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

ZUCLOMIPHENE

ROBERT B. RAMSEY and MICHAEL FREDERICKS

Department of Neurology, St. Louis University School of Medicine, St. Louis, MO 63104, and Institute of Medical Education and Research, St. Louis, MO 63104, U.S.A.

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Abstract—The hypocholesterolemic agent zuclomiphene was found to inhibit sterol formation from [2-^{14}C]mevalonic acid in cell-free preparations of developing rat brain. This inhibition was found if zuclomiphene were added to an incubation of normal brain. If the animals were pretreated for several weeks with the drug, but the brain tissue then incubated without the drug being added, the same reduction in sterol biosynthesis was seen. Incubation of brain tissue from pretreated animals, with addition of drug to the incubation as well, had reduced sterol biosynthetic activity, but the total labeled neutral lipid fraction was greater than without drug addition to the incubation. Intracerebral injection of [2-^{14}C]mevalonic acid into zuclomiphene-treated and control animals resulted in the isolation of much less labeled cholesterol from the drug-treated animal brains. Thin-layer chromatographic and radioactivity-monitored gas–liquid chromatographic analysis of the labeled free sterol fractions indicated more labeled lanosterol and zymosterol and less labeled desmosterol in the drug-treated brains. Approximately 20 per cent of the total free sterol radioactivity derived from the drug-treated brains had the mobility of $C_{27}^{\Delta^{5,7}}$-$C_{27}^{\Delta^{8,14}}$ sterols on silver nitrate thin-layer chromatography plates. On radioactivity-monitored gas–liquid chromatography the radioactive material derived from this thin-layer region was found to be contained in only one sterol. It had a retention time greater than $C_{29}^{\Delta^{8,14}}$, but less than would be expected for $C_{29}^{\Delta^{8,14,24}}$. Intraperitoneal administration of [3-^{14}C]-D-(−)-β-hydroxybutyrate to zuclomiphene and corresponding controls resulted in lower [^{14}C]sterol content in brain, spinal cord and liver of the treated animals. Silver nitrate thin-layer chromatography of the [3-^{14}C]-D-(−)-β-hydroxybutyrate labeled brain and spinal cord free sterol fractions again indicated increased radioactivity in the $C_{27}^{\Delta^{5,7}}$-$C_{27}^{\Delta^{8,14}}$ sterol region. One of the short-term effects of zuclomiphene pretreatment in the central vervous system seems to be blockage of sterol biosynthesis shortly after lanosterol formation.

The inhibition of cholesterol biosynthesis in the central nervous system by zuclomiphene (*cis* isomer of 2-[*p*-(2-chloro-1,2-diphenylvinyl)phenoxy]triethylamine; formerly called *trans*-clomiphene) has been demonstrated in the developing rat [1]. The reduction of the Δ^{24} bond of desmosterol appears to be most affected; indeed, in the other organs this is the only inhibition thus far reported [2]. In the developing nervous system another block, the isomerization of the Δ^{8} bond to the Δ^{7} position, is also partially inhibited by zuclomiphene[1].

Another hypocholesterolemic agent, AY-9944, has been shown to inhibit primarily the reduction of the Δ^{7} bond of $\Delta^{5,7}$ sterols [3–6]. In the brain, however, it has also been shown to generate an accumulation of Δ^{7} sterols as well as $\Delta^{5,7}$ sterols [7, 8]. With liver preparations *in vitro*, it also inhibits the reduction of the Δ^{14} bond [9, 10]. Because these hypocholesterolemic drugs may be truly multi-faceted in their inhibition of cholesterol biosynthesis, it was felt important to assess inhibition in short-term experiments as well as those stretching over a number of days. This has been done in the following work with the aid of radioactive tracers. A preliminary report of this work has been presented [11].

MATERIALS AND METHODS

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

Rats that were pretreated with drug were injected intraperitoneally with 50 mg/kg of zuclomiphene citrate dissolved in propylene glycol–water (50:50, v/v). Treatment was initiated at 4 days of age. A total of five injections was given before the animals were sacrificed at 20 days of age. Control animals were injected with propylene glycol–water only. Experimental animals exhibited no clinical neurological signs during the experiment.

Incubations. Cell-free preparations of brain, in the manner of Kelley *et al.* [12], involved hand homogenization of tissue in 67 mM sodium potassium phosphate buffer (pH 7.4). The homogenates were centrifuged at 3000 *g* for 10 min at 0°. The supernatant was decanted and used for metabolic studies.

