

Normal and inhibited cholesterol synthesis in the cultured rat embryo

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Abstract The Smith-Lemli-Opitz syndrome-affected fetus presents a deficiency in Δ^7 -dehydrocholesterol reductase, the last enzymatic step in the cholesterol biosynthesis pathway. Development of the abnormal human fetus takes place in a normal environment as the heterozygous mother's cholesterolemia remains normal. An animal model for this disease has been obtained from the offspring of pregnant rats treated with "distal" inhibitors of Δ^7 -dehydrocholesterol reductase, AY-9944 or BM15766. In the animal model, embryonic development occurs in a disturbed environment characterized by hypocholesterolemia and accumulation of Δ^7 -dehydrocholesterol and Δ^8 -dehydrocholesterol in the maternal serum. The purpose of the present study was to assess, in cultured rat embryos at early developmental stages, the relative contributions of exogenous and de novo synthesized cholesterol in the total embryonic cholesterol, according to the conditions of normal and altered de novo biosynthesis. Cultured rat embryos are able to synthesize cholesterol as shown by ¹³C-incorporation into cholesterol from ¹³C-labeled precursors added to the culture medium. De novo cholesterol biosynthesis is altered by addition to the culture medium of AY-9944 which inhibits the Δ^7 -dehydrocholesterol reductase and the Δ^8 - Δ^7 -sterol isomerase as suggested by the emergence of characteristic aberrant sterols in the embryonic tissues. Cholesterol-rich serum used for embryo culture alters the pattern in a way that confirms that the rat embryos are able to import exogenous cholesterol which down-regulates de novo cholesterol biosynthesis. Exogenous cholesterol substitutes for the deficit in a manner efficient enough to prevent the embryonic abnormalities induced by AY-9944.—Llibat, B., C. Wolf, F. Chevy, D. Citadelle, G. Berezziat, and C. Roux. Normal and inhibited cholesterol synthesis in the cultured rat embryo. *J. Lipid Res.* 1997. **38**: 22–34.

Supplementary key words Δ^7 -dehydrocholesterol reductase • Δ^8 - Δ^7 -sterol isomerase • AY-9944 • Smith-Lemli-Opitz syndrome • mass spectrometry • ¹³C-labeled precursors

Smith-Lemli-Opitz syndrome (SLO), an autosomal recessive multiple congenital malformation syndrome, was recently shown to be associated with reduced cholesterol biosynthesis (1, 2). High levels of Δ^7 -dehydrocholesterol, Δ^8 -dehydrocholesterol, and of the byprod-

uct nortrienol associated with low levels of cholesterol are found in serum, feces, and tissues of the affected children (1–5). This points to a deficiency in the last enzymatic step of the cholesterol biosynthesis pathway (6). The Δ^7 -dehydrocholesterol reductase defect was pointed out in liver microsomes preparation (7) and the isolated defect of this enzyme was characterized in cultured skin fibroblasts of homozygotes (8). A translocation breakpoint at 7q32.1 has been identified in one patient (9), but in the absence of gene cloning and sequence, the origin of the defect remains questionable.

The teratogenic activity of AY-9944 [*trans*-1,4-bis (2-dichlorobenzyl aminoethyl) cyclohexane dihydrochloride], a molecule inhibiting the last enzymatic step of cholesterol biosynthesis, was demonstrated 30 years ago (10). Administered to pregnant rats, AY-9944 decreases the maternal serum cholesterol level while Δ^7 -dehydrocholesterol and byproducts markedly increase (11, 12). When AY-9944 is given early in gestation, fetuses show holoprosencephalic type malformations, the less severe form of which is isolated pituitary agenesis (13). The occurrence of malformations was related to the timing and to the degree of maternal hypocholesterolemia (14, 15) below a threshold value of 30 mg/dl. BM 15766, another inhibitor of Δ^7 -dehydrocholesterol reductase, chemically unrelated to AY-9944, induces the same fetal malformations when given during the appropriate period of the gestation (16).

Abbreviations: Cholesterol, cholest-5-en-3 β -1; Δ^7 -dehydrocholesterol (7-DHC), cholesta-5,7-dien-3 β -ol; Δ^8 -dehydrocholesterol (8-DHC), cholesta-5,8-dien-3 β -ol; nortrienol, 19-nor-5,7,9(10)-cholestatrien-3 β -ol; epicoprostanol, 5 β -cholestan-3 α -ol; Δ^7 -lathosterol, cholest-7-en-3 β -ol; Δ^8 -lathosterol, cholest-8-en-3 β -ol; isodehydrocholesterol II (iDHC), cholesta-6,8-(9)-dien-3 β -ol; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; GLC-MS, gas-liquid chromatography coupled to mass spectrometry; TMS, trimethylsilyl; RRT, relative retention time; SLO, Smith-Lemli-Opitz syndrome.

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In the animal model the abnormal embryonic development occurs in a disturbed maternal sterol environment. In SLO, the cholesterol biosynthesis of the affected fetus is altered but the development takes place in the normal sterol environment provided by the normocholesterolemic heterozygous mother (17, 18). To delineate the respective role of the maternal environment and of the fetal deficit in the pathology, we assessed cholesterol metabolism in cultured embryos.

We studied the ability of cultured rat embryos to synthesize labeled cholesterol from ^{13}C -labeled precursors at early developmental stages. When embryo cholesterol biosynthesis was altered by AY-9944, the emergence of a characteristic pattern of aberrant sterols confirmed inhibition of Δ^7 -dehydrocholesterol reductase, and suggested the inhibition of Δ^7 - Δ^8 -sterol isomerase. In order to estimate the adaptation of the embryo's cholesterol metabolism to the environment, cultures were performed in normo- or hypercholesterolemic serum while cholesterol biosynthesis was inhibited by the drug. The data are discussed with regard to the ability of cultured embryos to substitute external cholesterol for the inhibited cholesterol synthesis pathway.

MATERIALS AND METHODS

Chemicals and reagents

[U- $^{13}\text{C}_2$]acetate and d-[U- $^{13}\text{C}_6$]glucose (isotopic enrichment 99%) were obtained from Euriso-Top (CEA Group, Saint-Aubin, F-91194). AY-9944 (*trans*-1,4-bis(2-chlorobenzylaminoethyl) cyclohexane dihydrochloride) was a generous gift from Ayerst Laboratories (New York, NY). Cholesterol, Δ^7 -dehydrocholesterol, epicoprostanol, Δ^7 -dehydrocholesterol, epicoprostanol, Δ^7 lathosterol were obtained from Sigma (St. Louis, MO).

Rat embryo culture

Female Wistar rats (Charles River France) were mated with males of the same strain. Rat embryos were explanted at 10 days +2 to 4 h within the intact visceral yolk sac and amnios and placed in a 25-ml round-bottom flask containing 2 ml of culture medium. Flasks were placed on a rotor in an incubator at 38°C. The gas phase was originally 5% O_2 , with 5% CO_2 in N_2 , and changed to increased O_2 concentrations at regular time intervals. When culture was performed for longer time than 48 h, embryos were transferred after 36 h of culture in 50-ml flasks containing 3 ml of medium. The culture medium was serum collected from adult Wistar rats, immediately centrifuged, pooled, and heat-inactivated. To increase the cholesterol concentration of serum, some adult rats were fed with additional choles-

terol to the normal diet (500 mg/kg suspended in olive oil given the evening and the next morning before killing). Cholesterol assayed by the enzymatic method was 60% higher than control (73 mg/dl vs. 45 mg/dl). These sera were used for embryo culture as cholesterol-rich medium.

