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Investigation of the Biogenetic Reaction Sequence of Cholesterol in Rat Tissues, Through Inhibition with AY-9944

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Abstract

An inhibitor of Δ^7-reductase, AY-9944 (trans-1,4-bis(2-dichlorobenzylaminomethyl cyclohexane dihydrochloride), was used to investigate the last steps of cholesterol formation in brain and liver of adult and newborn rats. The accumulation of different sterols in the two tissues of the same animals was observed. $\Delta^{5,7}$-Cholestadien-3β-ol, $\Delta^{7,24}$-cholestadien-3β-ol and $\Delta^{5,7,24}$-cholestatrien-3β-ol, which are not present in detectable amounts in control brains, were identified in brains of growing rats treated with AY-9944. An accumulation of $\Delta^{5,7}$-cholestadien-3β-ol only was found in adult rat tissues.

These differences in sterol accumulation are discussed in relation with the possible in vivo pathways of cholesterol biosynthesis.

Introduction

MANY STEROLS, either found in mammalian tissues or shown to be incorporated into cholesterol, are considered as possible intermediates in the conversion of lanosterol to cholesterol (1–9). Avigan and Steinberg (10) suggested two possible series of sterol precursors of cholesterol, one with a double bond in the side chain between the carbon atoms in position 24 and 25 and the other with a saturated side chain. Uncertainty still exists on the predominant physiological sequence leading to cholesterol. The reduction of Δ^{24}, an obligate step in cholesterol formation, is probably irreversible (11) and it is independent of structure of the the steroid nucleus (11,12); it seems to occur predominantly, at least in adult rat liver, at the stage of C 28 sterols (13).

In recent years pharmacological means have been used to investigate this problem. Drugs able to block selectively the Δ^{24}-reductase, such as Triparanol (14), 20,25-diazacholesterol (15,16), 22,25-diazocholestanol (17,18), induce in tissues an accumulation of desmosterol (5,24-cholestadien-3β-ol), the last possible cholesterol precursor with a double bond in the side chain.

A specific inhibitor of the 7-dehydrocholesterol-Δ^7-reductase has also been described. This compound, trans 1-4-bis (2-dichlorobenzylaminomethyl) cyclohexane dihydrochloride (AY-9944), was shown to inhibit this enzyme at low concentrations (19,20) and to be 100 times more active than Triparanol in this respect (21). In agreement with these data, 7-dehydrocholesterol was detected in plasma and liver (19, 22) of rats treated with AY-9944. The simultaneous administration of Triparanol and AY-9944 induces, in pig liver, an accumulation of 5,7,24-cholestatrien-3β-ol (23), a sterol already considered as a possible precursor of cholesterol (7,24).

It is interesting to observe that different cholesterol precursors are present in different mammalian tissues: 7-dehydrocholesterol in the small intestine of guinea pig (9), methostenol in several tissues of rat (1), desmosterol in brain of growing rat (25,26) and in fetal brain of guinea pig and man (26). A large number of sterol precursors of cholesterol were also found in skin of normal adult rat (27).

Such observations suggest tissue-dependent differences in the activity of enzymatic systems involved in the conversion of lanosterol to cholesterol; these findings also indicate that the use of specific enzyme inhibitors may be helpful in elucidating the physiological sequence leading to cholesterol, particularly when the effects on different tissues are compared.

In our experiments an attempt has been made to obtain evidence for possible differences in sterol composition of tissues in growing and mature rats after treatment with AY-9944.

TABLE I

Steroid Number Contributions for Functional Groups on Nonselective (SE-30) and Selevtive Phase (CNSi)

Functional groups	Steroid parent	SE-30		CNSi
		a	b	c
A/B cis	Coprostane/cholestane	—0.2	—0.2	.....
A/B cis	Coprostanol/cholestanol	—0.3	—0.4	—0.55
3 β-ol (eq.)	Cholestanol/cholestane	2.4	2.4	5.90
Δ^5	Cholesterol/cholestanol	—0.1	—0.1	0
Δ^7	Lathosterol/cholestanol	0.4	0.4	0.60
$\Delta^{5,7}$	7-Dehydrocholesterol/ cholestanol	....	0.3	0.95
Δ^8	Zymosterol/ cholestanol + Δ^{24}	....	0.7	0.70
Δ^{24}	Desmosterol/cholesterol	0.3	0.3	0.70
4,4',14-methyl	Lanosterol/zymosterol	....	0.8	0.30

[a] Vandenheuvel, W. J. A., and E. C. Horning, Biochim. Biophys. Acta *64*, 416, 1962.
[b] Our data—conditions: 2 m × 3 mm glass spiral tube, 1% SE-30 on 100–140 mesh Gas-Chrom P; 211C; N_2 1.7 kg/cm²; cholestane time 18.1 min.
[c] Our data—conditions: 4 m × 3 mm glass spiral tube, 1% CNSi on 100–120 mesh Gas-Chrom P; 213C; 1.8 kg/cm²; cholestane time 7.0 min.

