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Inhibition of Cholesterol Synthesis in Cultured Cells by 25-Azidonorcholesterol

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Summary: 25-Azidonorcholesterol, a side chain analogue of cholesterol, inhibits cholesterol biosynthesis in BHK 21 cells in tissue culture; but 3-azido-5-cholestene, the azido analogue of cholesterol has no inhibitory effect. Cell growth was unimpaired by the azido analogue, which showed only a minimal uptake by the cell, compared with that of cholesterol or 25-azidonorcholesterol. 25-Azidonorcholesterol is incorporated into the membranes of BHK 21 cells in place of cholesterol as demonstrated by isotope experiments. The incorporation of radioactive acetate is strongly inhibited by the side chain azido substituted cholesterol derivative; the incorporation of radioactive mevalonate is, however, unimpaired. We conclude that the inhibition occurs at the hydroxymethylglutaryl-CoA-reductase step. Cell growth is not significantly altered.

Hemmung der Cholesterin-Synthese in Zellen in der Gewebekultur durch 25-Azidonorcholesterin

Zusammenfassung: Das Seitenketten-Cholesterinanaloge 25-Azidonorcholesterin führt in Zellen in der Gewebekultur (BHK 21) zur Hemmung der Cholesterin-Synthese, während das 3-Azido-5-cholesten, eine Verbindung mit der Azidogruppe in der Ringstellung, keine Hemmung verursacht. In Isotopenversuchen wurde gezeigt, daß 25-Azidonorcholesterin an Stelle des Cholesterins in die Membranen der BHK-Zellen inkorporiert wird. Isotopenexperimente mit radioaktivem Acetat und Mevalonat zeigen auf, daß die Hemmung auf der Stufe der 3-Hydroxymethylglutaryl-CoA-Reduktase erfolgt. Das Zellwachstum wird nicht signifikant verändert.

Key words: 25-Azidonorcholesterol, 3α-azido-5-cholestene, inhibition of hydroxymethylglutaryl-CoA reductase, cell growth, membrane synthesis.

Enzymes:
Hydroxymethylglutaryl-CoA reductase (NADPH), mevalonate:NADP® oxidoreductase (CoA-acylating) (EC 1.1.1.34); Sterol 24-reductase (not yet registered).

Abbreviations:
Mesyl = methylsulfonyl; DBED = *N,N*-dibenzylethylenediammonium.

An attempt has been made to incorporate azido-labelled lipid precursors into the membranes of eucaryotic cells, which act as host cells for enveloped viruses. We recently demonstrated that phospholipids, labelled in their fatty acyl moieties with the photosensitive azido group, are suitable chemical probes for the determination of the nearest neighbour to the carbon segment of the lipid molecule because the aliphatic nitrene group generated by UV irradiation has a very short lifetime ($\sim 10^{-11}$ s), during which inter- or intra-molecular reactions occur. Intermolecular reactions (insertion into C-H-bonds and addition to double bonds) with adjacent fatty acyl chains and with polypeptide chains have been demonstrated[1]. During experiments aimed at the incorporation of the two photosensitive azido-labelled cholesterol derivatives ([25-^{3}H]25-azidonorcholesterol and [26-^{14}C]3α-azido-5-cholestene) into the membranes of baby hamster kidney (BHK 21) cells, it became apparent that the 25-azido compound inhibits the de novo synthesis of cholesterol, and that 3α-azido-5-cholestene lacks these properties. Isotope experiments suggest that 3-hydroxy-3-methylglutaryl-CoA synthesis is blocked. Concommittantly both compounds are incorporated into the membranes, but cell growth is barely inhibited.

Material and Methods

The syntheses of [26-^{14}C]3α-azido-5-cholestene and [25-^{3}H]25-azidonorcholesterol are described below. [2-^{14}C]Na-acetate (specif. radioactiv. 40 - 60 mCi/mmol) and [5-^{3}H]D, L-mevalonate (DBED salt, 5 mCi/mmol) were purchased from New England Nuclear, [5-^{14}C]-mevalonate from Amersham Buchler, Braunschweig and 26-norcholesten-3β-ol-25-one from Steraloids, Inc. Wilton, N.H.. BHK 21 cells were grown in Dulbecco's medium supplemented with 10% Bacto tryptose phosphate broth (Difco Laboratories, Inc. Detroit, Michigan) and 5% normal or lipoprotein free (d = 1.22 g/ml) fetal calf serum[2]. Monolayer cultures were grown in 12-cm Petri dishes, in a 5% CO_2 atmosphere at 37 °C in a humidified incubator.