Growth and morphological analysis of cultured rat embryos

Measurements of head length and the number of somites of cultured rat embryos were assessed as indicative of embryo's growth. Morphological scoring was performed according to Brown and Fabro (19). Data reported in Table 1 were analyzed statistically using the distribution free Wilcoxon test.

Lipid extraction and gas chromatography-mass spectrometry (GC-MS)

Embryos were scored, freed of amnios and yolk sac. Cephalic and caudal parts were separated at the level of the second branchial arch and transferred into chloroform-methanol 2:1 (vol/vol) 3 ml, with epicoprostanol as the internal standard (2 μg). Cephalic and caudal parts of embryos were pooled ($n = 2-6$) in order to get enough tissues for sterol analysis. Saponification of sterol esters was carried out in 0.5 N methanolic KOH (15 min at 56°C). Sterols extracted in hexane were derivatized to trimethylsilyl ether by BSTFA-TMCS 10% (Chrompack, Middleburg, Netherlands) (15 min at 56°C).

Sterol trimethylsilyl ethers were chromatographed on a bonded polar poly(ethylene glycol) capillary column (internal diameter 0.3 mm; length 30 m; film thickness 0.25 μm) [Supelcowax 10, Bellefonte, PA]. Two μl of TMS-sterol ethers stored in amber vials in *n*-heptan (100 μl) are injected in the splitless mode (Hewlett-Packard Automatic Sampler 6890 thermostated at 6°C). The conditions for chromatography are: inlet temperature 250°C, initial temperature 60°C, elution of the sterol derivatives along a linear gradient between 220 and 280°C (3°C/min). The helium as the carrier gas is pressured at 55 kPa to give a linear velocity of 30 cm/sec in this temperature range.

Sterol derivatives were detected by coupled gas chromatography-mass spectrometry (GC-MS) running in the electron impact mode (70 eV) with detection of the positive ion [Nermag R10-10 C, Rueil, France].

Identification of the sterols was obtained by comparison of the mass spectra collected with the data of the NIST library or published in the literature (7- and 8-DHC (4), nortrienol (5), Δ^7 and Δ^8 -lathosterol (20)).

Incorporation of ^{13}C -labeled precursors into cholesterol

Isotopic enrichment of cholesterol from ^{13}C -labeled precursors was followed by the ratio of ^{13}C -labeled cho-

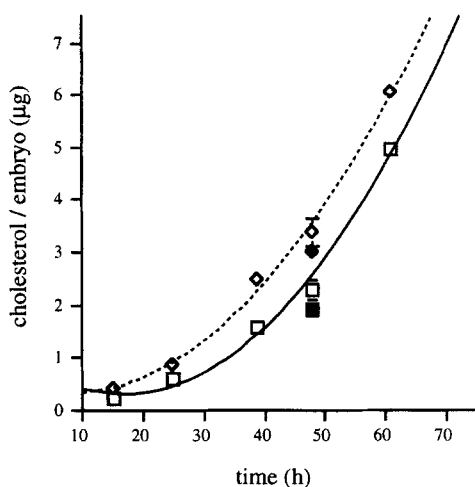


Fig. 1. Total cholesterol amount per embryo in the cephalic (\square) and the caudal (\diamond) part as a function of culture time. ^{13}C -labeled glucose (14 mM) was added to the culture medium except for control embryos (filled symbols) cultured in the absence of ^{13}C -labeled glucose. Error bars are SEM ($n = 5$).

lesterol to the sum of unlabeled and labeled cholesterol, i.e., the enrichment factor ($\text{e.f.} = \text{IL}/(\text{IU} + \text{IL})$) (21).

When $[\text{U-}^{13}\text{C}_2]\text{acetate}$ was used as precursor, ^{13}C -labeled cholesterol was estimated by the sum of the time-integrated ion currents for the even fragment-ions between m/z 370 to 380 and unlabeled cholesterol by the time-integrated ion current at m/z 368. When $[\text{U-}^{13}\text{C}_6]\text{glucose}$ was used as precursor, ^{13}C -labeled cholesterol was estimated by the sum of time-integrated ion currents for m/z 459 to 473 and unlabeled cholesterol by the time-integrated ion current for the molecular ion at m/z 458. ^{13}C -enrichment of cholesterol in the presence of labeled precursor was compared to cholesterol extracted from embryos cultured in the presence of unlabeled precursor to assess the natural isotopic abundance.

RESULTS

The amount of cholesterol in rat embryos increases during development in culture both in the cephalic and in the caudal parts (**Fig. 1**). In order to estimate *de novo* cholesterol biosynthesis in cultured embryos, the cholesterol precursors, acetate, or glucose uniformly labeled with ^{13}C , were added to the culture medium of the embryos explanted at day 10 of gestation. At the concentrations used (glucose 14 mM, acetate 7.9 μM), preliminary experiments demonstrated that these pre-

cursors did not affect the growth and the morphological scoring of cultured rat embryos compared to controls. These concentrations of precursors did not alter the final cholesterol amount per embryo (**Fig. 1**, filled symbols). The mass spectrum of the cholesterol extracted from the embryos cultured in the presence of ^{13}C -labeled glucose showed a pattern for ^{13}C -labeled ion-fragments in addition to the unlabeled fragment-ions at m/z 458 (M^+), 368 (458-silanol), 329 (458–129), 129 ($(\text{CH}_3)_3\text{SiO}-(\text{CHCH}_2\text{CH})$). ^{13}C -labeled fragment-ions show a Gaussian distribution between m/z 459 and 473, which results from the incorporation of a varying number of ^{13}C carbon atoms into cholesterol. When ^{13}C -labeled glucose was used as the cholesterol precursor, the most frequent incorporation was seven ^{13}C carbon atoms (at m/z 465), whereas it was two ^{13}C carbon atoms when labeled acetate was used as precursor.

The labeling of cholesterol was used to follow cholesterol synthesis in the cultured embryo. **Figure 2A** presents the enrichment factor (e.f.) (21) for cholesterol as a function of culture time with ^{13}C -labeled glucose as precursor. The labeled cholesterol incorporated 1 to 14 ^{13}C carbon atoms at a detectable level. Its total amount was estimated from the sum of the time-integrated ion-currents from m/z 459 ($\text{M} + 1$) to m/z 473 ($\text{M} + 15$). The unlabeled cholesterol was quantified by the time-integrated ion-current at m/z 458. ^{13}C -labeled cholesterol increased at a fast rate until 25 h of culture after which the labeling slowed down, while the amount of cholesterol in the embryos kept increasing at a fast rate (see **Fig. 1**). When ^{13}C -labeled acetate was used as the cholesterol precursor (**Fig. 2B**), continuous enrichment in ^{13}C -labeled cholesterol occurred for up to 72 h. The enrichment remained much lower in the cephalic part than in the caudal part of the embryo during the early culture times. The rate of incorporation from labeled acetate appeared to be especially low in the cephalic part of the embryo (**Fig. 2B**), whereas it proceeded with comparable fast rates in both parts of embryo from labeled glucose (**Fig. 2A**).