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TABLE II

Relative Retention Times to Cholestane of Sterols Present in Newborn Rat Brain and Liver After Treatment with AY-9944

	Free sterols	TMSi derivatives
Brain		
1st sterol	5.08	2.15
2nd sterol	6.09	2.53
3rd sterol	6.46	2.67
4th sterol	7.07	3.03
5th sterol	7.77	3.18
Liver		
1st sterol	5.08	2.15
2nd sterol	5.39	2.24
3rd sterol	6.46	2.67

Experimental conditions as in Table I (CNSi).

Materials and Methods

Experiments have been carried out in vitro and in vivo using normal adult, pregnant and newborn rats. Drugs employed were: AY-9944 (trans-1,4-bis (2-dichlorobenzylaminomethyl)-cyclohexane dihydrochloride, kindly supplied by Dr. D. Dvornik, Ayerst Labs., Montreal, Canada; and Triparanol [1-p-(β-diethylaminoethoxy)-phenyl-1-(p-tolyl)-2-(p-cholorophenyl) ethanol] supplied by Wasserman Co., Milan, Italy.

The Experimental Conditions

Treatment of Adult Animals with AY-9944. Sprague-Dawley male rats weighing 150 ± 30 g were given AY-9944 in water solution, at the daily doses of 2, 5 and 25 μmoles/kg by gastric intubation, while the controls received equal volumes of distilled water. After 1, 4, 7 and 28 days of treatment, the animals, fasted for 8 hours, were sacrificed by decapitation and blood, brain, aorta and liver rapidly removed.

Treatment of Adult Animals with AY-9944 and Triparanol. Adult male rats of the same strain and weight were used.

Animals were treated by gastric intubation at the following doses: AY-9944, 5 μmoles/kg/day (dissolved in distilled water); Triparanol, 50 μmoles/kg/day (dissolved in olive oil).

The animals were divided in 4 groups: vehicles alone for 5 days (1st group); Triparanol for 5 days (2nd group); AY-9944 for 4 days (3rd group); the first day of treatment, rats were given Triparanol alone, during the following 4 days they received Triparanol as well as AY-9944 (4th group).

At the end of treatment all animals were fasted for 8 hr, sacrificed by decapitation and brain and liver rapidly removed.

Treatment of Pregnant Rats. Pregnant rats were given 5 μmoles of AY-9944/kg/day by stomach intubation, during, respectively, the 3 and 6 days preceding delivery. Newborn rats were sacrificed by decapitation during the first day of life and brain and liver removed.

Tissue Preparation. Blood and tissues obtained from sacrificed animals were saponified in KOH 10% in ethanol-water 1:1 for 90 min at 70C. The unsaponifiable material was extracted with low-boiling petrol ether. The ether extract, washed with distilled water and dried over anhydrous Na_2SO_4, was used for qualitative and quantitative sterol analysis.

7-Dehydrocholesterol-Δ⁷-Reductase Determination. In liver from adult animals receiving 2 μmoles/kg/day of AY-9944 H, 7-dehydrocholesterol-Δ⁷-reductase activity was measured according to the method of Kandutsch (28) as previously described in detail (21).

Sterol Determination. Determination of total sterols in plasma and tissues was carried out according to Zlatkis et al. (29).

This colorimetric procedure gives, at 560 μ (corresponding to the maximal light absorption for cholesterol), an optical density for 7-dehydrocholesterol equal to one fifth of that of cholesterol. Therefore sterol concentrations were calculated from the colorimetric data corrected on the basis of cholesterol and 7-dehydrocholesterol percent composition of each sample, determined by gas chromatography.

Total sterol concentrations in tissues of newborn rats have been determined directly by gas chromatography adding to the samples known amount of 5-androsten-3β-ol-17-one (Southeastern Biochem. Inc., Georgia), as an internal standard.