Radioactive pulse experiments

The azidocholesterol derivatives, dissolved in 95% ethanol (5 mg/ml) and aqueous solutions of radioactive sodium acetate and mevalonate, were added as indicated in the legends to figures. The medium was decanted at appropriate times, the cell layer washed once with 5 ml medium containing 2% albumin, once with medium containing 5% serum and four times with phosphate buffered saline. Cells were released from the bottom of the dish with a rubber policeman, harvested in 4 ml phosphate buffered saline and sedimented by centrifugation at 250 × g for 10 min. The pellet was sonicated for 1 min and protein determined. The rest of the suspension was lyophilized in Sovirel tubes, and lipids were saponified with 2 ml 0.5N methanolic KOH for 1 h at 70 °C. Sterols were extracted three times with petroleum ether (30 - 60 °C). The extracts were taken to dryness in a stream of nitrogen at 40 °C, and the residue dissolved in 1 ml chloroform/methanol (1 : 1). Sterols were precipitated as digitonides[3]. The digitonides were pelleted, washed twice with 2 ml diethyl ether and dissolved in 0.5 ml soluene 350 and 10 ml toluene scintillator solution.

Gas liquid chromatographic analyses

A Packard gas-chromatograph model 805 was used. This was equipped with U-shaped 2-m glass columns packed with 1% OV 17 and operated at 250 °C. The instrument was connected to a Hewlett-Packard recorder integrator, model 3380 A. The effluent was split in a 1:1 ratio for mass and radioactivity determination of cholesterol, desmosterol, lanosterol and dihydrolanosterol. The latter and β-cholestane were used as standards. The Varian MAT CH5 mass spectrometer was used with the direct inlet and combined with gas-chromatographic separation (Varian model 1840).

Syntheses of radioactive azido-sterols

a) [25-^{3}H]25-Azidonorcholesterol (norcholest-5-en-3β-ol) 3β-Acetoxy-5-cholesten-25-one (I)

586.3 mg (1.5 mmol) *26-nor-5-cholesten-3β-ol-25-one* in 15 ml dry pyridine and 1.6 ml freshly distilled *acetic-anhydride* were heated at 60 - 70 °C for 1 h and left *overnight at room temperature. The solution was poured* into 500 ml ice water, the precipitate isolated by filtration, washed thoroughly with distilled water and dried over P_2O_5 in a desiccator. Yield: 587 mg (1.4 mmol, 93% of th.). The product proved to be pure in thin-layer chromatography (solvent system: petroleum ether/ether/methanol 70:30:7).

[25-^{3}H]3β-Acetoxy-5-cholesten-25-ol (II): 420 mg (1 mmol) *3β-acetoxy-5-cholesten-25-one (I)* was dissolved in 30 ml tetrahydrofuran/0.2M phosphate buffer pH 8.5 (8:1 v/v). A solution of 25 mCi (0.08 mmol) *NaB^3H_4* in 1 ml phosphate buffer pH 7 was added dropwise to the ketone. After 1 h additional 35.1 mg *$NaBH_4$ completed the reaction within 2 h.* The reaction mixture was adjusted to pH 6.0 with acetic acid and the solution poured into 400 ml ice water. The tritiated

product was extracted with ether, the ether solutions washed with water, dried over Na_2SO_4 and taken to dryness under reduced pressure. Yield: 420 mg (0.98 mmol) 98% of th.; specif. radioactiv. 7.7 mCi/mmol. The product proved to be homogeneous in thin-layer chromatography using the solvent system mentioned above.

[25-^{3}H]3β-Acetoxy-25-methylsulfonyloxy-5-cholestene-(III): To 200 mg (0.46 mmol) *[25-^{3}H]3β-acetoxy-5-cholesten-25-ol (II)* dissolved in 8 ml dry pyridine, 64 mg (0.55 mmol) *mesylchloride* was added and the mixture stirred at room temperature for 12 h. The solvent was evaporated under vacuum and the residue extracted six times with 50 ml diethyl ether. The ether extracts were washed with water, dried over Na_2SO_4 and then taken to dryness. Yield: 180 mg (0.36 mmol), 77% of th. Thin-layer chromatography showed, that the reaction had run essentially to completion.

[25-^{3}H]3β-Acetoxy-25-azido-5-norcholestene (IV): 180 mg (0.36 mmol) of the *mesyl derivative (III)* was dissolved in 20 ml dimethylformamide/water 10:1 (v/v) and 117 mg (1.8 mmol) sodium azide was added to this solution, which was subsequently stirred in the dark for 24 h. The mixture was concentrated at 70 °C under vacuum and the residue extracted five times with 50 ml chloroform. The combined extracts were washed with water, dried over Na_2SO_4 and taken to dryness. Yield: 165 mg (0.36 mmol). The product proved to be pure in thin-layer chromatography using the above system. IR-spectroscopy revealed the typical azido-absorption band at 2100 cm^{-1} and the ester band at 1730 cm^{-1}.