Embryo cholesterol synthesis can be altered by the presence of the Δ^7 -dehydrocholesterol reductase inhibitor AY-9944. Various doses of AY-9944 (0.01 up to 0.8 $\mu\text{g}/\text{ml}$) were added to the embryo culture medium, and the synthesis of cholesterol was followed for 48 h with ^{13}C glucose as precursor.

Figure 3 shows that the inhibition of ^{13}C -incorporation into cholesterol, assayed after 48 h of culture, was clearly observed from the lowest doses of AY-9944 added to the culture medium. At 0.01 $\mu\text{g}/\text{ml}$ AY-9944, the ^{13}C -labeled cholesterol diminished by 43%, and inhibition was complete at concentrations above 0.1 $\mu\text{g}/\text{ml}$. This demonstrates that AY-9944 was able to enter into the embryo tissues and to inhibit *in situ* cholesterol biosyn-

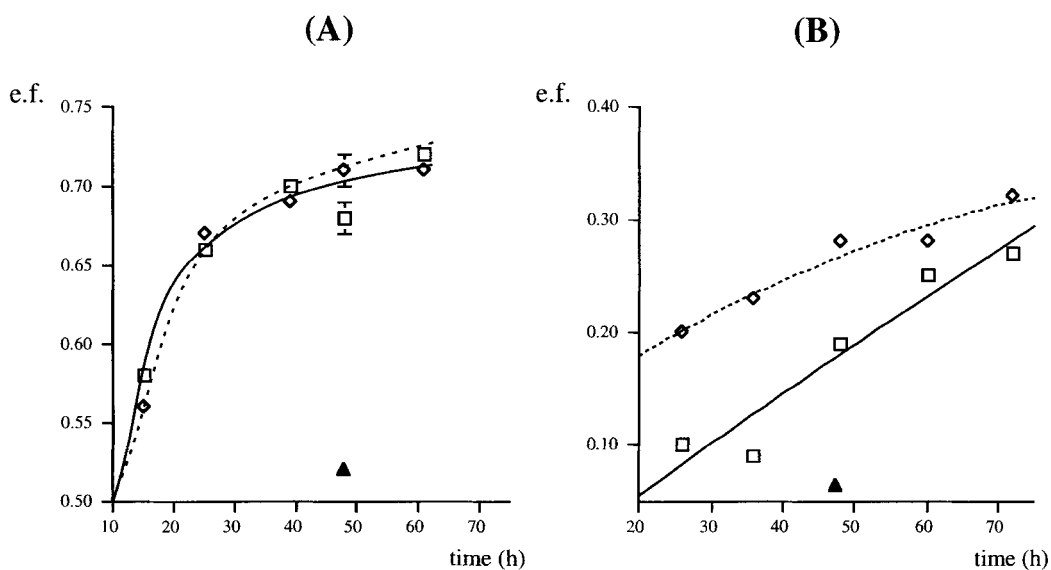


Fig. 2. Cholesterol ^{13}C -enrichment as a function of culture time, in cephalic (□) and caudal (◇) part of cultured rat embryos. Isotopic enrichment factor (e.f.) is estimated by the ratio of ^{13}C -labeled cholesterol over the sum of labeled and unlabeled cholesterol (21). A: ^{13}C -glucose was used as labeled precursor. ^{13}C -enriched cholesterol was estimated as the sum of ion currents between m/z 459 and m/z 473, and unlabeled cholesterol was estimated as the ion current at m/z 458. B: ^{13}C -acetate was used as the labeled precursor. ^{13}C -enriched cholesterol was estimated as the sum of ion currents between m/z 370 and m/z 380 at even intervals, and unlabeled cholesterol was estimated as the ion current at m/z 368. In A and B: (▲) represent the natural isotopic abundance in cholesterol extracted from embryo tissues after 48 h of culture.

thesis. The total amount of cholesterol of embryos cultured 48 h in the presence of AY-9944 (Fig. 4) was considerably decreased compared to controls. However, the total amount of sterols including cholesterol, Δ^8 -lathosterol, and Δ^7 -dehydrocholesterol remained unchanged up to AY-9944 0.1 $\mu\text{g}/\text{ml}$. The average cholesterol was 3.6 $\mu\text{g}/\text{embryo}$ for inhibitor concentrations between 0.01 and 0.1 $\mu\text{g}/\text{ml}$ versus 5.7 $\mu\text{g}/\text{embryo}$ for

controls. At higher concentrations of AY-9944 (0.5 or 0.8 $\mu\text{g}/\text{ml}$), the cholesterol decreased dramatically down to 1.6 $\mu\text{g}/\text{embryo}$. Although inhibition of cholesterol biosynthesis was complete with AY-9944 from 0.1 $\mu\text{g}/\text{ml}$, the embryo's cholesterol level remained higher at AY-9944 concentration of 0.1 $\mu\text{g}/\text{ml}$ than at higher concentrations (0.5–0.8 $\mu\text{g}/\text{ml}$). This result might be explained by the cholesterol influx from the culture

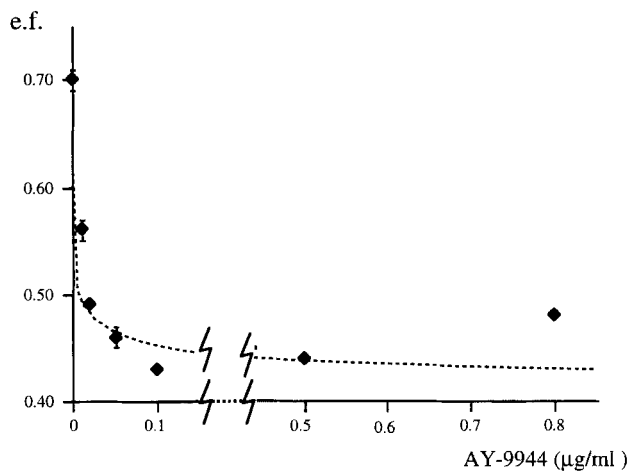


Fig. 3. Inhibition of ^{13}C -incorporation from ^{13}C -glucose in embryo cholesterol by the cholesterol synthesis inhibitor AY-9944 added to the culture medium. The ^{13}C -enrichment of embryo cholesterol is assayed after 48 h of culture as a function of AY-9944 concentration.

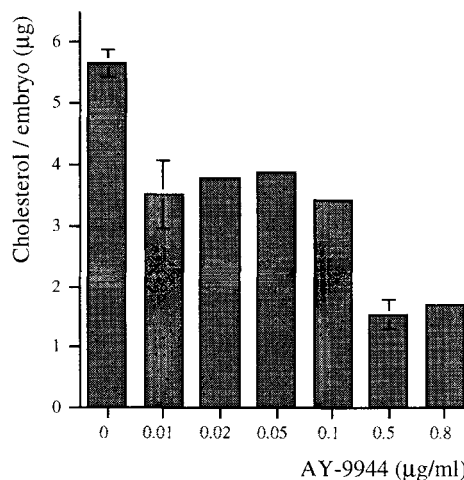


Fig. 4. Total tissue cholesterol assayed by GC-MS in embryos cultured for 48 h in a normocholesterolemic serum with the cholesterol synthesis inhibitor, AY-9944. Error bars are SEM.