Gas Chromatographic Determination of Sterols. The gas chromatograph used was Factovap Model C (C. Erba, Italy), equipped with a flame ionization detection system. The columns were glass spiral tubes (4 m × 3 mm and 2 m × 3 mm). Packing materials were prepared according to Horning et al. (30). The support was Gas-Chrom P 100–120 mesh (Applied Science Laboratories, Inc.), the stationary phases were SE-30 (dimethyl-siloxane polymer, General Electric Co.) and CNSi (cyanoethyl methyl silicone, General Electric Co.). All columns were coated with 1% of stationary phase. Working conditions are stated in Table I.

The SE-30 column was used for identification purposes only, CNSi column for routine work as well as for identification. The CNSi column showned 5,000 theoretical plates for cholesterol. This high column efficiency is required to separate sterols with very similar chemical structure, accumulated in the tissues after the above-mentioned treatments. Sterols were

TABLE III

Steroid Number of Newborn Rat Brain and Liver Sterols on a Selective Stationary Phase (CNSi)

Tissue	Steroid number	Reference compounds
Brain[a]		
1. Sterol A	32.90	Cholesterol
2. Sterol B	33.60	Desmosterol
3. Sterol C	33.80	7-Dehydrocholesterol
4. Sterol D	34.15	
5. Sterol E	34.50	
Liver[a]		
1. Sterol F	32.90	Cholesterol
2. Sterol G	33.10	
3. Sterol H	33.80	7-Dehydrocholesterol

[a] Sterol patterns after treatment with AY-9944.

TABLE IV

Steroid Number of Newborn Rat Brain and Liver Sterols on a Nonselective Stationary Phase (SE-30)

Tissue	Steroid number	Reference compounds
Brain		
1. Sterol A	29.3	Cholesterol
2. Sterol B + C	29.7	Desmosterol + 7-dehydrocholesterol
3. Sterol D + E	30.0	
Liver		
1. Sterol F	29.3	Cholesterol
2. Sterol G[a]	29.3	
3. Sterol H	29.7	7-Dehydrocholesterol

[a] It shows as a shoulder of cholesterol peak.

TABLE V

7-Dehydrocholesterol-Δ⁷-Reductase Activity in Liver Homogenates of Rats Treated Orally with AY-9944 (2 μmoles/kg/day)

Treatment		7-Dehydrocholesterol metabolized (μmoles /g fresh tissue/hr)	Percentage of inhibition
Controls	(5)	0.890 ± 0.13	....
1 dose		0.070	92
1 dose		0.160	82
1 dose		0.160	82
1 dose		0.100	89
1 dose		0.0	100
1 dose		0.0	100
4 doses	(5)	0.0	100

TABLE VI

Cholesterol and Total Sterol Content (mg/100 g fresh tissue or mg/100 ml) of Adult Rat Tissues After Oral Treatment with AY-9944 for 7 Days

AY-9944 μmoles/kg/day	2			5			25		
Tissue		Cholesterol	Total sterols		Cholesterol	Total sterols		Cholesterol	Total sterols
Serum controls	(5)	75 ± 2	75 ± 2	(5)	76 ± 8	76 ± 8	(5)	75 ± 2	75 ± 2
Treated	(5)	65 ± 5 [a]	67 ± 5 [a]	(4)	45 ± 2 [b]	60 ± 4 [b]	(5)	15 ± 4 [b]	35 ± 9 [b]
Liver controls	(5)	304 ± 19	304 ± 19	(5)	306 ± 22	306 ± 22	(5)	304 ± 28	304 ± 28
Treated	(5)	320 ± 23	348 ± 23	(4)	206 ± 15 [b]	276 ± 12	(5)	105 ± 11 [b]	367 ± 38
Aorta controls				(5)	152 ± 7	152 ± 7	(5)	182 ± 6	182 ± 6
Treated				(4)	120 ± 8 [b]	135 ± 9 [b]	(5)	122 ± 6 [b]	154 ± 9 [b]
Brain controls				(10)	1120 ± 211	1120 ± 211	(10)	1150 ± 111	1150 ± 111
Treated				(4)	1060 ± 75	1093 ± 75	(5)	1050 ± 21	1120 ± 20

[a] $p < 0.05$.
[b] $p < 0.001$.

run both in free form and as trimethyl-silylether derivatives (TMSi), prepared according to Luukkainen et al. (31). Samples were dissolved in CS_2 for the gas chromatographic analyses.

Identification of the Sterols

The identification of the sterols present in biological samples was carried out using a gas-chromatographic procedure, according to Vandenheuvel and Horning (32) on nonselective (SE-30) and selective (CNSi) stationary phases.