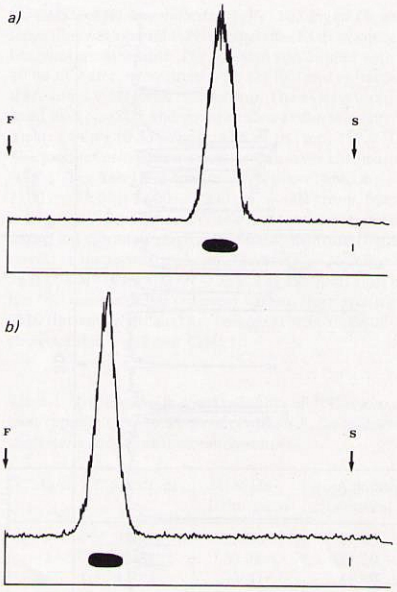


Fig. 1. Radio thin-layer chromatogram of a) [25-^{3}H]25-azidonorcholesterol, b) [26-^{14}C]3α-azido-5-cholestene. Solvent system: petroleum ether/ether/methanol 70:30:15 (V/V); S = start; F = front.

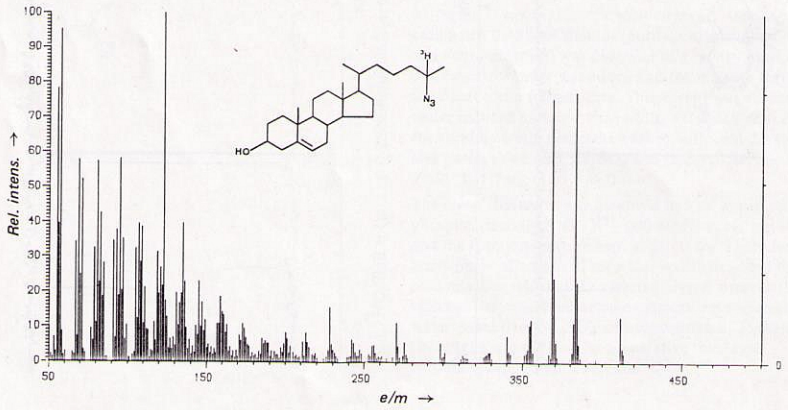
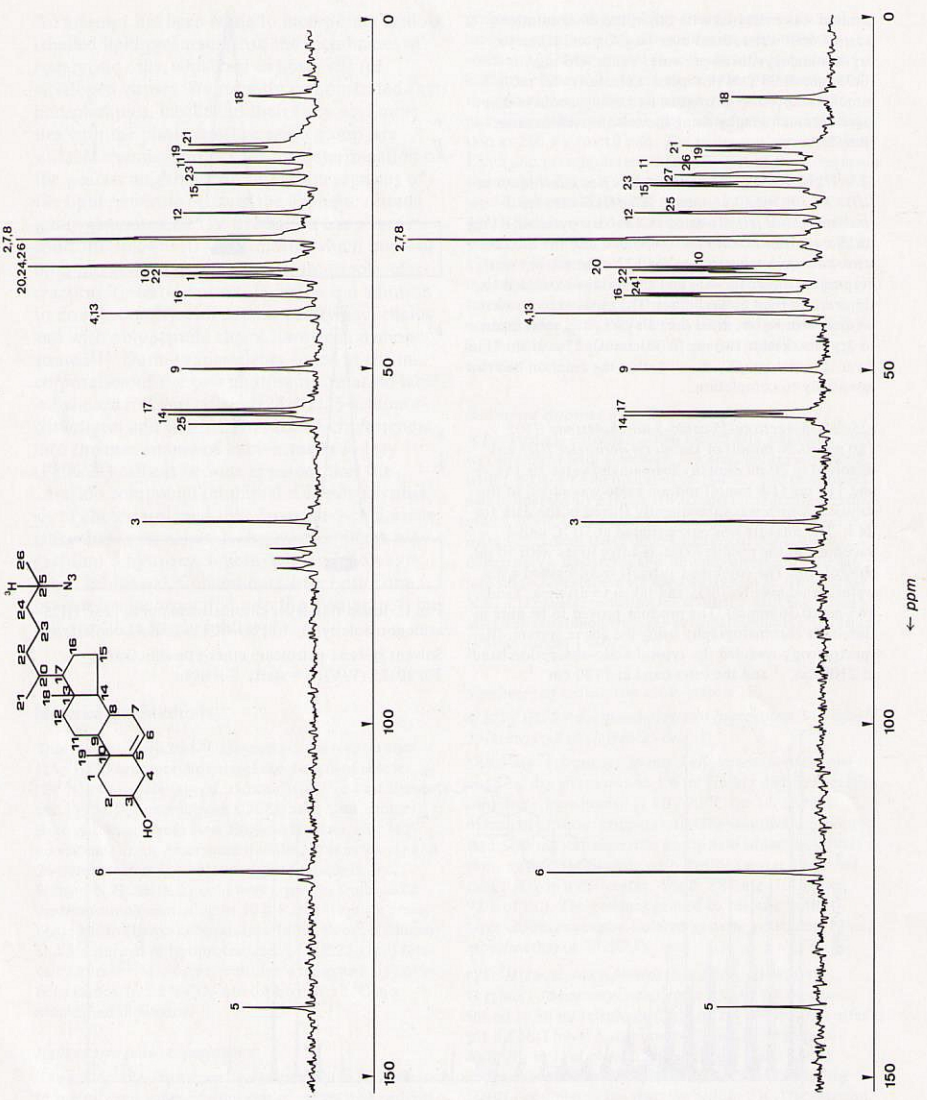


