μM, growth was arrested at a cell concentration corresponding to 60% of control, this effect being almost totally prevented by simultaneous addition of 50 μM cholesterol and the drug (Fig. 3). Similarly, when cells were grown in serum-containing medium, which corresponds approximately to 10^{-4} M cholesterol, the drug had no effect. When growth-arrested cells were washed, at day 3, and incubated further in delipidated medium with 50 μM cholesterol, the cells remained at the plateau even in the absence of 2-aza-2,3-dihydrosqualene (Fig. 4). In contrast when the washed cells were reincubated in presence of total serum, the cells resumed growth even when 1 μM drug was present in the medium (Fig. 4).

Effect of 2-aza-2,3-dihydrosqualene on cellular cholesterol

The results obtained above indicate that the inhibition of the C_{27}-sterols biosynthesis by 2-aza-2,3-dihydrosqualene markedly affected cell growth. In

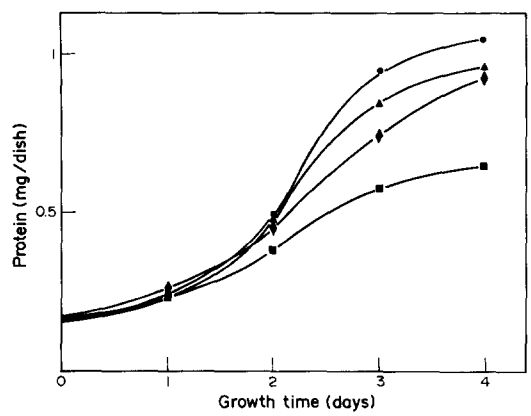

Fig. 3. Influence of 2-aza-2,3-dihydrosqualene on growth. Protection by cholesterol. 3T3 cells were seeded at a density of 3.10^5 cells/60 mm dish and cultured in 5 ml of a delipidated growth medium (see Materials and Methods) in the absence (—●—) or presence (—■—) of 1 μM 2-aza-2,3-dihydrosqualene. Growth curve with 50 μM (—▲—) or 25 μM (—◆—) cholesterol and 1 μM drug co-administered at time zero. Dishes were harvested in duplicate every day, and cell growth determined.

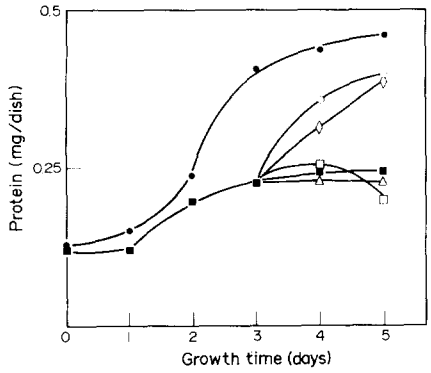

Fig. 4. Reversion of the effect of 2-aza-2,3-dihydrosqualene on cell growth by fetal calf serum. 3T3 cells were plated at a density of 1.5×10^5 cells/60 mm-dish and cultured in 5 ml of delipidated growth medium in the presence of 1 μM 2-aza-2,3-dihydrosqualene (—■—). On day 3, the medium was drained off the dishes and after rinsing with phosphate-buffered saline (pH 7.4) the cells were given new media (5 ml): delipidated growth medium containing 50 μM cholesterol supplemented (—□—) or not (—△—) with 1 μM drug and normal growth medium, i.e. containing 10% (v/v) fetal calf serum, supplemented (—◇—) or not (—○—) with 1 μM drug. Control growth curve (—●—) in delipidated growth medium, in the absence of drug. The dishes were harvested in duplicate daily, and cellular growth determined.