Steroid number (SN) of some available sterols, known to be precursors of cholesterol, were determined both on SE-30 and CNSi, and SN-contributions for functional groups were then established (Table I).

The clear decrease in relative retention times to cholestane, shown by the compounds under investigation, when run as TMSi derivatives on CNSi, suggests the presence of alcoholic functions. Their gas-chromatographic behaviour was that of sterols (Table II). Whenever possible, sterol identification was achieved by using known reference compounds. In this way we could demonstrate that adult rat tissues contain cholesterol and 7- dehydrocholesterol after AY-9944 treatment, and cholesterol and desmosterol after Triparanol treatment.

In the case of newborn rats, liver and brain controls contain only cholesterol and cholesterol and desmosterol, respectively. After administration of AY-9944, other sterols, which do not correspond to any of the available reference compounds, were observed. A tentative identification of these unknown sterols is suggested on the basis of their gas-chromatographic behavior. The SN of the newborn rat brain and liver sterols was calculated from data obtained with CNSi, which has selective retention effects for the functional groups (Table III). The three first peaks show SN corresponding to those of cholesterol, desmosterol and 7-dehydrocholesterol, respectively. The last two peaks (D and E) are due to unknown sterols with SN 34.15 and 34.50, respectively. When brain sterols are run on SE-30, a stationary phase able to separate compounds mainly on the basis of molecular size with small retention effects for functional groups, only three peaks are obtained (Table IV).

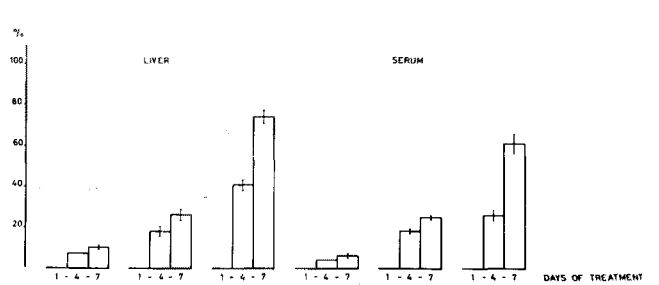

FIG. 1. 7-Dehydrocholesterol content in adult rat tissues after AY-9944 treatment (as percentage of total sterols ± SE).

The third peak on SE-30 (SN 30.0) corresponds to the sterols D and E separated on CNSi and which are not resolved on this phase. Of these two sterols, E is quantitatively the most important and attempts have been made to identify its structure. An SN 30.0 on SE-30 excludes the possibility that E may correspond to lanosterol (SN 31.2), 24-dihydrolanosterol (30.9), zymosterol (30.4) and lathosterol (29.8).

Partially demethylated sterols from lanosterol and from 24-dihydrolanosterol are ruled out because the corresponding SN should be greater than those of zymosterol (30.4) and zymostenol (30.1), respectively. C 28 and C 29 sterols with double bonds in position 7 and 24 are excluded because their SN should be greater than 30.1, calculated for 7,24-cholestadien-3β-ol. Sterols with 29 carbon atoms and a double bond in position 7 are also excluded because their SN should be greater than 30.0, which is the SN of methostenol (4α methyl-7-cholesten-3β-ol) on SE-30. Therefore methostenol remains the only possibility to be considered among the C 28 and C 29 sterols known to be precursors of cholesterol.

When the SN are calculated also for the CNSi column the following structures may account for the unknown sterols D and E:

	CNSi	SE-30
Nucleus	27.00	27.0
3β-ol (eq.)	5.90	2.4
Δ^7	0.60	0.4
Δ^{24}	0.70	0.3
	34.20	30.1

7,24-cholestadien-3β-ol

	CNSi	SE-30
Nucleus	27.00	27.0
3β-ol (eq.)	5.90	2.4
$\Delta^{5,7}$	0.95	0.3
Δ^{24}	0.70	0.3
	34.55	30.0

5,7,24-cholestatrien-3β-ol

	CNSi	SE-30
Nucleus	27.00	27.0
3β-ol (eq.)	5.90	2.4
Δ^8	0.70	0.7
	33.60	30.1

8-cholesten-3β-ol (zymostenol)

	CNSi	SE-30
Nucleus	27.00	27.0
3β-ol (eq.)	5.90	2.4
Δ^7	0.60	0.4
4α-methyl	0.10(?)	0.2(?)
	33.60	30.0

4α-methyl, 7-cholesten-3β-ol (methostenol)

The SN obtained from the two phases exclude zymostenol and methostenol, while the data calculated for 7,24-cholestadien-3β-ol and 5,7,24-cholestatrien-3β-ol correspond to the SN of compounds D and E, respectively.