Fig. 2. Mass spectrum of 25-azidonorcholesterol.


HO
N3
2,7,8
20,24,26
4,13
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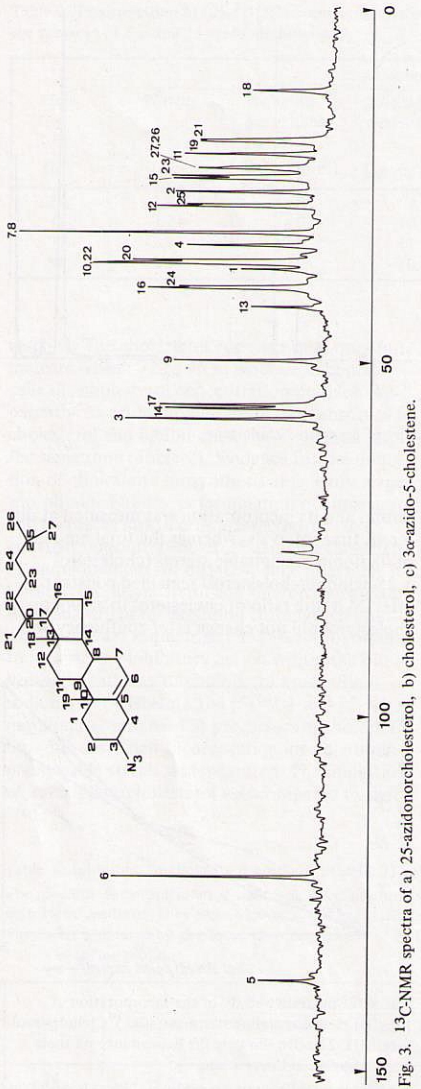


Fig. 3. ^{13}C-NMR spectra of a) 25-azidonorcholesterol, b) cholesterol, c) 3α-azido-5-cholestene.

[25-^3H]25-Azidonorcholesterol (V): 165 mg of *IV* was saponified with 60 m*l* 0.5N methanolic KOH at room temperature overnight. The solution was diluted with 40 m*l* of water, neutralized with 6N HCl and extracted three times with 50 m*l* chloroform. The extracts were dried over Na_2SO_4 and concentrated under vacuum. Yield: 144 mg (0.35 mmol), 97% of th., mp. 119.5 °C. The product proved to be pure in thin-layer chromatography (Fig. 1a). IR spectrum: absorption bands at 2100 cm^{-1}-N_3; 3 600 – 3 200 cm^{-1}-OH group. Specif. radioactiv. 7.7 mCi/mmol. The compound deteriorates during gas-chromatography. The mass spectrum (Fig. 2) reveals characteristic peaks at: 413 $M^{\oplus}$; $^m/_e$ $M^{\oplus}$-(H_2+N_2) 383; $^m/_e$ $M^{\oplus}$-(CH_3+H_2+N_2) 368. The chemical shift of the ^{13}C resonance lines of every carbon atom relative to TMS (tetramethylsilane) are compared with those of cholesterol in Fig. 3 and Table 1.

Table 1. Differences in chemical shifts of ^{13}C resonance lines (ppm relative to TMS) of cholesterol, 3α-azido-5-cholestene and 25-azidonorcholesterol.