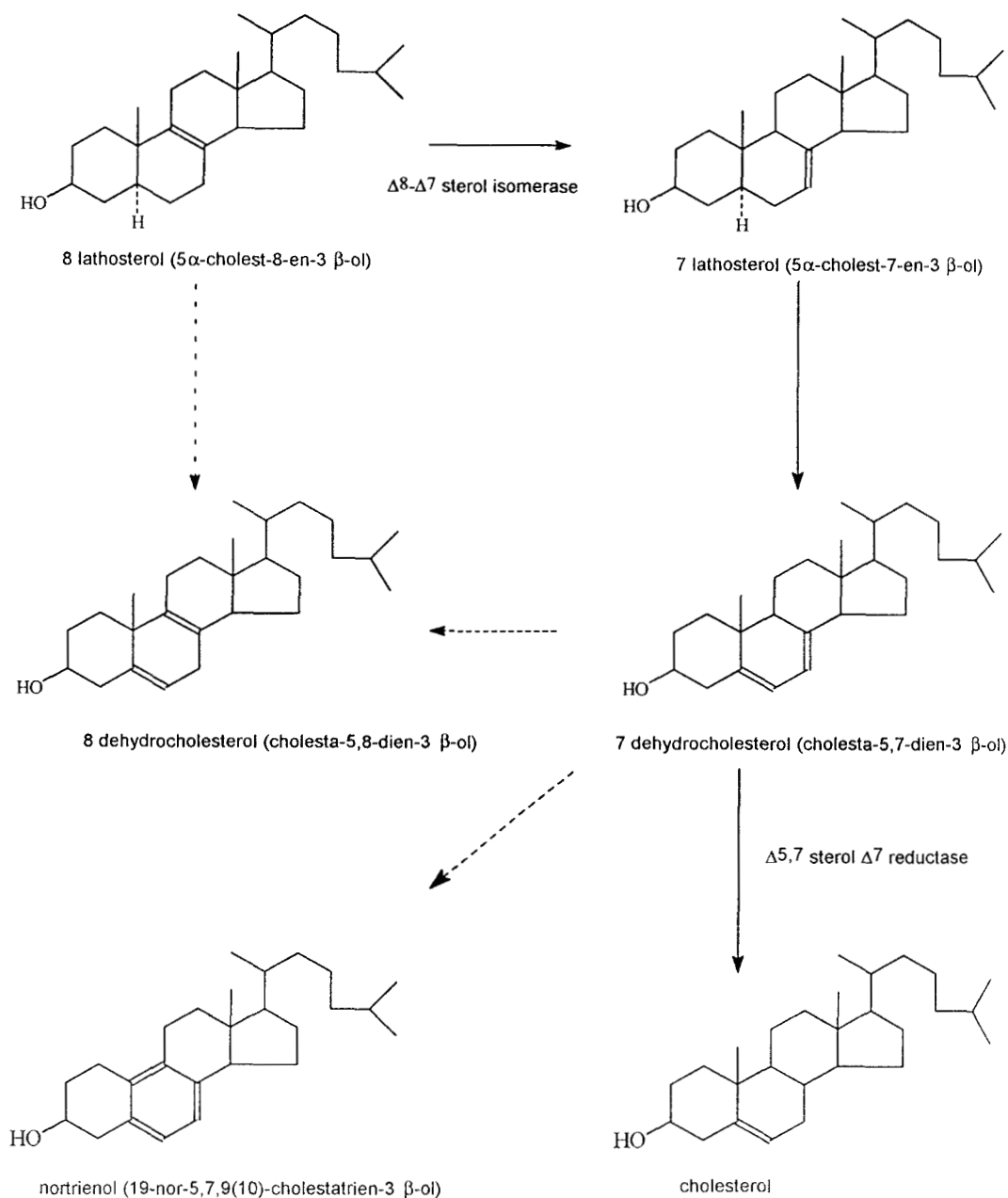


Fig. 5. Structures of the sterols observed in tissues of embryos cultured in the presence of the cholesterol synthesis inhibitors AY-9944.

medium into the embryo which compensated for the deficit in biosynthesis, a phenomenon that will be illustrated further.

The emergence of the aberrant sterols (Fig. 5) was detected in the tissues of rat embryos cultured 48 h in the presence of the inhibitor AY-9944. Δ^7 -Dehydrocholesterol, Δ^8 -dehydrocholesterol, Δ^8 -lathosterol, Δ^7 -lathosterol, nortrienol, and trienols were detected and identified by their GC retention times and mass

spectra (Fig. 6) as published previously (12). As the mass spectra of these abnormal sterols showed high labeling in [^{13}C]glucose-supplemented culture (data not shown) we concluded that they were newly synthesized in embryos, only after the addition of AY-9944. In untreated embryos, among aberrant sterols, only traces of Δ^8 -lathosterol, a by-product of the synthesis at step located upstream, were detected after 48 h of culture. Emergence of aberrant sterols and inhibition of chole-