Each incubation contained cell-free supernatant equivalent to 0.5 g wet wt of brain tissue and 1 μCi [2-^{14}C]-DL-mevalonic acid (as the dibenzylethylenediamine salt, sp. act. 5.80 mCi/m-mole, New England Nuclear Corp., Boston, MA). The following cofac-

tors were added in 1.0 ml buffer: ATP (0.9 mM), NADH (1.4 mM), $MgCl_2$ (4.9 mM), glucose 6-phosphate (3.6 mM) and glutathione (3.3 mM). When zuclomiphene was added to incubations, it had to be added in 0.1 ml propylene glycol–water (50:50, v/v). Other incubations received 0.1 ml of propylene glycol–water only. Total incubation volume was 3 ml. Incubations were carried out for 5 hr.

Studies in vivo. In order to explore the effects of zuclomiphene on lipid metabolism *in vivo*, 20-day-old treated and control rats were injected intracerebrally [13] with 5 μCi (0.05 ml) [2-^{14}C]mevalonic acid, specific activity as above, or intraperitoneally with 2 μCi (0.1 ml) [3-^{14}C]-D-(−)-β-hydroxybutyrate (sp. act., 32.3 mCi/m-mole, New England Nuclear Corp., Boston, MA). Animals were sacrificed 5 hr after injection.

Lipid analysis. Lipids were extracted by the method of Folch *et al.* [14]. Extracts were taken to dryness under nitrogen. Lipid extracts derived from [2-^{14}C]mevalonate experiments were redissolved in petroleum ether and applied to an alumina column [12]. The column was eluted with petroleum ether (squalene fraction), benzene (steryl ester and squalene oxide fraction) and ethanol (free sterol and isoprenoid alcohol fraction). The lipid extracts derived from [3-^{14}C]-D-(−)-β-hydroxybutyrate experiments were redissolved in chloroform and applied to a silicic acid column [15]. The column was eluted with chloroform (neutral lipid fraction), acetone (galactolipid fraction) and methanol (phospholipid fraction). All column eluates were taken to dryness on a rotary evaporator. All alumina column fractions and the neutral lipid fractions from silicic acid column chromatography were redissolved in ethyl acetate from which aliquots were taken for further characterization. The galactolipid and phospholipid fractions were redissolved in chloroform–methanol (2:1, v/v).

Steryl digitonides were formed by the method of Sperry and Webb [16]; cholesterol was purified by the dibromide method of Schwenk and Werthessen [17].

Neutral lipids were separated on thin-layer chromatography (t.l.c.) using petroleum ether–ethyl ether–acetic acid (82:18:1, v/v) as a solvent system. Galactolipids and phospholipids were separated by thin-layer chromatography using chloroform–methanol–water (70:30:4, v/v) as a developing solvent. Silica gel H was used for both types of chromatography.

Free sterols were fractionated into 4,4-dimethyl, 4α-methyl and 4-demethyl sterols using the solvent system of Rahman *et al.* [18]. Free sterols were also chromatographed on 7% silver nitrate–Silica gel G thin-layer plates. Thin-layer plates were first developed in a 7% aqueous silver nitrate solution, then activated and the samples applied. Thin-layer plates were then developed in chloroform–acetone (95:5, v/v).

Reference neutral and complex lipids were visualized with iodine or sprayed with sulfuric acid–water (50:50, v/v) and then heated at 110° to develop color. Plates were then scraped and radioactivity was determined, or in the case of the silver nitrate thin-layer plates, the labeled sterols were eluted with ethyl ether.

Table 1. Thin-layer chromatographic properties of free sterols on Silica gel G-silver nitrate*

Compound	R_f
Lanosterol	0.47
Dihydrolanosterol	0.52
4,4-Dimethyl-5α-cholesta-8,14-dien-3β-ol	0.32
4α-Methyl-5α-cholest-7-en-3β-ol	0.44
5α-Cholesta-8,24-dien-3β-ol	0.32
5α-Cholest-7-en-3β-ol	0.35
Cholesta-5,7-dien-3β-ol	0.06
5α-Cholesta-8,14-dien-3β-ol	0.20
Desmosterol	0.25
Cholesterol	0.32
Cholesteryl palmitate	0.89
Squalene	0.08

* The solvent used was acetone–chloroform (95:5, v/v). Silica gel G plates were first developed in a 7% solution of silver nitrate, air dried and activated at 110° for 1 hr and then the samples were spotted and the plate was developed in the above solvent system at room temperature.