C atom	Cholesterol	3α-Azido-5-cholestene	25-Azidonorcholesterol
2	31.58	25.80	31.45
3	71.37	57.86	71.29
4	41.98	33.31	41.89
24	39.18	39.18	35.20
25	27.83	27.61	57.62
26	22.39	22.39	35.20

b) [26-^{14}C]3α-Azido-5-cholestene

81 mg (0.21 mmol) *[26-^{14}C]cholesterol*[4] (specif. radioactiv. 0.198 mCi/mmol (purified by bromination and debromination) was dissolved in 4 m*l* dry pyridine, 30 µ*l* *mesylchloride* was added and the mixture stirred for 2 h at room temperature. The solvent was evaporated under reduced pressure, the residue extracted with ether, the combined ether extracts washed with cold 2N HCl and water, dried over Na_2SO_4 and taken to dryness. Yield: 101.7 mg (100% of th.).

The *mesyl derivative* was dissolved in 5 m*l* hexamethylphosphotriamide (HMPT)[5], 200 mg *Na-azide* was added and the reaction mixture kept at 80 °C for 3 h under an atmosphere of argon. 10 m*l* water was then added to the cold mixture, which was extracted several times with hexane. The combined hexane extracts were washed with water, dried over Na_2SO_4 and concentrated. 3α-Azido-5-cholestene was purified by preparative thin-layer chromatography, mp. 107 - 108 °C.

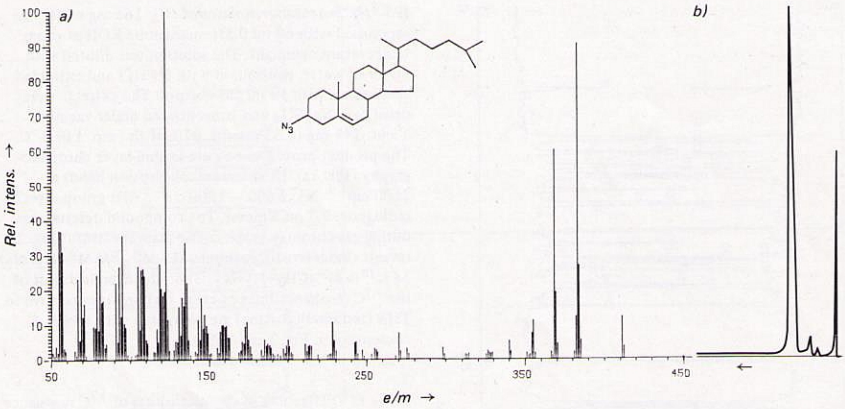


Fig. 4. Combined gas liquid chromatography-mass spectroscopy of 3α-azido-5-cholestene.

36.5 mg [26-^{14}C]3α-azido-5-cholestene (42% of th.) was separated from 3,5-cholestadiene and obtained in pure form. The product was characterized by gas-chromatography-mass spectroscopy (Fig. 4). The IR spectrum revealed the characteristic absorption bands at 2080 - 2120 cm^{-1}-N_3, C=C: 800 cm^{-1}. The chemical shifts of the ^{13}C resonance lines can be taken from Fig. 3 and Table 1. It proved to be radiochemically pure in radio thin-layer chromatography, (Fig. 1b).

Results

Incorporation of [25-^{3}H]25-azidonorcholesterol into BHK 21 cells

BHK 21 cells were grown for 24 h in media supplemented with either lipoprotein-containing serum or with lipoprotein-free serum, and the uptakes of [26-^{14}C]cholesterol and [25-^{3}H]25-azidonorcholesterol were compared during this period. Cells take up both sterols linearly, depending on the concentration in the medium (Fig. 5). In the concentration range up to 10 μg of cholesterol and 25-azidonorcholesterol/ml the incorporation of both sterols was distinctly higher, when lipoprotein-free serum was used as a supplement. [25-^{3}H]25-Azidonorcholesterol, at a concentration of 5 μg/ml medium, was also added to BHK 21 cell cultures growing in the logarithmic phase in medium supplemented with 5% normal fetal calf serum, and its incorporation was measured at different time intervals. Whereas the total amount of digitonin precipitable sterols (cholesterol + 25-azidonorcholesterol) remained constant after 24 h, the ratio of cholesterol to azidonorcholesterol did not change after confluency was

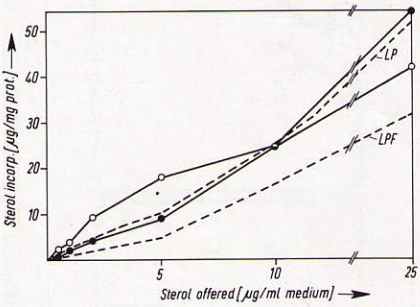


Fig. 5. Comparative study of the incorporation of [25-^{3}H]25-azidonorcholesterol and [26-^{14}C]cholesterol into BHK 21 cells, showing the dependence on their concentration in the medium.