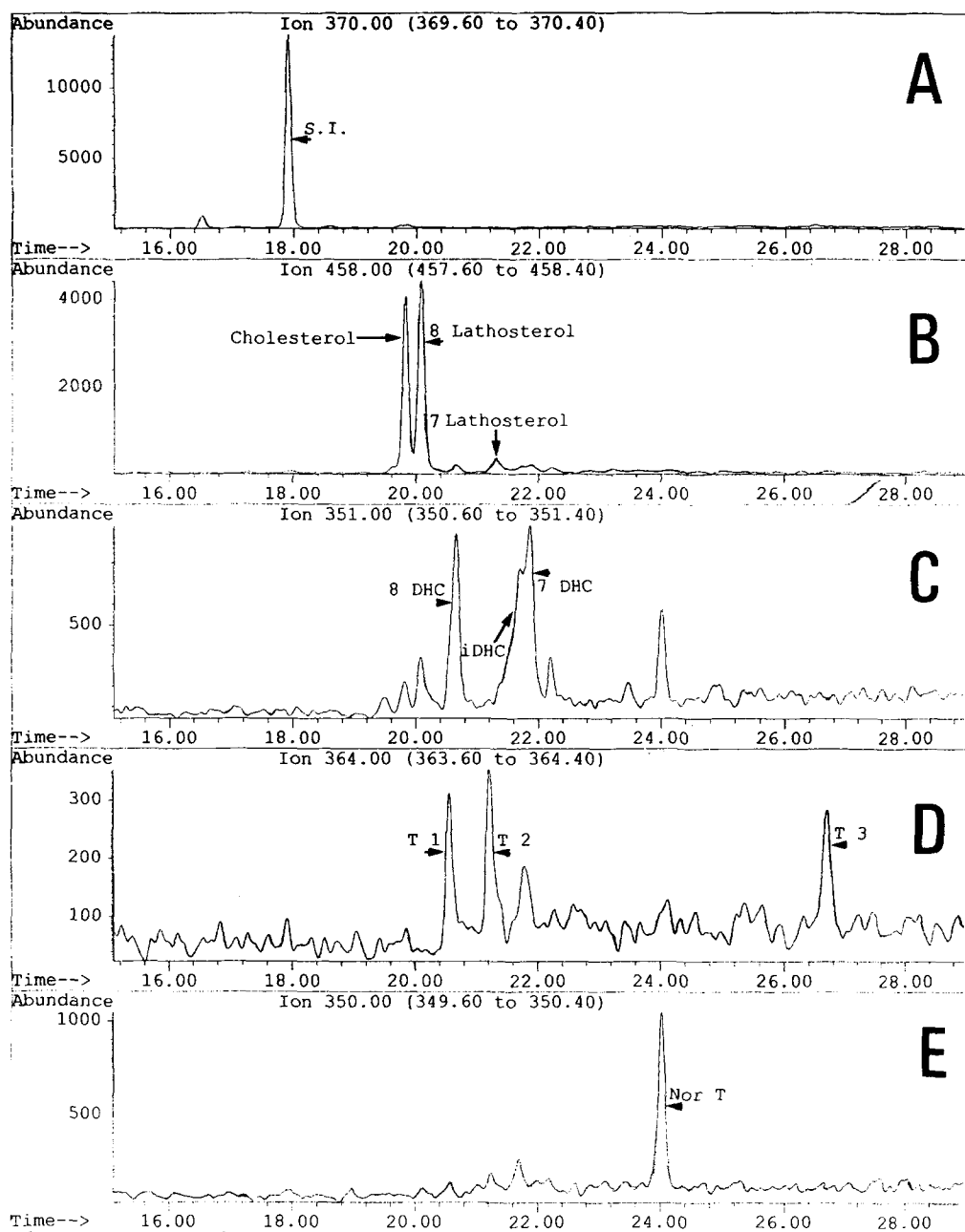


Fig. 6. Chromatograms of the neutral sterols extracted from rat embryo cultured in the presence of the cholesterol synthesis inhibitor AY-9944 (1 $\mu\text{g}/\text{ml}$). Peaks are identified by the ions currents: (A) at m/z 370: 5- β -cholestan-3 α -ol (SI, internal standard; RRT = 0.904); (B) at m/z 458: cholesterol (RRT = 1; RT = 19.83 min), Δ^8 -lathosterol (RRT = 1.013), and Δ^7 -lathosterol (RRT = 1.075); (C) at m/z 351: Δ^7 -dehydrocholesterol (8 DHC, RRT = 1.042), isodehydrocholesterol II (38) (iDHC, RRT = 1.094), and Δ^7 -dehydrocholesterol (7 DHC, RRT = 1.103); (D) at m/z 364: trienol I (T1, RRT = 1.036), trienol II (T2, RRT = 1.070), and trienol III (T3, RRT = 1.346); (E) at m/z 350: nortrienol (norT, RRT = 1.212).

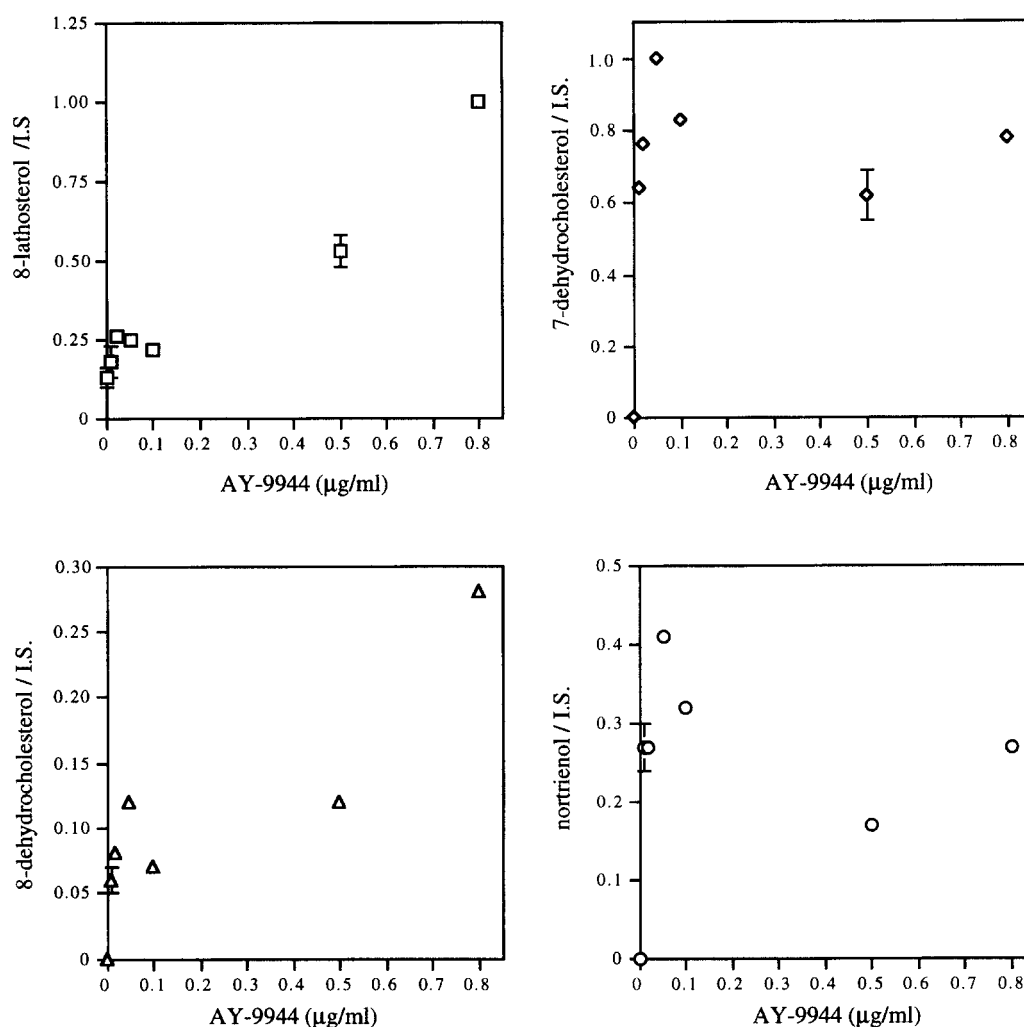


Fig. 7. Aberrant sterols in 48-h cultured rat embryos as a function of AY-9944 concentration. Sterols are quantified by the peak area under the characteristic ion current relative to internal standard (I.S., epicoprostanol); (□); Δ^8 -lathosterol; (◇); Δ^7 -dehydrocholesterol; (▲) Δ^8 -dehydrocholesterol; (○); nortrienol.

sterol biosynthesis by AY-9944 confirmed the inhibition at the Δ^7 -dehydrocholesterol reductase step in vitro, as previously described for adults in vivo (12). In addition, an important accumulation of Δ^8 -sterols was noted, suggesting inhibition of the Δ^7 - Δ^8 -isomerase step as previously described for AY-9944 in plants (22). The variation of sterols in embryos displayed two types of dependence as a function of AY-9944 concentrations (**Fig. 7**): Δ^7 -dehydrocholesterol and by-product nortrienol levels rose sharply with very low concentrations of AY-9944 to reach a plateau, whereas Δ^8 -sterols (Δ^8 -lathosterol and Δ^8 -dehydrocholesterol) accumulated gradually with increasing AY-9944 concentrations. In the presence of AY-9944 concentrations of 0.5 µg/ml or above, Δ^8 -lathosterol became as abundant as Δ^7 -dehydrocholesterol. The different rates of accumula-

tion in embryos of Δ^8 -sterols compared to Δ^7 -sterols suggests a higher sensitivity to the inhibitor AY-9944 of the enzyme Δ^7 -dehydrocholesterol reductase compared to Δ^8 - Δ^7 -isomerase.