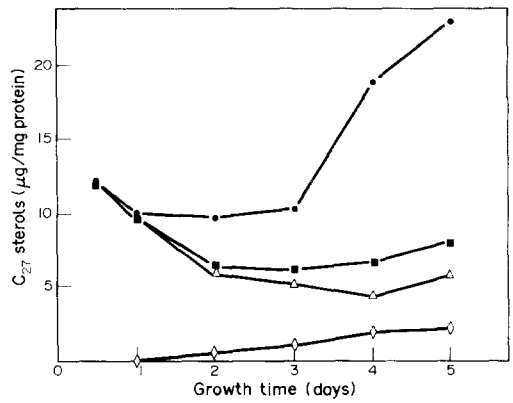

Fig. 5. Evolution of cholesterol and desmosterol content during cell growth. 3T3 cells were seeded at a density of 3×10^5 cells/60 mm dish and cultured in a delipidated growth medium, in the absence or presence of 1 μM 2-aza-2,3-oxidosqualene. Every day dishes (duplicates) were harvested, i.e. the medium was drained off and the cells carefully washed with phosphate-buffered saline. The C_{27}-sterol fraction, obtained by thin layer chromatography (system I) from the nonsaponifiable lipid extract, was analyzed by gas chromatography as described in Materials and Methods. (—●—) Cholesterol content in control cells, (—△—) cholesterol content in treated cells and (—■—) total C_{27}-sterols (cholesterol and desmosterol). (—◇—) Desmosterol in treated cells. The results are the average of 4 experiments.

order to correlate such effects, we have studied the influence of the drug on cellular cholesterol content during cell growth. The C_{27}-sterol fraction was analyzed by gas chromatography, and as shown in Fig. 5, a drastic decrease in cellular sterol could be observed, e.g. less than 40% of control was measurable when cells were grown 4 days in delipidated medium containing 1 μM 2-aza-2,3-dihydrosqualene. After 2–3 days of culture in the presence of the drug, analysis of the cellular C_{27}-sterol fraction by GC revealed an additional peak. This new compound, which gradually accumulated at the expense of cholesterol, was identified as desmosterol by mass spectrometry.

Cellular toxicity of 2-aza-2,3-oxidosqualene and of 2-aza-2,3-oxidosqualene N*-oxide*

When given to 3T3 fibroblasts at high concentrations (≥ 10 μM), 2-aza-2,3-oxidosqualene and its *N*-oxide provoked, within 2–6 hr depending on the dose, the detachment of the cells and their lysis as evidenced by trypan blue uptake. Moreover, the cells could only marginally be protected from this toxic effect of the drug by addition of cholesterol (50 μM) or by complete serum. This deleterious action cannot therefore be ascribed solely to the inhibition of the biosynthesis of cholesterol. Due to the amphiphilic structure of these compounds a membrane perturbation could be expected. When estimated with a fluorometric method [32, 33], both compounds did not form micelles at the highest concentration tested (10^{-4} M); however, they were found to induce leakage in large unilamellar liposomes composed of phosphatidylcholine/cholesterol (1:1), the threshold level being about 5 μM (Fig. 6).

DISCUSSION

The aza-derivatives of squalene, designed to inhibit 2,3-oxidosqualene cyclase [16–19] have been found to be excellent inhibitors of cholesterol biosynthesis in 3T3 fibroblasts grown in delipidated medium. The potency of 2-aza-2,3-dihydrosqualene and of its *N*-oxide, i.e. $IC_{50} = 0.3$ μM (0.1 μg/ml), compares favorably with other known cyclase inhibi-

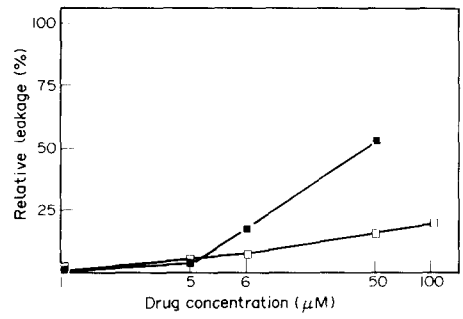

Fig. 6. Leakiness of 5(6)-carboxyfluorescein loaded liposomes induced by 2-aza-2,3-dihydrosqualene and its *N*-oxide. Suspension of unilamellar liposomes composed of egg phosphatidylcholine/cholesterol (molar ratio 1:1) (16 μM phospholipid) having encapsulated 40 mM 5(6)-carboxyfluorescein were incubated in phosphate-buffered saline (pH 7.4) at 25° with increasing concentrations of 2-aza-2,3-dihydrosqualene (—■—) and 2-aza-2,3-dihydrosqualene *N*-oxide (—□—). The fluorescence was determined after 5 min and the values are given as percent of total fluorescence measured in the presence of detergent (see Materials and Methods).

