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EFFECT OF HYPOCHOLESTEROLEMIC AGENTS ON CENTRAL NERVOUS SYSTEM CHOLESTEROL BIOSYNTHESIS—II.

AY-9944 AND AY-9944 PLUS ZUCLOMIPHENE

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Abstract—When the hypocholesterolemic agent AY-9944 was examined *in vitro* and *in vivo* for its influence on developing brain sterol biosynthesis, multiple effects were exhibited. Addition of AY-9944 to incubations of cell-free preparations resulted in a decrease in [^{14}C]sterol biosynthesis from [2-^{14}C]mevalonic acid. Pretreatment of animals with AY-9944 and subsequent incubation of their brain tissue *in vitro* resulted in greater labeled sterol synthesis than in control incubations. Pretreatment of animals with AY-9944 followed by intracerebral injection of [2-^{14}C]mevalonic acid resulted in comparable labeled sterol biosynthesis in control and drug-treated brains, but reduced [^{14}C]cholesterol formation by the AY-9944-treated animals. Examination of the labeled free sterol fractions by thin-layer chromatography and radioactivity-monitored gas chromatography indicated several labeled sterols present in the AY-9944-treated brain tissue that were not seen in controls. These [^{14}C]sterols were: 4,4-dimethyl-5α-cholesta-8,14,24-trien-3β-ol; 5α-cholesta-7,24-dien-3β-ol; 5α-cholesta-8,14,24-trien-3β-ol; cholesta-5,7,24-trien-3β-ol; cholesta-5,7-dien-3β-ol; and an unknown 4α-methyl sterol. Another set of developing rats was pretreated with a combination of AY-9944 and zuclomiphene, another hypocholesterolemic agent. Intracerebral administration of [2-^{14}C]mevalonic acid resulted in lower [^{14}C]sterol and labeled cholesterol synthesis in the brain tissue of drug-treated animals than in controls. Thin-layer and radioactivity-monitored gas chromatographic analysis again revealed [^{14}C]sterols other than those present in controls. Two labeled Δ^7 sterols, 5α-cholesta-7,24-dien-3β-ol and cholesta-5,7,24-trien-3β-ol, a 4α-methyl sterol of unknown structure, previously seen in the AY-9944-treated brain tissue, and a 4,4-dimethyl sterol, also of unknown structure, were present in the brains of the drug-treated animals. The present findings indicate not only that both drugs block central nervous system sterol synthesis at several points, but also that use of these hypocholesterolemic agents in various combinations may further elucidate the pathway(s) and control of neural sterol biosynthesis.

A number of studies have explored the biochemical and morphological changes brought about in the nervous system by AY-9944 [*trans*-1,4-*cis*(2-chlorobenzylaminomethyl) cyclohexane dihydrochoride] [1–11]. Much less information is available regarding the effect of zuclomiphene (*cis*-2-[*p*-(2-chloro-1,2-diphenylvinyl) phenoxy]-triethylamine) on the nervous system [12–14]. Administration of zuclomiphene over a period of several weeks to developing rats results in desmosterol, and to a lesser extent zymosterol (5α-cholesta-8,24-dien-3β-ol) accumulation in the brain and spinal cord [12]. An accumulation of $C_{27}\Delta^7$ sterols also occurs when developing neural tissue is treated with AY-9944 [7, 10].

Radioactive tracer studies have indicated that AY-9944 treatment reduces brain cholesterol formation [1]. Incubation of liver cell-free preparations in the presence of AY-9944 and various labeled sterols or sterol precursors demonstrated that the drug can block Δ^{14} double bond reduction [15]. These short-term radioactive substrate studies reveal sterol biosynthetic inhibitions that long-term studies do not. The present study was conducted to assess short-term inhibition of AY-9944 in the nervous system. In addition, the effect of combined treatment with AY-9944 and zuclomiphene on brain sterol biosynthesis was also examined.

MATERIALS AND METHODS

Animals. Wistar rats of both sexes were used. Nursing rats were left with mothers.

Rats which were pretreated with drugs were injected intraperitoneally with 50 mg/kg body wt AY-9944 in saline, or with 50 mg/kg body wt zuclomiphene citrate plus 5 mg/kg body wt AY-9944 dissolved in propylene glycol–water (50:50, v/v). Control animals received the appropriate vehicle only. Treatment, in all cases, was initiated when the rats were 4 days of age. A total of five injections was given before the animals were sacrificed at 20 days of age. During the course of the experiment the test animals exhibited no clinical neurological signs.

Incubations. Cell-free preparations of brain were prepared as described before [16] and the incubation conditions and contents were as described in the preceding paper [14]. Incubations were continued for 5 hr.