Defects in the offspring have been described as a result of an alteration in cholesterol synthesis, both in the human SLO syndrome and in the animal disease model. **Table 1** presents the morphological scoring and the growth measurements of 48-h cultured rat embryos explanted at day 10 and treated with AY-9944. Embryos treated with AY-9944 below 0.1 µg/ml were similar to controls. From AY-9944 0.5 µg/ml, embryos showed growth and differentiation retardation (**Table 1, Fig. 8**). At AY-9944 0.5 µg/ml, both head length and morphological score were significantly decreased (respectively, $P < 0.02$ and $P < 0.04$), whereas the number of somites

TABLE 1. Growth measurements and morphological scoring (19) of explanted 10-day-old rat embryos after 48 h under various culture conditions, in normocholesterolemic rat serum (45 mg/dl) or in hypercholesterolemic rat serum (73 mg/dl)

Embryos	Culture in Normocholesterolemic Serum		
	Head length	Number of Somites	Morphological Score
	mm		
Control (n = 18)	2.3 ± 0.1	26.8 ± 0.7	47.2 ± 1.0
Treated with AY-9944			
0.01 µg/ml (n = 3)	2.1 ± 0.2	25.7 ± 3.3	45.9 ± 3.4
0.02 µg/ml (n = 3)	2.3 ± 0.1	25.7 ± 0.9	46.0 ± 1.9
0.05 µg/ml (n = 3)	2.5 ± 0.2	27.3 ± 1.2	48.0 ± 3.3
0.1 µg/ml (n = 3)	2.4 ± 0.1	28.0 ± 0	49.6 ± 1.0
0.5 µg/ml (n = 6)	1.9 ± 0.2 ^a	22.5 ± 1.9 ^a	39.4 ± 2.5 ^a
1.0 µg/ml (n = 12)	1.2 ± 0.2	indistinct	29.4 ± 2.9 ^d
	Culture in Hypercholesterolemic Serum		
Control (n = 5)	2.5 ± 0.2	28.8 ± 0.6	50.0 ± 0.4
Treated with AY-9944			
0.5–0.8 µg/ml (n = 5)	2.3 ± 0.1 ^b	27.8 ± 0.7 ^b	49.0 ± 0.7 ^b
1.0 µg/ml (n = 5)	1.7 ± 0.2 ^c	19.0 ± 1.7	32.9 ± 1.1 ^c

Data (mean ± SEM) were statistically analyzed with the Wilcoxon test.

^aRat embryos treated with AY-9944, 0.5 µg/ml, are compared with controls: $P < 0.02$, $P < 0.14$, $P < 0.04$, respectively, for head length, number of somites, and morphological score.

^bRat embryos treated with AY-9944, 0.5–0.8 µg/ml, cultured in hypercholesterolemic rat serum are compared to controls: $P < 0.79$, $P < 0.5$, $P < 0.69$, respectively, for head length, number of somites, and morphological score.

^cRat embryos treated with AY-9944, 1 µg/ml, cultured in hypercholesterolemic rat serum are compared to embryos treated with a similar concentration of AY-9944 but cultured in normocholesterolemic serum: $P < 0.04$, $P < 0.34$, respectively, for head length and morphological score.

^dScoring was achievable in 9 of 12 embryos.

appeared to be less altered ($P < 0.14$). Abnormalities in the cephalocaudal flexion were frequently observed. Maturation of olfactory, optic and otic systems was delayed (Fig. 8B) by AY-9944, but no defect was detected in the neural tube closure. Although telencephalic evaginations were poorly developed, no malformation of the holoprosencephalic type was seen by the external morphological observation. This malformation was described as the landmark of the teratogenic effect of the distal cholesterologenesis inhibitor given in early pregnancy (at day 4–6) but it is absent in rats treated later in vivo (15).

To study the effect of cholesterol level in the serum serving as the culture medium, embryos were cultured for 48 h in serum collected from rats recently fed with an excess of cholesterol added to the diet. Serum cholesterol levels increased by 60% under these conditions as compared to rats receiving a normal diet. Rat embryos cultured in high cholesterol medium showed a 13% decrease in the ¹³C enrichment of cholesterol as a result of the inhibition of the biosynthetic pathway. As the total cholesterol remained similar to the amount in embryos cultured in standard serum (6.39 vs 5.7 µg/embryo), this showed that the exogenous cholesterol could enter embryo tissues and inhibit synthesis. Exoge-

nous cholesterol substituted for the cholesterol normally biosynthesized by the embryo.

The addition of AY-9944 to cholesterol-rich medium led to the emergence of aberrant sterols and to a decrease in cholesterol in embryonic tissues. However, the amount of these aberrant sterols was significantly lower compared to the controls cultured in normocholesterolemic serum (Fig. 9). In cholesterol-supplemented culture medium, for AY-9944 0.8 µg/ml, the aberrant sterols were decreased by 50% whereas the cholesterol was increased by 30% as compared to non-supplemented medium. These experiments showed that excess cholesterol in the culture medium was able to partially suppress the metabolic alterations induced by AY-9944 in embryos.

Table 1 shows that this partial correction of the sterol pattern in a cholesterol-rich culture was sufficient to prevent the occurrence of abnormalities in growth and morphological features of rat embryos. The statistical analysis of morphological data showed that the embryos cultured in hypercholesterolemic serum were not affected up to AY-9944 0.8 µg/ml (n = 5) as compared to untreated controls ($0.5 < P < 0.79$). The conditions of embryos exposed to the highest concentration of AY-9944 (1 µg/ml) were also largely improved in chole-