On the basis of the gas-chromatographic analysis, it may be therefore concluded that the five sterols present in newborn rat brain after AY-9944 (Table III) are as follows: cholesterol (A), desmosterol (B), 7-dehydrocholesterol (C) and, tentatively, 7,24- cholestadien-3β-ol (D), 5,7,24-cholestatrien-3β-ol (E). The sterols present in newborn rat liver after AY-9944

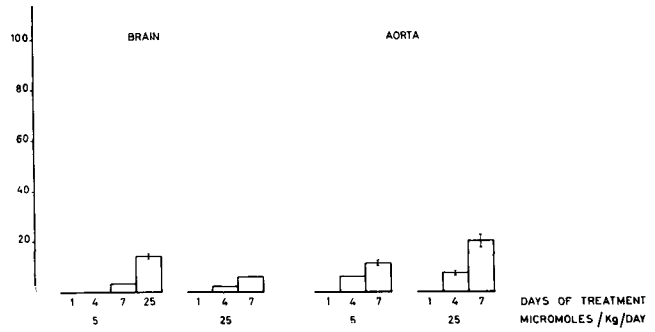

FIG. 2. 7-Dehydrocholesterol content in adult rat tissues after AY-9944 treatment (as percentage of total sterols ± SE).

treatment have been identified as follows: F as cholesterol, H as 7-dehydrocholesterol. Sterol G is imperfectly resolved from cholesterol on SE-30 and has an SN of 33.10 on CNSi. Its behaviour on SE-30 indicates that G is a C 27 sterol, but its chemical structure cannot be postulated on the basis of the available SN.

In order to obtain additional evidence on the structure of the sterols present in our biological samples, a mass-spectrometric analysis was carried out using an Atlas CH4 mass-spectrometer modified in order to allow a direct combination with a gas chromatograph (33). The injection of relatively large samples resulted in slightly less chromatographic resolution than in a conventional column using smaller samples.

Using a 6 f. 1% SE-30 column only two partially resolved peaks were obtained from the liver sterol fraction. Mass-spectra of these two peaks were recorded in such a way as to minimize the interference by the neighbouring peaks.

The fragmentation pattern of the first peak compared with the mass-spectrum of an authentic sample of cholesterol confirmed the identification of this sterol. The mass-spectrum of the second peak shows a molecular ion of mass 384 which is in accord with the proposed GLC identification of this compound as 7-dehydrocholesterol. Other peaks in the spectrum support this identification; in particular the fragments m/e 271 and 225 can be tentatively assigned to M-113 and M-(113 + 18), respectively, and correspond to the loss of the side chain and to the loss of the side chain + water. This indicates that the side chain is saturated.

Three partially resolved peaks were obtained from newborn rat brain sterols. The mass-spectra were recorded in such a way as to minimize the overlapping of spectra from adjacent GLC peaks. The mass-spectra of the first two peaks were identical to those of the peaks obtained from the liver sterols, confirming that the first peak is due to cholesterol and the second one

TABLE VII

Total sterol Content of Newborn Rat Tissues

Treatment		Total sterols	
Liver			
Controls	(10)	227 ± 6	(a)
Treated 3 days	(10)	183 ± 9	(b)
6 days	(9)	200 ± 7	(c)
Brain			
Controls	(10)	412 ± 13	(d)
Treated 3 days	(10)	370 ± 13	(e)
6 days	(9)	370 ± 12	(f)

a–b $p < 0.001$
a–c $p = 0.01$
d–e $p < 0.05$
d–f $p < 0.05$
Figures into parentheses represent the number of animals.
Data are expressed in mg/100 g of fresh tissue ± SE.
Pregnant mothers treated with AY-9944 (5 μmoles/kg/day by stomach intubation) 3 and 6 days before delivery.

is mainly due to 7-dehydrocholesterol. A positive identification of the third peak through the mass-specrum was not possible because of the partial superimposition of two sterols, one of which, moreover, is present in large excess over the other. Because of the occurrence of a certain degree of pyrolysis in the transfer system between GLC and mass-spectrometer and because of the presence of two substances in this peak, the molecular ion could not be positively identified. However, the peak of highest mass in this region is the m/e 382, excluding the possibility that the peak corresponds to a C 28 sterol and suggesting that the main component is a C 27-triene sterol, in accord with the gas-chromatographic identification.