Studies in vivo. Twenty-day-old control and drug-treated animals were injected intracerebrally [17]

with 2 μCi (0.02 ml) [2-^{14}C]mevalonic acid (as dibenzyl-ethylenediamine salt, sp. act. 5.80 mCi/m-mole, New England Nuclear Corp., Boston, MA). Animals were sacrificed after 5 hr.

Lipid analysis. Extraction of lipids, alumina column chromatography, sterol digitonide formation and cholesterol dibromide assay were conducted by the methods previously cited [14].

Free sterols were separated by thin-layer chromatography (t.l.c.) into 4,4-dimethyl, 4α-methyl and 4-demethyl sterols using the solvent system of Rahman *et al.* [18]. Free sterols were also fractionated on 7% silver nitrate Silica gel G thin-layer chromatography plates [14] using chloroform–acetone (95:5, v/v) as a developing solvent. Visualization of sterols and elution were as before [14].

Radioactive sterols were also separated by radioactivity-monitored gas–liquid chromatography (g.l.c.) [19] on 3% OV-17 on Gas ChromQ (100/120 mesh, Applied Science Laboratories, Inc., State College, PA) with the bath temperature at 265°. Sterol peaks were identified by comparison to standard sterol retention times [19, 20]. Quantitation of peaks was by triangulation.

RESULTS

The sterols present in the brain tissue of the drug-treated animals and controls at 20 days of age are shown in Table 1. The sterol composition of the brain tissue of AY-9944-treated animals was dominated by the presence of cholesta-5,7-dien-3β-ol. When the animals were treated with both drugs, a total of five sterols was present in the brain, only two of which, cholesterol and desmosterol, were present in the normal brain tissue.

When brain tissue was incubated with [2-^{14}C]mevalonic acid, in the presence of AY-9944, the overall [^{14}C]neutral isoprenoid lipid content was no different than controls (Table 2). There was, however, a decrease of labeled free sterol synthesis, as measured by sterol digitonide formation. If tissue from animals which had been pretreated with AY-9944 was incubated in the same manner, total ^{14}C-neutral lipid was comparable to control, labeled squalene content reduced and sterol digitonide elevated. If the brain tissue of pretreated animals was incubated in the presence of AY-9944, the incorporation data were quite like control values.

Table 1. Endogenous sterol content of control, AY-9944- and AY-9944 plus zuclomiphene-treated 20-day-old rat brain*

	Control		AY-9944		AY-9944 plus zuclomiphene	
	1	2	1	2	1	2
Sterol	(% of total sterol)					
Cholesterol	91.4	90.7	22.7	26.3	26.0	24.2
Desmosterol	8.6	9.3			7.9	5.1
5α-Cholesta-8,24-dien-3β-ol					8.6	6.0
Cholesta-5,7-dien-3β-ol			77.3	73.7	20.1	29.9
Cholesta-5,7,24-trien-3β-ol					37.4	34.8
Total sterol content (mg/g dry wt tissue)	42.2	42.9	40.4	41.9	41.8	42.0

* Sterols were separated by silver nitrate t.l.c. Sterols were quantitated by means of g.l.c. using 5α-cholestane as an internal standard.

Table 2. Incorporation *in vitro* of [2-^{14}C]mevalonic acid into brain isoprenoid lipids of AY-9944 and control rats*

Experiment	Total neutral lipids	Squalene	Steryl esters and squalene oxide	Free sterols and isoprenoid alcohols	Digitonide
	(dis./min 10^{-4}/g wet wt)				
Controls	518 ± 18	48 ± 2	170 ± 3	300 ± 19	238 ± 33
Controls plus AY-9944 in incubation	540 ± 26	49 ± 11	190 ± 39	301 ± 49	105 ± 28
Pretreated with AY-9944	516 ± 22	22 ± 1	183 ± 34	314 ± 31	320 ± 23
Pretreated with AY-9944 plus AY-9944 in incubation	560 ± 27	47 ± 3	255 ± 4	259 ± 22	187 ± 2

* Each incubation contained 2.5 μCi [2-^{14}C]mevalonic acid, 0.5 g wet wt of tissue (20-day-old animals) cofactors, and AY-9944 (1×10^{-4} M), where indicated. Incubations were carried out for 5 hr. Pretreated animals were given AY-9944 intraperitoneally over a period of 15 days prior to sacrifice. Results are expressed as mean ± S. E. M. of four experiments.