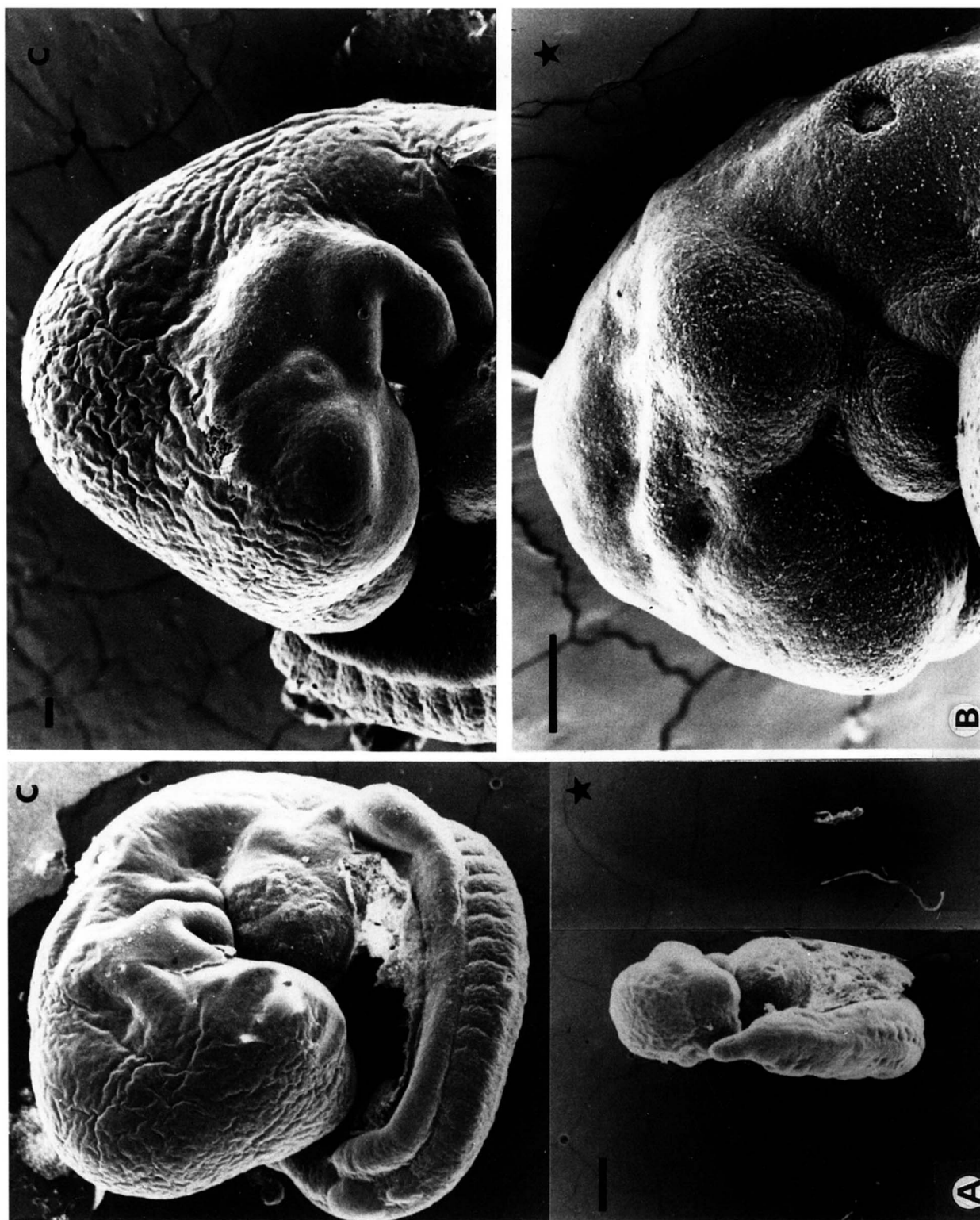


Fig. 8. Scanning electron microscopy images of control and AY-9944-treated embryos after 48 h of culture. A At low magnification the growth retardation is observed for AY-9944 (0.8 $\mu\text{g}/\text{ml}$)-treated embryo (*) versus control (c). Scale bar: 250 μm . B Detailed cephalic view of AY-9944 (1 $\mu\text{g}/\text{ml}$)-treated embryo (*) versus control (c). Differentiation retardation is evident: otic vesicle is still open, maxillary process is overdeveloped, there is no olfactory pit visible, and eyes are underdeveloped. Scale bar: 100 μm .

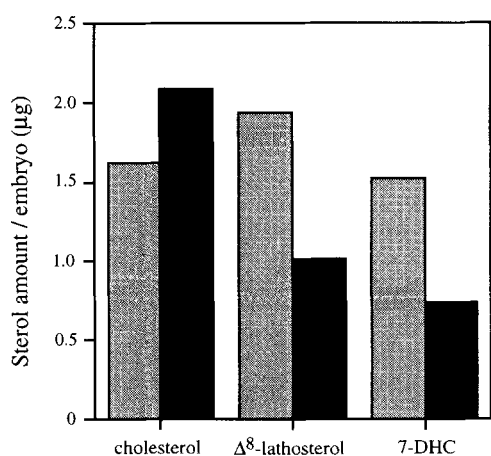


Fig. 9. Comparison of cholesterol, Δ^8 -lathosterol, Δ^7 -dehydrocholesterol (7-DHC) after 48 h in rat embryos cultured in normocholesterolemic (45 mg/dl) (▨) or hypercholesterolemic (73 mg/dl) (■) in the presence of the cholesterol synthesis inhibitor AY-9944 (0.8 μ g/ml).

terol-rich culture ($n = 5$), as regard to the head length ($P < 0.04$ versus treated embryos cultured in normocholesterolemic serum), but not as regard to the morphological score which remained significantly low.

DISCUSSION

The amount of cholesterol in tissues of rat embryos explanted at day 10 increases during development in vitro. The embryo's cholesterol is derived from that of rat serum in the culture medium or from de novo biosynthesis. Incorporation of ^{13}C carbon atoms into cholesterol from labeled precursors added to the culture medium demonstrates that cholesterol biosynthesis is active in embryos. As a function of time, the rate of ^{13}C -enrichment is initially faster with glucose (14 mM) serving as the precursor than with acetate (7.9 μM). However, the rate obtained with glucose decreases after 1 day in culture while it remains steady for 72 h with acetate. Labeled glucose is presumably used more efficiently than acetate for cholesterol synthesis, probably due to a more favorable ratio of labeled to unlabeled acetyl groups. This could result from the high concentration of glucose and its fast conversion to acetylCoA, as compared to the slow rate of activation of acetate. A consequence of different metabolization rates of the precursors is that mass spectra of de novo synthesized cholesterol show a mean incorporation of seven ^{13}C carbon atoms when glucose is the labeled precursor instead of only two ^{13}C carbon atoms with acetate.

The present study shows that acetate is used as the

preferential cholesterol precursor in the caudal part compared with the cephalic part, whereas glucose is used equally in both parts. This suggests that during rat embryonic development, the activation of acetate for cholesterologenesis occurs at a different rate within the different tissues. This allows us to observe that the redistribution and remixing of the produced cholesterol is negligible between the different parts of the embryo. Noticeably in fetal human brain, glucose was also shown to be used preferentially over acetate for cholesterol biosynthesis (23).

From preimplantation stages (24) and early post-implantation stages on, rat embryos are able to synthesize cholesterol. Accordingly, HMG-CoA reductase, the committed step in cholesterol biosynthesis, is widely expressed in embryonic tissues at these developmental stages (25). In vivo, rat embryos at days 12–13 have been found to be 60–70% dependent on the maternal cholesterol supply (26). Under culture conditions, embryos adapt to a different environment. The cholesterol biosynthetic rate has been found to be nearly 2-fold higher in culture (from radioactive labeled acetate) than in utero for equivalent developmental stages (27). The present study shows also that when the culture medium was enriched in cholesterol, the isotopic enrichment of embryo cholesterol was decreased, which demonstrates that exogenous cholesterol has entered into the embryo and exercised a backward inhibition of cholesterol anabolism. We conclude that cholesterol de novo synthesis rate is probably modulated as regard to the external supply.