LP = lipoprotein containing medium, LPF = lipoprotein free medium. —— 25-Azidonorcholesterol, – – – cholesterol.

Table 2. Incorporation of [25-^{3}H]25-azidonorcholesterol into BHK 21 cells grown on lipoprotein-free medium in the presence of 5 μg/ml 25-azidonorcholesterol.

Time	+ 25-Azidonorcholesterol				Control	
	Protein	Digitonin-precipitable sterols	25-Azidonor-cholesterol	Cholesterol	Protein	Cholesterol
[h]	[mg]	[μg/mg prot.]	[μg/mg prot.]	[μg/mg prot.]	[mg]	[μg/mg prot.]
24	2.92	16.4	9.7	6.7	1.83	6.6
48	3.74	14.3	10.8	3.5	2.61	8.8
72	2.92	17.1	11.4	5.7	1.53	11.6
96	2.31	17.2	10.4	6.8	1.25	13.5

reached. The cholesterol concentration does not increase within 72 to 96 h, whereas in control cells the cholesterol concentration doubles. Apparently 25-azidonorcholesterol can substitute for cholesterol and inhibit cholesterol synthesis at the same time (Table 2). Evidence for the inhibition of cholesterol biosynthesis at an early stage was obtained by the determination of cholesterol and its precursors in cells after exposure to a constant concentration of 25-azidonorcholesterol (5 μg/ml medium for different time intervals). Cholesterol, desmosterol and dihydrolanosterol were quantified by gas liquid chromatography. The results are summarized in Table 3. In order to localize the inhibitory action within the biosynthetic pathway of cholesterol biosynthesis sodium-[2-^{14}C]acetate and [5-^{14}C]- and [5-^{3}H]-mevalonate were used as precursors in cholesterol biosynthesis. Their incorporation into digitonin-precipitable sterols was measured. The inhibition of 25-azidonorcholesterol was compared to that of cholesterol (Fig. 6). The regulatory function of cholesterol at the level of 3-hydroxymethyl-glutaryl-CoA reductase is well established[6].

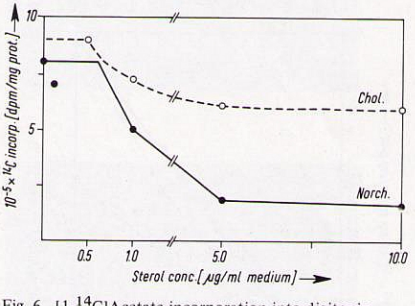


Fig. 6. [1-^{14}C]Acetate incorporation into digitonin-precipitable sterols of BHK 21 cells in the presence of 25-azidonorcholesterol (●——●) and cholesterol (○– – –○).

Table 3. Inhibition of cholesterol synthesis in BHK 21 cells in monolayer culture.

The medium was supplemented with 5 μg 25-azidonorcholesterol/ml medium. Cells were grown in 10-cm Petri dishes with 10 ml medium. They were harvested and the sterol fraction isolated as described under Methods and Materials. They were separated by gas liquid chromatography (2 m 1% OV-1 column; temperature 245 °C; 30 ml/argon/min. Values in μg/mg protein.

[h]	+ 25-Azidonorcholesterol			Control		
	Cholesterol	Desmosterol	Dihydro-lanosterol	Cholesterol	Desmosterol	Dihydro-lanosterol
24	14.4	1.2	1.3	16.1	2.1	–
48	11.2	1.9	0.3	18.8	1.4	–
72	8.6	1.7	–	21.5	1.3	–

Fig. 6 indicates that 25-azidonorcholesterol is a strong inhibitor of cholesterol biosynthesis, causing 50% inhibition at a concentration of 1 - 2 μg/ml medium (2.5×10^{-6} M). Whereas cholesterol, at a concentration of 10 μg/ml medium (2.5×10^{-5} M), reduces the incorporation of [^{14}C]acetate by 40% of the control experiment, 25-azidonorcholesterol, at the same concentration, inhibits cholesterol biosynthesis by more than 85%. The inhibition by 25-azidonorcholesterol did not lead to the accumulation of sterol intermediates such as desmosterol, dihydrolanosterol and lanosterol, as can be seen in the gas-chromatographic analyses of the sterol fraction. Therefore radioactive mevalonate was supplied as precursor of cholesterol synthesis to the medium of BHK 21 cells. Cells were incubated in the presence and absence of the inhibitory 25-azidonorcholesterol (5 μg/ml) and the medium supplemented with [5-^{14}C]mevalonate (0.2 μCi/ml). Cells were harvested at time intervals indicated in Fig. 7. The incorporation of radioactive