Table 3. Incorporation *in vivo* of [2-^{14}C]mevalonic acid into brain isoprenoid lipids of AY-9944 and zuclomiphene plus AY-9944-treated and control rats*

Treatment	Total neutral lipids	Squalene	Steryl esters and squalene oxide	Free sterols and isoprenoid alcohols	Digitonide	Cholesterol dibromide
	(dis./min 10^{-3}/g wet wt)					
AY-9944-treated						
Expt. 1	674	3.76	89.2	581	376	7.91
2	697	5.54	142	549	356	7.66
Controls						
Expt. 1	614	8.48	70.3	535	334	57.0
2	622	2.60	65.0	555	401	59.0
3	668	3.03	64.7	599	347	69.9
Zuclomiphene plus AY-9944-treated						
Expt. 1	456	4.67	64.8	386	204	5.07
2	453	5.71	102	345	202	4.88
3	396	2.35	90.0	304	194	4.53
Controls						
Expt. 1	381	1.45	21.5	358	248	56.9
2	421	1.76	44.3	374	282	47.0

* Each 20-day-old animal was injected intracerebrally with 2 μCi [2-^{14}C]mevalonic acid and sacrificed 5 hr later. Lipid extraction was with chloroform–methanol (2:1). Separation into general isoprenoid lipid classes was by means of alumina column chromatography.

The distribution of labeled neutral isoprenoid lipids after intra-cerebral injection of [2-^{14}C]mevalonic acid into control and AY-9944-treated animals yielded no significant differences between the two groups except for [^{14}C]cholesterol content, as determined by cholesterol dibromide (Table 3). The drug-treated animals had considerably less labeled cholesterol.

When the animals which had been previously pretreated with the combination of drugs were injected with labeled mevalonic acid, again the total labeled neutral isoprenoid lipid was comparable to control values (Table 3). Labeled squalene and steryl ester–squalene oxide fractions were more highly labeled in the drug-treated animals than controls, but the [^{14}C]sterol digitonide was lower in radioactivity. In this case, too, the production of [^{14}C]cholesterol was reduced considerably by the AY-9944 and zuclomiphene treatment.

Examination of the distribution of the labeled free sterols by silver nitrate-impregnated t.l.c. indicated several differences from the controls (Table 4). Both types of drug treatment, AY-9944 and AY-9944 plus zuclomiphene, resulted in greater labeled sterol in the lanosterol and 7-dehydrocholesterol (cholesta-5,7-dien-3β-ol) regions. The cholesterol and desmosterol regions, accordingly, contained less radioactivity than controls.

Chromatography of the [^{14}C]sterols derived from preparative silver nitrate t.l.c. on radioactivity-monitored g.l.c. revealed numerous [^{14}C]-sterols present in the samples derived from the brains of drug-treated animals that were not found in the sterol fractions isolated from control brains (Table 5). Using the thin-layer regions of Table 4 as reference points, the lanosterol region derived originally from the AY-9944-treated brain tissue contained [^{14}C]lanosterol and an

Table 4. Silver nitrate thin-layer chromatographic distribution of labeled free sterols after intracerebral injection of [2-^{14}C]mevalonic acid into AY-9944- or AY-9944 plus zuclomiphene-treated animals and controls*

Reference sterols	R_f of area scraped	Treatment: AY-9944 1	AY-9944 2	AY-9944 + zuclomiphene 1	AY-9944 + zuclomiphene 2	Control 1	Control 2
		(% of total ^{14}C-free sterol)					
Lanosterol	0.55–0.38	23.4	18.7	36.8	37.9	11.6	8.8
Cholesterol	0.38–0.28	35.2	37.0	40.6	39.8	78.3	80.0
Desmosterol	0.28–0.23	7.4	5.6	3.0	3.2	10.1	11.2
7-Dehydrocholesterol	0.23–0.03	32.0	38.7	19.6	19.1		
Total dis./min × 10^{-3}/g wet wt tissue		581	549	345	304	535	358

* Duplicate aliquots of individual free sterol fractions derived from alumina column chromatography were fractionated on 7% $AgNO_3$ thin-layer plates as described in Materials and Methods. Areas were scraped according to reference compounds.

Table 5. Composition of labeled free sterol fractions as determined by radioactivity-monitored gas–liquid chromatography*

Sterol	Treatment: AY-9944 (Per cent of total radioactivity)	(t_R)	AY-9944 + zuclomiphene (Per cent of total radioactivity)	(t_R)	Control (Per cent of total radioactivity)	(t_R)
Lanosterol	15.6	4.01	28.7	3.98	7.3	3.98
4,4-Dimethyl-5α-cholesta-8,14,24-trien-3β-ol	18.1	4.26				
Unknown 4,4-dimethyl sterol			12.9	4.09		
4α-Methyl-5α-cholesta-8,24-dien-3β-ol			3.7	3.34	2.9	3.34
Unknown 4α-methyl sterol	5.4	3.81	4.1	3.81		
5α-Cholesta-8,24-dien-3β-ol	20.5	2.99	32.4	3.00	34.2	3.01
5α-Cholesta-7,24-dien-3β-ol	14.4	3.30	7.4	3.29		
5α-Cholesta-8,14,24-trien-3β-ol	7.7	3.08				
Cholesta-5,7,24-trien-3β-ol	6.4	3.34	6.4	3.34		
Cholesta-5,7-dien-3β-ol	4.1	2.76				
Desmosterol	6.4	2.87	3.1	2.86	10.2	2.86
Cholesterol	4.1	2.38	1.3	2.38	45.4	2.38