The only sterol, other than cholesterol, present in brain of untreated newborn rats, was confirmed as desmosterol by comparison of its mass-spectrum with that of an authentic sample of desmosterol.

Results

Experiments were carried out in vivo by administering AY-9944 to adult rats by stomach intubation for short periods of time, and by giving the same compound to pregnant rats. The sterol composition and the total sterol content of tissues of adult animals as well as of newborn rats were evaluated. Other experiments were designed to show the effect of treatment with two drugs (Triparanol + AY-9944) on brain and liver sterols of adult rats.

Effect of AY-9944 in Adult Rats

At the dose selected, AY-9944 inhibits completely the 7-dehydrocholesterol Δ^7-reductase activity in homogenates of livers obtained from treated rats. Over 85% blocking of this enzyme system is obtained after a single dose, this is constantly 100% after 4 doses (Table V). The inhibition of 7-dehydrocholesterol-Δ^7-reductase activity occurs also in vivo as shown by the determinations of total sterol concentration and cholesterol percentage in rat tissues after a 7-day treatment with AY-9944 at different dose levels. Total

TABLE VIII

Sterol Composition of Newborn Rat Brain and Liver

Treatment		Cholesterol	7-dehydro-cholesterol	Desmosterol	7,24-Cholestadien-3β-ol	5,7,24-Cholestatrien-3β-ol	Unknown C 27 sterol
Brain							
Controls	(10)	72.6 ± 0.5	0	27.4 ± 0.5	0	0	0
Treated 3 days	(10)	46.6 ± 0.7	25.1 ± 0.7	10.8 ± 0.3	4.0 ± 0.1	13.5 ± 0.4	0
Treated 6 day	(9)	29.0 ± 1.1	37.2 ± 1.3	4.7 ± 0.2	4.3 ± 0.1	24.8 ± 0.5	0
Liver							
Controls	(10)	100	0	0	0	0	0
Treated 3 days	(10)	60.0 ± 1.9	40.0 ± 1.3	0	0	0	tr.
Treated 6 days	(10)	25.6 ± 1.0	59.8 ± 0.8	0	0	0	14.6 ± 1.2

Pregnant mothers were treated by stomach intubation with AY-9944 (5 μmoles/kg/day) for 3 and 6 days before delivery.
Figures represent the percentage composition of total sterols ± SE.
In parenthesis, the number of animals.

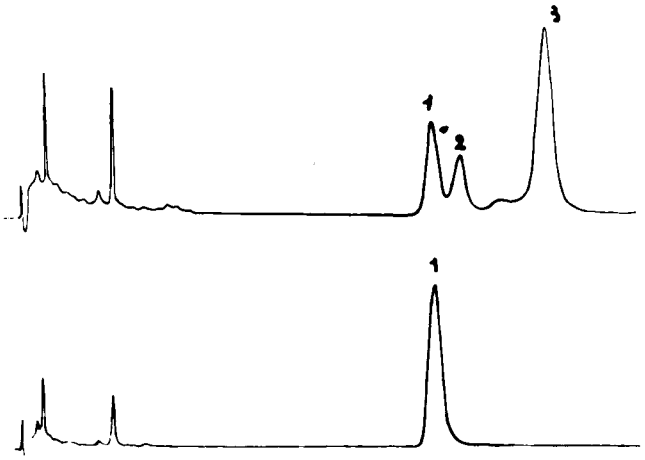

FIG. 3. Sterol composition of newborn rat liver. Lower chromatogram, control rats; upper chromatogram, AY-9944 rats (6-day treatment—see text). Peak 1, cholesterol; peak 2, unknown sterol with 27 carbon atoms; peak 3, 7-dehydrocholesterol. Sterols were run in the free form on CNSi column, at the experimental conditions stated in Table I.

sterols are sharply decreased in serum and aorta, but practically unchanged in liver and brain. Cholesterol percentage is even more clearly decreased with the exception of brain, indicating that sterol precursors of cholesterol do accumulate in the tissues (Table VI). After treatment for 7 days with 25 μmoles/kg/day of AY-9944, serum total sterols decrease for about 50% and in addition cholesterol decreases from 100% to about 40% of total sterols. As a consquence, total cholesterol concentration decreases from 75 to 15 mg per 100 ml.

Total sterols in liver do not change significantly, but cholesterol decreases again from 100% to about 30%.