tors which have been tested in tissue culture cells, e.g. 4,4,10β-trimethyl-*trans*-decal-3β-ol [34], 3β-[2-(diethylamino-ethoxy]andost-5-en-17-one [35, 36], chloroquine [37] or in yeast, e.g. 2,3-iminosqualene [38]. 2,3-Oxidosqualene and 2,3:22,23-dioxidosqualene were the major lipids accumulated at the expense of cholesterol when 3T3 cells were treated with 2-aza-2,3-dihydrosqualene, indicating that a primary cellular target of the drug in the cholesterol biosynthetic pathway is indeed the 2,3-oxidosqualene cyclase. Similar squalene epoxides have been reported with other cyclase inhibitors [34, 36, 37, 39–41]. Sterols more polar than cholesterol were also detectable in cultured cells exposed to cyclase inhibitors [34, 36, 41]; the major one corresponding to 24(*S*),25-oxidocholesterol, was characterized [27, 42]. This compound, which most probably results from the cyclization of 2,3(*S*):22(*S*),23-dioxidosqualene [27], a product of the oxidation of 2,3-oxidosqualene by squalene epoxidase [43, 34], was recently proposed as a regulator of HMG-CoA reductase [28, 44]. A polar sterol presenting similar characteristics was detected in 3T3 cells treated with 2-aza-2,3-dihydrosqualene. Besides squalene epoxides a small quantity of squalene also accumulated in the cells grown in the presence of the drug. A similar observation was made in cultured plant cells (P. Benveniste, personal communication). Since treatment of 3T3 cells with U 18666 A, a potent 2,3-oxidosqualene inhibitor [16, 36], did not lead to an increased incorporation of label into squalene (not shown), the result obtained with 2-aza-2,3-dihydrosqualene might be ascribed to a marginal inhibition by the drug of squalene epoxidase. Related to this observation is the known weak inhibition of this enzyme of mammalian origin by allylamines [45].

The inhibition of cholesterol biosynthesis by 2-aza-2,3-dihydrosqualene in 3T3 cells is dependent on concentration and on duration of incubation. The drug has a very low solubility in aqueous media and because of its hydrophobicity is certainly gradually taken-up and accumulated by the cells. This accumulation and the fact that 2-aza-2,3-dihydrosqualene does not readily diffuse out of cells, most probably explains why the drug only slowly reaches its maximal effect and why its effect cannot be completely reversed in chase experiments.

When mammalian cells are grown in delipidated medium, the induction of enzymes involved in the sterol biosynthesis, mostly the rate-limiting HMG-CoA reductase, is generally observed [46] leading to an active synthesis of cholesterol. Under such conditions, inhibition of cholesterol biosynthesis at the cyclase step affects normal cell growth and leads to an arrest of division at drug dose-dependent densities. The observation that this effect could be fully reversed by free cholesterol or complete serum when co-administered with the drug suggests that 2-aza-2,3-dihydrosqualene is specific for the cholesterol biosynthesis pathway and that growth inhibition is due to sterol deficiency rather than to squalene epoxide accumulation. Effect on cell growth by 2,3-oxidosqualene cyclase inhibitors is in part distinct from that attributable to HMG-CoA reductase inhibitors; in this latter case, besides the inhibition of cholesterol, the effect on growth could also be related to metabolites of mevalonate other than cholesterol directly involved in DNA replication [10, 47]. The observation that cyclase inhibitors block cell growth [34, 38], corroborates the fact that in tissue culture, cholesterol is an absolute requirement for cellular proliferation [4]. The biochemical basis for cholesterol accumulation in dividing cells is not fully understood, it is probably related to its role as membrane constituent or to some regulatory function in some event of the cell cycle.