* Remainder of free sterol fractions was separated preparatively on 7% $AgNO_3$ thin-layer plates as was described previously. Common regions were pooled in each type of experiment. This provided adequate radioactive material for radioactivity-monitored g.l.c. Sterol retention time (t_R) is given relative to 5α-cholestane. The retention time of cholestane was 4.0 min.

unidentified 4α-methyl sterol. The cholesterol region contained three radioactive sterols: cholesterol, 5α-cholesta-7,24-dien-3β-ol and 5α-cholesta-8,24-dien-3β-ol. Only [^{14}C]desmosterol was found in the desmosterol thin-layer region. The 7-dehydro-cholesterol region contained four labeled sterols: cholesta-5,7-dien-3β-ol, cholesta-5,7-24-trien-3β-ol, 5α-cholesta-8,14,24-trien-3β-ol and 4,4-dimethyl-5α-cholesta-8,14,24-trien-3β-ol. The ^{14}C content of the last sterol was equal to the total of the other three sterols and this sterol was second only to 5α-cholesta-8,24-dien-3β-ol in total radioactive content in the sample.

Analyzing the [^{14}C]sterols recovered from the brains of animals treated with both AY-9944 and zuclomiphene in the same manner, lanosterol, 4α-methyl-5α-cholesta-8,24-dien-3β-ol and an unidentified 4α-methyl sterol, as seen with AY-9944 treatment only, were identified (Table 5). The cholesterol region contained the same three labeled sterols present after AY-9944 treatment. Only [^{14}C]desmosterol was seen in the desmosterol t.l.c. region. The 7-dehydrocholsterol region had two sterols, cholesta-5,7-24-trien-3β-ol and an unidentified 4,4-dimethyl sterol. The presence or absence of methyl groups at C-4 in the sterols recovered from the silver nitrate t.l.c. was confirmed by further chromatography of each recovered silver nitrate t.l.c. region in the thin-layer system designated in Materials and Methods for such separation. This chromatography confirmed the number of C-4 methyl groups originally assigned to each labeled sterol after radioactivity-monitored g.l.c. examination.

DISCUSSION

It is now quite apparent that there are numerous points of action of the hypocholesterolemic agents studied, AY-9944 and zuclomiphene, on brain cholesterol biosynthesis. Some inhibition may be exercised prior to lanosterol and desmosterol. The two drugs seem to act differently *in vitro*. Zuclomiphene inhibited [^{14}C]sterol biosynthesis, whether it was merely added to the incubation, or the incubation contained brain tissue from an animal previously treated with the drug [14]. AY-9944 pretreatment stimulated sterol formation; addition to the incubation of AY-9944 did, however, inhibit sterol synthesis. Further kinetic studies will be needed in order to define these effects more precisely.

The experiments *in vivo* demonstrated clearly that AY-9944 blocks the reduction of the Δ^{14} double bond. Previously, Gibbons and Mitropoulos [15] have been able to show that AY-9944 can cause accumulation of 5α-cholesta-8,14-dien-3β-ol in liver incubations. Since sterol demethylation at position C-4 is much slower in brain than in liver [21–23] and the unsaturated side chain is reduced only at the penultimate step of cholesterol biosynthesis [24, 25], an accumulation of 4,4-dimethyl-5α-cholesta-8,14,24-trien-3β-ol was found in the present AY-9944 investigation. Other C_{27} sterols with conjugated double bonds were also found, which is in line with the previous observations.

With the limited information available thus far, it is difficult to speculate about the possible structure of the unknown [^{14}C]-4,4-dimethyl sterol which accumulated as a result of zuclomiphene treatment. The fact that it was labeled in the brain tissue of rats treated with AY-9944 plus zuclomiphene and the 4,4-dimethyl-5α-cholesta-8,14,24-trien-3β-ol was not, suggests that this unknown sterol may be a precursor of the $C_{29}{}^{\Delta^{8,14,24}}$ sterol. It is possible, however, that insufficient AY-9944 was present to bring about an accumulation of the $C_{29}{}^{\Delta^{8,14,24}}$ compound. Since the brain tissue contained liberal amounts of endogenous $\Delta^{5,7}$ sterols, lack of adequate amounts of AY-9944 seems unlikely.

Some of the properties of the unknown 4,4-dimethyl sterol have been defined and discussed in the preceding paper [14]. Unfortunately, not enough information is yet available to derive a probable structure.

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