The decrease of cholesterol percentage corresponds to an accumulation of 7-dehydrocholesterol in peripheral tissues, in agreement with the observations of Dvornik et al. (19) and Chappel et al. (22) (Fig. 1). It must be emphasized that even with the highest dose of AY-9944, a prolonged treatment is necessary to induce some accumulation of 7-dehydrocholesterol in the nervous tissues of the adult animals (Fig. 2). These findings are consistent with the very low rate of cholesterol biosynthesis (34). In addition to this, it was shown in this laboratory that even in the case of Triparanol and 20,25-diazacholesterol treatments, 10 days were required to induce an accumulation of desmosterol in mature rat brain, corresponding to 4% and 8% of total sterols, respectively (16).

Effect of AY-9944 in Newborn Rats

Brain and liver sterol composition is affected in a different way when AY-9944 is administered to rats during the last days of pregnancy and the newborn animals are sacrificed immediately after birth. Total sterols are slightly decreased in liver and practically unchanged in brain (Table VII). In the brain of control newborn rat (Table VIII), cholesterol represents 73% of total sterols, the remainning 27% being desmosterol, as already observed in this laboratory (26).

After a 6-day treatment with AY-9944 of the pregnant rat, cholesterol represents only 29% of total sterols and desmosterol less than 5% in the brain of newborn rats. 7-Dehydrocholesterol is the major component of brain sterols (37%) and two other sterols, nonpresent in measurable amounts in brain of control animals, are detectable. The first, identified as 7,24-cholestadien-3β-ol, is a minor component, less than 5% of total sterols. The second, identified as 5,7,24-cholestatrien-3β-ol, represents about 25% of the total sterols.

Sterol composition of the livers from the same animals shows a different pattern.

Cholesterol in control livers is the only sterol present in measurable amounts, but after treatment for 6 days of the pregnant mother with AY-9944, its proportion falls to 26%, while 7-dehydrocholesterol represents 60% of total sterols (Table VIII and Fig. 3).

A third sterol is also present in considerable amounts (15%); its identity is still unknown.

Effect of Combined Treatment of Adult Rats With Triparanol and AY-9944

Brain and liver sterols have also been examined in adult rats, after combined treatment with Triparanol and AY-9944. The accumulation of 7-dehydrocholesterol in liver (expressed as percentage of total sterols) is not changed in comparison with rats treated with AY-9944 alone (Table IX), but the percentage of desmosterol is greatly decreased in comparison with liver of rats treated with Triparanol alone (6.5% against 43%).

After the combined treatment the sterol pattern in adult rat liver is comparable to that observed in the brain of newborn animals, delivered by rats treated with AY-9944 alone. The sterol 7,24-cholestadien-3β-ol, is present in trace amounts, but 5,7,24-cholestatrien-3β-ol, represents 33% of total sterols.

Brain sterols of adult rats treated with both drugs, contain only trace amounts of desmosterol and 7-dehydrocholesterol (Table IX), indicating a greater stability of the sterol composition in comparison with growing brain and peripheral tissues of growing and adult animals.

Discussion

The two possible series of sterol intermediates between lanosterol and cholesterol with unsaturated and saturated lateral chain respectively, may be represented as follows (Fig. 4).

TABLE IX

Sterol Composition of Adult Rat Brain and Liver (as Percentage of Total Sterols ± SE)

Treatment		Cholesterol	Desmosterol	7-dehydrocholesterol	5,7,24-Cholestatrien-3β-ol
Brain					
Controls	(10)	100.0	.....	.....	.....
Mer-29	(5)	94.9 ± 0.2	5.1 ± 0.2	.....	.....
AY-9944	(4)	100.0	.....	tr.	.....
Mer-29 + AY-9944	(5)	100.0	tr.	tr.	.....
Liver					
Controls	(10)	100.0	.....	.....	.....
Mer-29	(5)	56.8 ± 2.7	43.2 ± 2.7	.....	.....
AY-9944	(4)	81.7 ± 1.7	.....	18.3 ± 1.7	.....
Mer-29 + AY-9944	(5)	43.1 ± 4.2	6.5 ± 0.5	17.2 ± 1.0	33.2 ± 3.6

Treatments: Mer-29 50 μmoles/kg/day per 5 days (stomach intubation). AY-9944 5 μmoles/kg/day per 4 days (stomach intubation).

The series of sterols with unsaturated side chain (I) is certainly present in mammalian tissues, because lanosterol represents the first product of squalene ciclyzation (35) and the last possible compound of the series, desmosterol, is accumulated in blood and tissues after treatment with inhibitors of the Δ^{24}-reductase (14–18).

