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EFFECT OF HYPOCHOLESTEROLEMIC AGENTS ON CENTRAL NERVOUS SYSTEM CHOLESTEROL BIOSYNTHESIS III. ZUCLOMIPHENE IN COMBINATION WITH AY9944 AND TRIPARANOL*

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Abstract—Treatment of developing rats with a combination of hypocholesterolemic agents, zuclomiphene in combination with AY9944 and Triparanol, resulted in the accumulation of Δ^7 sterols in the brain. Abnormal levels of desmosterol and zymosterol were also present in the brain tissue of the treated animals. Pre-treatment of the animals with the combination of drugs followed by intracerebral injection of [2-^{14}C]mevalonic acid yielded comparable labeling of free sterols in treated and control animals. There was a significant increase in the labeled squalene oxide–steryl ester fraction derived from the brains of animals receiving drug treatment, but a significant decrease in labeled cholesterol. Examination of the labeled free sterol fraction by means of silver nitrate-impregnated thin-layer chromatography and radioactivity-monitored gas–liquid chromatography indicated that sterol biosynthesis was impeded in the brains of the treated animals when compared to control animals. There was an increase in labeled sterols having a C-4 methyl group in test animals' brain in comparison to controls. [^{14}C]zymosterol was also elevated in brain of drug treated animals.

The inhibition of sterol biosynthesis in the developing central nervous system by the hypocholesterolemic agent, Triparanol, has been investigated by several researchers in the last few years [1–3]. Another hypocholesterolemic drug that has been used more extensively in this same regard is AY-9944 [4–8]. Chronic treatment of developing rats with these drugs results in the accumulation of abnormal amounts of desmosterol and C_{27} Δ^7-sterols in the central nervous system of Triparanol-treated animals, and the build-up of 7-dehydrocholesterol with AY-9944 treatment.

Another hypocholesterolemic drug that we have utilized in examining cholesterol biosynthesis in the rat developing central nervous system is zuclomiphene (*cis* isomer of 2-(*p*-(2-chloro-1,2-diphenylvinyl) phenoxy)-triethylamine). This drug causes an accumulation of desmosterol in most tissues of the rat [9], but induces a build-up of both desmosterol and zymosterol in the developing central nervous system [10, 11].

Investigation of short-term (5 hr) sterol biosynthesis in the central nervous system of animals pre-treated with these hypocholesterolemic agents by means of radioactive tracers indicated that these drugs may affect sterol formation at a number of points in the biosynthetic sequence [8, 12]. In addition to testing the effect of these compounds on central nervous system sterol biosynthesis individually, two of the drugs, AY-9944 and zulcomiphene have been tested in combination [8, 13]. The purpose of these multi-drug types of experiments is 2-fold: (1) to test the relationship between the type of sterol being accumulated in the nervous system and the formation of membranous cytoplasmic inclusion bodies [13], and (2) by providing multiple blocks to nervous system cholesterol formation, cause the accumulation of quickly metabolized intermediates which would not normally be detected, thereby providing further delineation of the pathway of cholesterol biosynthesis in this tissue [8, 12]. In a continuation of that approach a third hypocholesterolemic agent, Triparanol, has been utilized in combination with these other two drugs. A preliminary report of this work has been presented [14].

MATERIALS AND METHODS

Animals. Wistar strain rats of both sex were used. Nursing rats were left with mothers during the course of the experiment. Animals were maintained on standard laboratory diet.

Rats that were pretreated with the combination of drugs were injected intraperitoneally with 30 mg/kg body wt of zuclomiphene citrate, 30 mg/kg Triparanol and 3 mg/kg AY-9944, all of which was dissolved in propylene glycol–water (3:1, v/v). Treatment was begun at 4 days of age. Animals were injected twice weekly. A total of 5 injections were given before the animals were sacrificed at 20 days of age. Control animals were injected with the propylene glycol–water mixture only. Treated animals generally had a thinner coat of hair and were smaller in stature than controls.

Administration of labeled precursor. Control and drug-treated rats were injected intracerebrally with 4 μCi (0.05 ml) of [2-^{14}C]-DL-mevalonic acid (specific activity 5.80 mCi/m-mole, as DBED salt, New England Nuclear Corp., Boston, MA) at 20 days of age. Animals were sacrificed 5 hr after injection.

Lipid analysis. Lipids were extracted by the method of Folch *et al.* [15]. Extracts were taken to dryness under nitrogen, redissolved in petroleum ether, and

* Previous paper in the series is reference [8].

applied to an alumina column [16]. The column was eluted with petroleum ether (squalene fraction), benzene (steryl ester and squalene oxide fraction) and ethanol (free sterol fraction). All column eluates were taken to dryness on a rotary evaporator and then redissolved in ethyl acetate for further characterization.

Steryl digitonides were formed by the method of Sperry and Webb [17]. Cholesterol purification was accomplished by the dibromide method of Schwenk and Werthessen [18].