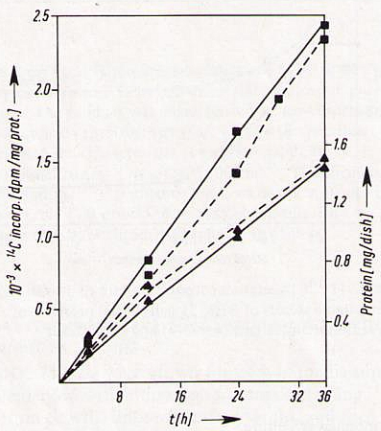


Fig. 7. [5-^{14}C]Mevalonate incorporation into digitonin-precipitable sterols of BHK 21 cells.
Digitonin-precipitable sterols: ■——■ Control, ■- - -■ in the presence of 25-azidonorcholesterol (5 μg/ml). Protein: ▲——▲ control, ▲- - -▲ in the presence of 25-azidonorcholesterol (5 μg/ml).

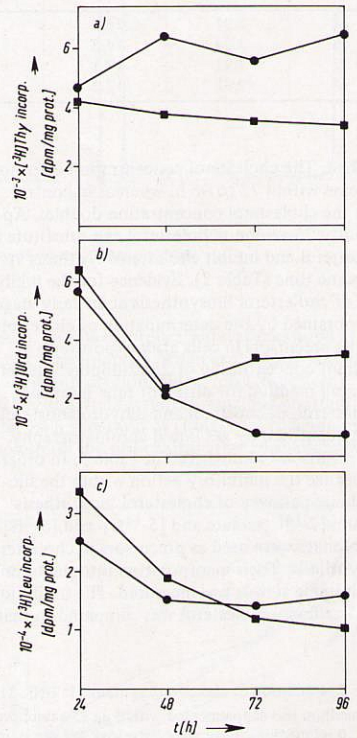
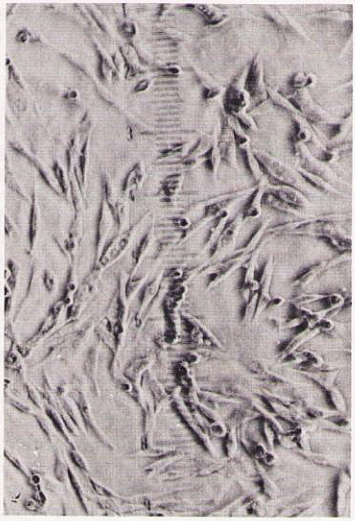


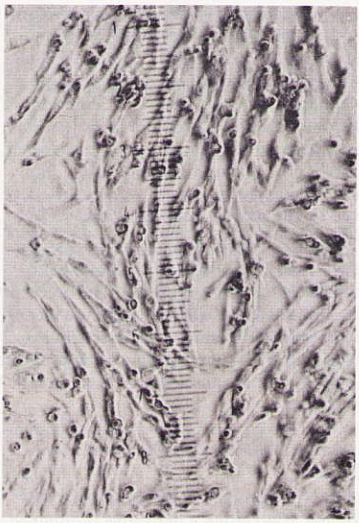
Fig. 8. Incorporation of a) [^{3}H]thymine (3 μCi/ml), b) [^{3}H]uridine (3 μCi/ml), c) [^{3}H]leucine (2 μCi/ml) into BHK 21 cells growing in the absence ●——● and presence ■——■ of 25-azidonorcholesterol (5 μg/ml).

Fig. 9. Morphology of BHK 21 cells growing 48 h (a, c) and 72 h (b, d), respectively, in the absence (a, b) and presence (c, d) of 25-azidonorcholesterol. ▷

Lipoprotein-free medium was used. Magnification approx. 1000-fold.



a)



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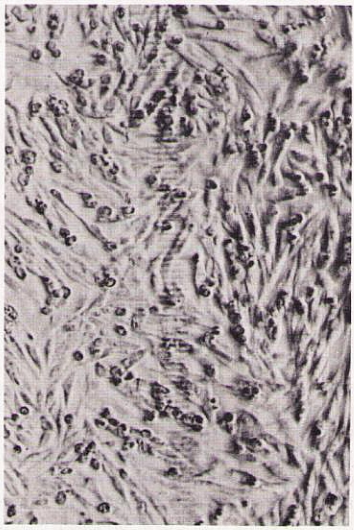
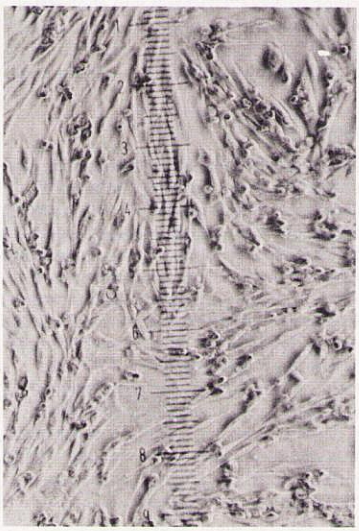