Each sterol of the series with saturated lateral chain (II) may derive from the corresponding compound of the first series; however, the main site of saturation of the Δ^{24} bond may well occur before the formation of $\Delta^{5,7}$. Several experimental data indicate this possibility: $\Delta^{5,7}$ is a sterol normally present in the small intestine of guinea pig (9); its immediate precursor with saturated lateral chain, lathosterol, is present in large amounts in normal rat skin (5) and it is irreversibly converted to cholesterol through the formation of $\Delta^{5,7}$ (36,4,6); $\Delta^{5,7}$ itself is readily transformed into cholesterol (28).

In addition to this, Goodman et al. have shown that the main pathways of Δ^{24} saturation in rat liver may occur, before the complete demethylation of lanosterol (13).

The two squences exposed in Figure 4, explain many findings reported after treatment with drugs interfering with the last steps of cholesterol biosynthesis. When the Δ^{24}-reductase is blocked with Triparanol, an accumulation of $\Delta^{5,7,24}$ in addition to that of $\Delta^{5,24}$ takes place in the small intestine of guinea pig (24). In pig liver, after combined treatment with Triparanol and AY-9944, Dvornik et al. (23) showed an accumulation of $\Delta^{5,7,24}$. Our experimental results are also consistent with the possibility that the Δ^{24} reduction may occur before the formation of $\Delta^{5,7}$.

Treatment of adult rats with AY-9944 induces a decrease of total sterols in serum and aorta, but not in the liver, where only cholesterol is decreased, but a compensatory accumulation of $\Delta^{5,7}$ occurs.

In brain of newborn rats, where desmosterol is normally present, AY-9944 administration induces a decrease of cholesterol and desmosterol content and a simultaneous accumulation of $\Delta^{5,7}$, $\Delta^{7,24}$ and $\Delta^{5,7,24}$. From these findings it may be deduced that in newborn rat brain in vivo cholesterol precursors of both series are synthesized. In mature brain, even after a prolonged treatment with AY-9944, accumulation of $\Delta^{5,7}$ only is observed, indicating that the in vivo cholesterol biosynthetic pathway in rat brain is age-dependent.

In liver of newborn rats, AY-9944 treatment induces only accumulation of $\Delta^{5,7}$ and not of $\Delta^{5,7,24}$. This is a direct demonstration that brain and liver of the same animals accumulate different sterols after in vivo treatment with a drug blocking a specific enzyme active in cholesterol biosynthesis. In adult rats, submitted to a combined treatment with AY-9944 and Triparanol, an accumulation of $\Delta^{5,7}$, $\Delta^{5,7,24}$ and, to a lesser extent, $\Delta^{5,24}$ sterols is observed, confirming that both biosynthetic routes are possible in the adult mammalian liver.

The two series of compounds proposed in Figure 4 are both significant for cholesterol biosynthesis in the intact animal, but it is still uncertain at what step the reduction of Δ^{24} double bond occurs.

It may be supposed that in tissues where the Δ^{24}-reductase activity is relatively more efficient, as in rat liver, the saturation may take place earlier in the sterol intermediate sequence. In other tissues, such as growing rat brain, the Δ^{24}-reductase seems to be relatively less efficient and an accumulation of

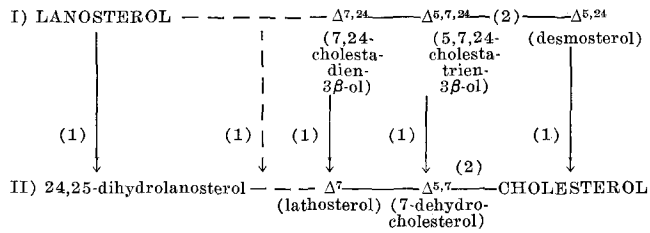

FIG. 4. In this figure, 1 indicates sites of action of Δ^{24}-reductase; 2, site of action of Δ^{7} reductase; dotted lines refer to intermediates from C 30 sterols to Δ^{7} and $\Delta^{7,24}$ sterols with 27 carbon atoms.

the latest precursors of cholesterol with unsaturated side chain occurs normally.

Keeping in mind the relative differences in Δ^{24}-reductase activity in brain and liver of growing rats, it will be not surprising to observe an accumulation of different sterols in different tissues of the same animals, treated with an inhibitor of other specific enzymes of cholesterol biosynthesis.

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