Free sterols were fractionated into 4, 4-dimethyl, 4α-methyl and 4-demethyl sterols by means of the thin-layer chromatographic (t.l.c.) system of Rahman *et al.* [19]. Free sterols were also chromatographed on 7% silver nitrate–silica gel G t.l.c. plates [12]. Visualization, and elution of sterols, as well as R_f values of reference sterols on silver nitrate t.l.c., have been described previously [12].

Labeled and unlabeled free sterols derived from t.l.c. were examined further by means of radioactivity-monitored gas–liquid chromatography (g.l.c.), as has been described [20]. Sterols were separated on a column packed with 3% OV-17 on Gas Chrom Q (100/120 mesh, Applied Science Laboratories, Inc., State College, PA), which was operated at 265°C. Identification of sterol peaks was made by comparison to standard sterol retention times previously derived. Quantitation of peaks was by triangulation. Endogenous sterol content was determined by use of an internal standard, 5α-cholestane.

RESULTS

The endogenous sterol content of drug-treated and control brains of animals at 20 days of age is presented in Table 1. The brain tissue of animals treated with the combination of hypocholesterolemic drugs, zuclomiphene, AY-9944 and Triparanol, was rich in cholesterol-precursor sterols, particularly sterols having $\Delta^{5,7}$ double bonds. Desmosterol, a normal constituent of developing brain, was markedly elevated in the brain tissue derived from the drug treated animals. Overall sterol content also appeared to be reduced in drug-treated animals.

The total labeled neutral isoprenoid lipid derived from the brains of animals injected intracerebrally with [2-^{14}C]mevalonic acid after pretreatment with the hypocholesterolemic drug mixture was quite similar to that obtained from control animal brain (Table

Table 1. Endogenous sterol content of control animal brain and of brain tissue derived from animals treated with zuclomiphene, AY-9944 and triparanol

	Control		Treated	
	1	2	1	2
Sterol	(% of total sterol)			
Cholesterol	95.9	96.1	31.4	29.4
Desmosterol	4.1	3.9	15.0	22.2
5α-Cholesta-8,24-dien-3β-ol	—	—	4.6	5.3
5α-Cholesta-7,24-dien-3β-ol	—	—	2.5	0.5
Cholesta-5,7-dien-3β-ol	—	—	15.9	14.6
Cholesta-5,7-24-trien-3β-ol	—	—	30.5	28.0
Total Sterol Content (mg/g dry wt tissue)	38.6	43.8	30.3	34.4

Sterols were separated by means of $AgNO_3$ t.l.c. and quantitated by g.l.c. using 5α-cholestane as an internal standard.

Table 2. *In vivo* incorporation of [2-^{14}C]mevalonic acid into brain isoprenoid lipids of zuclomiphene, triparanol and AY-9944 treated and control rats

Experiment	Total neutral isoprenoid lipids	Squalene	Steryl esters and squalene oxide	Free sterols	Digitonide	Cholesterol dibromide
	(dpm × 10^{-3}/g tissue)					
Controls	765 ± 137	4.60 ± 2.45	44.2 ± 8.41	708 ± 39	373 ± 60	180 ± 9.1
Treated with zuclomiphene + Triparanol + AY-9944	752 ± 121	7.44 ± 1.56	112 ± 50.7*	568 ± 164	317 ± 50	32.6 ± 4.0†

Each 20-day-old animal was injected intracerebrally with 4 μCi of [2-^{14}C]mevalonic acid and sacrificed 5 hr later. Lipids were extracted and separated into general isoprenoid lipid classes by means of alumina column chromatography. Results are expressed as mean ± S.D. of three experiments.

* $P < 0.01$.

† $P < 0.001$.

Table 3. Distribution of labeled free sterols after intracerebral injection of [2-^{14}C]mevalonic acid into animals treated with the combination of hypocholesterolemic agents and corresponding controls

Reference sterols	Area scraped R_f	Control	Treated
		(% of total ^{14}C-free sterol)	
Lanosterol	0.55–0.38	27.7 ± 4.2	51.0 ± 3.6
Cholesterol	0.38–0.28	61.6 ± 5.0	41.3 ± 2.9
Desmosterol	0.28–0.23	8.0 ± 1.0	2.7 ± 0.58
7-Dehydrocholesterol	0.23–0.03	2.7 ± 0.58	4.7 ± 1.5

Aliquots of free sterol fractions were further fractionated on 7% $AgNO_3$ thin layer plates. Areas were scraped according to reference compounds. Results are expressed as mean ± S.D. of three experiments.

2). Labeled squalene, free sterol and sterol precipitable by digitonide values were also comparable in both types of tissue. There was a significant difference with regard to radioactive material in the steryl ester–squalene oxide and cholesterol dibromide fractions. The labeled free cholesterol, as determined by the dibromide procedure, was very depressed in the brains of drug-treated animals. The elevated radioactive content of the steryl ester–squalene oxide fraction isolated from the brains of drug-treated animals may reflect an increase of squalene oxide rather than steryl ester. However, since the two components were not separated, this cannot be stated with certainty as yet.