AY-9944 is an inhibitor of Δ^7 -dehydrocholesterol reductase and its teratogenicity has been demonstrated in vivo (10). In higher plants (*Zea mays*) and in *Chlorella*, AY-9944 is also able to inhibit Δ^8 - Δ^7 -sterol isomerase, upstream in the cholesterol biosynthesis, depending on the conditions of treatment (22). When AY-9944 was added in vitro, the decrease of the cholesterol ^{13}C -enrichment in cultured rat embryos demonstrated the inhibition of cholesterol synthesis. This was observed at concentrations of AY-9944 that were very much lower than those usually given to adult pregnant rats for the purpose of experimental pathology (>75 mg/kg). There is a noteworthy discrepancy between the complete inability of embryos to synthesize cholesterol in the presence of AY-9944 (the synthesis is suppressed from 0.1 $\mu\text{g}/\text{ml}$) and the relatively preserved cholesterol content of the embryos (-37% at 0.1 $\mu\text{g}/\text{ml}$, but -72% at 0.5 $\mu\text{g}/\text{ml}$). At AY-9944 0.1 $\mu\text{g}/\text{ml}$, the compensation in cholesterol content should originate from the alternative source of cholesterol, that is the influx from the culture medium. AY-9944 at higher concentrations, would make the embryos unable to import the exogenous cholesterol bound to lipoproteins, ex-

plaining the deficit of the compensation. Interestingly, the inhibition of low density lipoprotein (LDL) internalization and the subsequent degradation by human fetal lung fibroblasts in culture has been previously shown to be inhibited by AY-9944 at concentrations above 1.5 $\mu\text{g}/\text{ml}$ (28). This suggests that AY-9944, in addition to the inhibition of the embryonic cholesterol synthesis, would also interfere with the compensatory influx of exogenous lipoprotein cholesterol.

The pattern of aberrant sterols in embryo tissues confirmed the inhibition by AY-9944 of Δ^7 -dehydrocholesterol reductase. Indeed, we observed the same metabolites identified previously in adult rats treated with BM 15766 (18, 12) or in the human defect of the enzyme, the Smith-Lemli-Opitz syndrome: Δ^7 -dehydrocholesterol, Δ^8 -dehydrocholesterol (4), and nortrienol (5). These abnormal sterols, in the presence of labeled glucose, display a very high ^{13}C -enrichment, meaning that they are not synthesized prior to the addition of the inhibitor. A noteworthy difference with adult rats treated with AY-9944 (12) is the emergence of quantities of Δ^8 -lathosterol. Δ^7 -lathosterol, the isomer produced by the conversion of Δ^8 -lathosterol, is the precursor of Δ^7 -dehydrocholesterol and is commonly found in the amniotic fluid of normal human fetus. It was shown to be increased in tissues and amniotic fluid during the Smith-Lemli-Opitz syndrome (29). The present study shows that the accumulation rates of Δ^7 -dehydrocholesterol and of Δ^8 -lathosterol as a function of AY-9944 concentration are very different, suggesting that two different enzyme steps are targeted by the inhibitor. Δ^7 -Dehydrocholesterol reductase is inhibited by low concentrations of AY-9944, explaining that Δ^7 -dehydrocholesterol accumulates rapidly. By contrast, Δ^8 -lathosterol increases gradually with AY-9944 concentration, suggesting a higher IC_{50} for Δ^8 - Δ^7 -lathosterol isomerase as compared with Δ^7 -dehydrocholesterol reductase. Previous studies have suggested that Δ^8 - Δ^7 conversion could become rate-limiting under conditions of accelerated cholesterol production (20). Δ^8 - Δ^7 -sterol isomerase inhibition could significantly reinforce the sterol reduction in AY-9944-treated embryos.

The teratogenic activity of the "distal" cholesterol synthesis inhibitor AY-9944 was evidenced 30 years ago (10). Given to rats on the fourth day of gestation, it induces fetal malformations of the holoprosencephalic type. When AY-9944 is added to the culture medium, the abnormalities of embryos are mainly growth and differentiation retardation. Because occurrence of holoprosencephaly is determined before day 10 of the gestation (30), it is not surprising that older rat embryos cultured in a serum containing AY-9944 do not present such malformations but mostly show growth retardation. In vivo the growth retardation is superimposed on the characteristic defects determined early as a conse-

quence of the permanent metabolic alteration. In the human SLO syndrome, as in the animal model, growth retardation constantly accompanies the characteristic craniofacial and neural defects.

The induction by AY-9944 of embryonic morphological alterations displays a threshold at 0.5 $\mu\text{g}/\text{ml}$ when embryos are cultured in normocholesterolemic serum. The threshold is shifted at 1 $\mu\text{g}/\text{ml}$ when embryos are cultured in hypercholesterolemic serum. Similarly, in vivo, a threshold value of the maternal hypocholesterolemia (30 mg/dl) was described in the Wistar rat for the occurrence of pituitary agenesis in fetuses (14). This threshold can be shifted in treated rats by feeding them with a cholesterol-rich diet (31). Together with the fact that exogenous cholesterol can enter the embryo tissues, these findings suggest that the teratogenic activity of distal cholesterol synthesis inhibitors results from an imbalanced inhibition of fetal cholesterol biosynthesis by the compensatory cholesterol influx from the mother.

Apolipoprotein B, expressed in the yolk sac of rodents, plays a critical role in maternal-fetal lipid transport (32). Therefore, in apoB knockout embryos, tissue cholesterol level is decreased. Previously, mice were shown to be resistant to the teratogenic activity of cholesterol synthesis inhibitors such as BM 15766. A targeted apoB gene modification (33) made them become sensitive to the teratogenic effect of this drug (34). This recent information suggests that transfer of cholesterol from yolk sac to embryo requires appropriate apoB synthesis and secretion, especially under conditions where the synthesis of cholesterol in the embryo is depressed.

Cholesterol from the culture medium corrects the metabolic abnormalities, not only by increasing the amount of cholesterol, but also by decreasing the amount of aberrant sterols. Aberrant sterols are putative competitors of the physiological activities assumed by cholesterol, and their decrease participates in the normal development of treated embryos. The role of these aberrant sterols in the offset of the malformations remains until now questionable.

In vivo, cholesterol supplementation or hypercholesterolemia-provoking diets given to pregnant rats treated with AY-9944 have already been shown to be efficient in preventing the occurrence of the fetal malformations (11, 31). In vivo, AY-9944 alters cholesterol metabolism of the pregnant rat (e.g., the embryonic environment). In vitro, it affects only the cholesterol biosynthesis of the embryo. We have taken opportunity of the in vitro model where the sterols supplied in the culture medium can be modified independently to observe directly the effect of a compensatory increase of cholesterol influx in treated embryos. We found that exogenous cholesterol complements the cholesterol

supply to the embryo tissues and down-regulates the biosynthesis of aberrant sterols.

Embryos whose last step in cholesterol biosynthesis is inhibited are dependent exclusively on the maternal supply. In SLO, heterozygous mother have normal cholesterol levels (17, 18) but severe perturbations are observed in the affected fetus. The normal maternal supply is apparently not sufficient under these circumstances to overcome the embryo defect. The permeability of placenta to placenta to cholesterol has been confirmed (35), but the blood-brain barrier that develops after organogenesis remains to impede cholesterol diffusion to the human fetal brain. This matter has previously been much debated (23, 36, 37). We do not know yet whether dietary supplementation to pregnant mothers could overcome the malformations seen in human offspring. One requirement would be that the supply of cholesterol from the mother is not delayed by the placental development. The results obtained in rats suggest a favorable effect of an early dietary cholesterol supplementation.

Finally, this leads us to suggest that the SLO condition could result from embryo cholesterol synthesis deficit exceeding the possible compensatory supply of lipid from the mother. This combination of causes could eventually explain the highly variable clinical features and severity of the syndrome. It prompts also additional studies of lipid transfer between mother and affected fetus. ■

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