c)



d)

mevalonate into the digitonin precipitable sterol fraction was identical in control cells and those growing on the azido derivative. Therefore the inhibition must occur at the level of hydroxymethylglutaryl-CoA reductase. The inhibition of cholesterol synthesis by 25-azidonorcholesterol does not influence cell growth. Growth curves of control cells (Fig. 7) and those growing in the presence of 25-azidocholesterol were identical, as indicated by the protein determination.

[^{3}H]Thymine, [^{3}H]uridine and [^{3}H]leucine were also added to BHK 21 cells with inhibited and non-inhibited cholesterol synthesis. The precursors of DNA, RNA and protein synthesis were incorporated at very similar rates in both sets of experiments (Fig. 8). The morphological appearance of the cells is normal and unaltered even after 96 h (Fig. 9a - d).

Incorporation of 3α-azido-5-cholestene into BHK 21 cells

If the 3-hydroxy group of cholesterol is substituted by the azido group a derivative is obtained, which, in the concentration range between 0 and 6×10^{-5} M, has no inhibitory action on the incorporation of Na-[^{14}C]acetate into cholesterol. Its uptake into the cell is minimal, as compared to cholesterol and 25-azidonorcholesterol. Cell growth remained unimpaired by this compound.

Discussion

The studies reported here indicate that 25-azidonorcholesterol is a very effective inhibitor of cholesterol biosynthesis in BHK 21 cells. Isotope experiments prove that the biosynthetic sequence is inhibited at the level of hydroxymethylglutaryl-CoA reductase, because mevalonate releases the block of cholesterol synthesis. 25-Azidonorcholesterol not only inhibits cholesterol synthesis but is also incorporated in the membranes of the cell linearly with time. Kandutsch et al.[7,8] have demonstrated that cholesterol-derived sterols, with additional oxygen containing functional groups, depress cholesterol synthesis at the level of hydroxymethylglutaryl-CoA reductase. These additional hydroxyl or carbonyl groups may either be in the sterane ring system (C-6, C-7, C-15) or in the side chain (C-20, C-22, C-25). 7-Oxocholesterol has been studied in detail by Erickson et al.[9] in perfused rat liver and intact rat liver. These authors demonstrated that hydroxymethylglutaryl-CoA reductase is inhibited only for a brief period of time. The synthesis of oxygenase is then enhanced and the inhibitor is quickly transformed into more polar products, which are excreted in the bile. The azido group, as a substituent in the side chain of cholesterol, lends the sterol molecule properties which lead to an effective inhibition of the regulatory enzyme of cholesterol biosynthesis, hydroxymethylglutaryl-CoA reductase. No polar derivatives could be detected during the period of incubation with BHK 21 cells. By following DNA, RNA and protein synthesis with appropriate precursors, we observed that a minor deviation in thymine incorporation into DNA could be correlated with a slightly slower growth of the cells growing on 25-azidonorcholesterol. [^{3}H]Uridine and [^{3}H]-leucine incorporation resulted in specific activities of RNA and protein (dpm/mg protein) which were quite comparable in cells with and without inhibition of cholesterol synthesis. The appearance of both types of cells in light microscopy showed no morphological differences. This was also observed for 3α-azido-5-cholestene. Therefore the two azido derivatives, one of which is labelled in the side chain (25-azidonorcholesterol), the other in the nucleus (3α-azido-5-cholestene), are not toxic for cell growth in the concentration range studied here.

The low incorporation of 3α-azido-5-cholestene into the membranes of BHK 21 cells underlines the importance of the 3-hydroxy group in the 3 position. On the other hand substitution of a CH_3 group of the side chain of cholesterol by an azido group does not reduce its uptake compared with that of cholesterol. Whereas the 3α-azido-5-cholestene does not decrease cholesterol biosynthesis, 25-azidonorcholesterol has an inhibitory effect, similar to that of cholesterol on the de novo synthesis of cholesterol.

On the basis of isotope studies ([^{14}C]acetate and [^{14}C]mevalonate) this inhibition occurs at the level of hydroxymethylglutaryl-CoA reductase. Further in vitro studies are planned for the elucidation of the inhibition mechanism (inhibition of enzyme or enzyme synthesis). Photoaffinity labelling with this azido-labelled sterol may further help to determine the site of inhibition.

We acknowledge gratefully the support of this study by the *Deutsche Forschungsgemeinschaft*.

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