Examination of the labeled free sterol fractions by silver nitrate impregnated t.l.c. indicated greater labeling of methyl sterols by the brain tissue of treated animals than by controls (Table 3). There was a corresponding lower labeling of sterols chromatographing in the cholesterol and desmosterol regions. Drug treatment in this instance did not result in appreciable labeling of sterols having conjugated double bond systems, such as $\Delta^{5,7}$ and $\Delta^{8,14}$ double bond-containing sterols, which would generally be found in the area having an R_f range of 0.03–0.23.

Radioactivity-monitored gas–liquid chromatography of the free ^{14}C-sterols obtained from preparative silver nitrate t.l.c. revealed few appreciable differences in the types of labeled sterols found in normal and drug-treated brain tissue (Table 4). Two exceptions were the identification of radioactive 4,4-dimethyl-5α-cholesta-8,24-dien-3β-ol and 7-dehydrodesmosterol (cholesta-5,7-24-trien-3β-ol) in brains of treated animals, but not in controls. Although the sterols labeled were generally similar between the two types of animals, the relative distribution of radioactivity among these sterols was dissimilar. It was indicated earlier that the [^{14}C]cholesterol content of brain tissue of the drug-treated animals was reduced appreciably. In addition to this difference, the tissue from drug-treated rats had reduced labeled desmosterol, but increased labeling of all other sterols. The increase in labeled lanosterol was minimal in the drug-treated tissue, but the other two methyl sterols present, 4,4-dimethyl-5α-cholesta-8,24-dien-3β-ol and 4α-methyl-5α-cholesta-8,24-dien-3β-ol, resulted in an overall greater content of labeled methyl sterol in the brains derived from drug-treated rats.

DISCUSSION

Of the various regiments of hypocholesterolemic drugs tested thus far, the combination of zuclomi-

Table 4. Composition of labeled free sterol fractions as determined by radioactivity monitored-gas liquid chromatography

Sterol	Control	Treated
	(% of total radioactivity)	
Lanosterol	23.6	27.9
4,4-Dimethyl-5α-cholesta-8,24-dien-3β-ol	—	14.6
4α-Methyl-5α-cholesta-8,24-dien-3β-ol	4.1	8.4
Zymosterol (5α-cholesta-8,24-dien-3β-ol)	24.5	36.1
7-Dehydrodesmosterol	—	4.5
Desmosterol	8.6	2.9
Cholesterol	39.2	5.6

Remainder of free sterol fractions were separated preparatively on 7% $AgNO_3$ thin-layer plates, as described in Table 3. Regions were scraped, eluted with anhydrous ethyl ether and common regions pooled in each type of experiment. Samples were pooled so that adequate radioactive material would be available for the radioactivity monitored gas–liquid chromatography. Radioactive sterol peaks were identified by comparison of retention times (relative to 5α-cholestane) of the unknowns to reference sterol retention times, as described in the material and methods section.

phene, AY-9944 and Triparanol yielded the most complex mixture of endogenous brain sterols [8, 10–14]. As with the other experiments utilizing AY-9944, Δ^7-sterols constituted a large portion of the total sterol. Except for the presence of 5α-cholesta-7,24-dien-3β-ol in the drug-treated brain tissue, the same cholesterol-precursor sterols were identified in brain tissue derived from animals treated with a combination of zuclomiphene and AY-9944 [8, 13]. The build-up of this particular sterol may be attributable to the addition of Triparanol to the combination of hypocholesterolemic agents being used [3].

The *in vivo* experiments utilizing [2-^{14}C]mevalonic acid demonstrated that cholesterol is being synthesized at a much reduced rate in the brain of the drug treated animals. The total quantity of [^{14}C]sterol being formed, however, was not significantly different than control values. Although a slight increase was apparent in the labeling of the steryl ester–squalene oxide fraction of animals pre-treated with zuclomiphene and AY-9944 only [8], the difference between control and experimental animals was much more obvious in the present study. Since it has been previously demonstrated that Triparanol can cause a blockage of the conversion of squalene oxide to lanosterol in *S. cerevisiae*, and hence result in the accumulation of squalene oxide [21], a portion of the increase radioactivity in this fraction may be attributable to an increase in labeled squalene oxide. This compound has not, however, been specifically isolated in this set of experiments, and so its accumulation is only speculation at the moment.

Previous work involving cholesterol biosynthesis from [2-^{14}C]mevalonic acid in animals previously treated with AY-9944 has resulted in the formation of appreciable labeled sterol having $\Delta^{5,7}$ or $\Delta^{8,14}$ conjugated double bond systems [8, 12]. The present investigation, although it too utilized AY-9944, demonstrated little labeling of this type of sterol. Actually, the labeled sterols isolated from the brain tissue 5 hr after administration of the sterol precursor, [2-^{14}C]mevalonic acid, were not too unlike those recovered from the control tissue. This shift in the pattern of sterol labeling must be attributed, at least in part, to the third hypocholesterolemic agent used, Triparanol. In order to verify this, experiments utilizing Triparanol alone, or in combination with AY-9944 or zuclomiphene, must be conducted so that the specific contribution of Triparanol to the inhibition of brain cholesterol biosynthesis can be asserted.

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