After 3 days of treatment with 1 μM 2-aza-2,3-dihydrosqualene the division of the 3T3 cells was arrested and cellular cholesterol content had reached its minimum (5.2 $\mu g/10^6$ cells). This confirms that cholesterol biosynthesis is efficiently blocked by this concentration of drug and that cells had probably made use of their residual cholesterol and (or) of that left in the medium to undergo, at a slow rate, some divisions. Interestingly at day 3, and in contrast to untreated cells, the cells started to accumulate desmosterol, indicating an inhibition of the sterol side-chain reduction. The Δ^{24}-reductase is a classical target of amino-containing compounds such as U 18666 A [35] and the 25-aza-sterol series [48]. The side-chain reduction seems to be a rather limiting step in our cell line; indeed when 3T3 fibroblasts, cultured for 48 hr in delipidated medium, were pulse-labeled with [^{14}C]acetate, the labeled C_{27}-sterol fraction consisted in almost equal proportions of desmosterol and cholesterol. Under these conditions the effect of 2-aza-2,3-dihydrosqualene on the Δ^{24}-sterol reductase is very marked, indicating that it is probably another important target of the drug. It remains to be confirmed that 2-aza-2,3-dihydrosqualene is *in vitro*, an inhibitor of the desmosterol Δ^{24}-reductase. The fact that desmosterol becomes detectable only 3 days after exposure to the inhibitor, i.e. when cells had stopped dividing, probably indicates that the bulk of available cholesterol was first used in residual divisions and that the sterol neosynthesis, which gradually led to the accumulation of desmosterol, was unable to sustain cell growth. When free cholesterol was given on day 3 to the treated cells, which had stopped dividing, no effect on growth could be observed; in contrast, the cells resumed dividing when complete serum was added to the medium. The reasons for this difference remain unexplained; it is possible that cells when starving for cholesterol, which increases their LDL receptor number [49], can efficiently channel LDL-associated cholesterol into compartments relevant to the regulation of cell growth. On the other hand the metabolization of the drug by the 3T3 cells remains to be determined.*

Whereas 2-aza-2,3-dihydrosqualene and its *N*-oxide at low concentrations had a cytostatic effect on 3T3 fibroblasts, at higher concentrations they became cytotoxic. The occurrence of this toxicity which is quite fast has certainly little relevance to the primary mode of action of these drugs and is probably related to cell membrane disturbance. The compounds were indeed found to cause leakiness in

* 2-Aza-2,3-dihydrosqualene was found to be oxidized into its *N*-oxide derivative by pig liver microsomal fractions (Cattel *et al.*, unpublished).

phosphatidylcholine/cholesterol (1:1) single-layered liposomes and interestingly the threshold concentrations for the observance of this phenomenon was in the same range as with cells.

In conclusion 2-aza-2,3-dihydrosqualene proved to be an interesting and potent new cholesterol biosynthesis inhibitor in cultured mammalian cells. Its main target is the cyclization of 2,3-oxidosqualene and it is also able to inhibit the Δ^{24}-sterol reductase. This drug allows the isolation of cells which are somewhat depleted in sterols and whose remaining C_{27}-sterol content is very enriched in desmosterol. Although the 2,3-oxidosqualene cyclase is not considered to be a rate determining step in the biosynthesis of cholesterol, its inhibition results in cell growth inhibition. Moreover this enzyme is an attractive target for the design of inhibitors, i.e. in addition to the direct reduction of cholesterol synthesis, inhibition of the cyclase induces the accumulation of 2,3:22,23-dioxidosqualene, a precursor of 24,25-epoxycholesterol which is a known repressor of HMG-CoA reductase [44].

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