The R_f values on silver nitrate t.l.c. of available reference sterols and squalene are shown in Table 1.

Labeled free sterols were further fractionated by means of radio-activity-monitored gas–liquid chromatography (g.l.c.), as has been described [19, 20]. Sterols were separated on a column packed with 3% OV-17 on Gas Chrome (100/120 mesh, Applied Science Laboratories, Inc., State College, PA). The column bath temperature was 265°. Identification of sterol peaks was made by comparison to standard sterol retention times previously derived. Quantitation of peaks was by triangulation.

RESULTS

The initial experiments carried out using zuclomiphene were to test the effect of the drug on sterol biosynthesis *in vitro* (Table 2). Addition of zuclomiphene to the incubation of normal brain tissue increased the labeling of the total neutral lipid and the steryl ester–squalene oxide fractions but depressed labeled sterol formation as measured by digitonide. If the animals were pretreated with zuclomiphene and then the brain tissue was incubated, incorporation of [2-^{14}C]mevalonic acid into all fractions examined was reduced. If the pretreated brain tissue was incubated in the presence of drug, the label distribution was much like that seen when control tissue was incubated with zuclomiphene.

Isoprenoid lipid formation *in vivo* was examined in control and drug-treated rats by intracerebral injection of [2-^{14}C]mevalonic acid (Table 3). The label distribution was similar for both control and test animals, with the exception of the cholesterol fraction. Cholesterol labeling, as determined by cholesterol dibromide, was very depressed in the zuclomiphene-treated animals.

The distribution of radioactivity after silver nitrate t.l.c. of the free sterol fractions indicated that more than just cholesterol formation was disturbed in the drug-treated animals (Table 4). In comparison to controls, the brain lanosterol fraction of the drug-treated animals contained much more radioactive material, the cholesterol and desmosterol regions much less.

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

Experiment	Total neutral lipids	Squalene	Steryl esters and squalene oxide	Free sterols and isoprenoid alcohols	Digitonide
	(dis./min × 10^{-3}/g wet wt)				
Controls	1160 ± 74	178 ± 12	192 ± 18	802 ± 72	41.2 ± 10.6
Controls + zuclomiphene in incubation	1690 ± 122	184 ± 5.2	790 ± 60	724 ± 72	7.92 ± 2.0
Pretreated with zuclomiphene	818 ± 56	88.2 ± 11.2	70.8 ± 6.4	670 ± 52	8.28 ± 0.88
Pretreated with zuclomiphene + zuclomiphene in incubation	1434 ± 46	116 ± 9.4	490 ± 35.2	838 ± 44	6.50 ± 0.80

* Each incubation contained 1 μCi of [2-^{14}C]mevalonic acid, 0.5 g wet wt of tissue (20-day-old animals) cofactors and zuclomiphene (1×10^{-4} M), where indicated. Incubations were carried out for 5 hr. Pretreated animals were given *trans*-clomiphene 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 zuclomiphene-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^{-4}/g wet wt)					
Zuclomiphene						
Expt. 1	274	1.78	14.1	258	146	2.49
Expt. 2	232	1.66	22.3	208	128	4.22
Controls						
Expt. 1	206	0.86	21.7	183	138	39.6
Expt. 2	282	1.28	27.3	253	189	48.1
Expt. 3	256	0.97	14.4	241	167	38.2

* Each 20-day-old animal was injected intracerebrally with 5 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.

Table 4. Distribution of labeled free sterols after intracerebral injection of [2-^{14}C]-mevalonic acid into zuclomiphene-treated and control rats*

Reference sterols	R_f Area scraped	Treatment: Zuclomiphene 1	Zuclomiphene 2	Control 1	Control 2
		(% of total ^{14}C-free sterol)			
Lanosterol	0.55–0.38	47.2	45.9	34.1	34.7
Cholesterol	0.38–0.28	27.6	29.8	43.8	44.1
Desmosterol	0.28–0.23	3.1	3.9	17.7	16.0
7-Dehydrocholesterol	0.23–0.03	22.1	20.4	4.4	5.2
Total dis./min × 10^{-4}/g wet wt tissue		258	208	183	253

* Duplicate aliquots of individual free sterol fractions derived from alumina column chromatography were further fractionated on 7% $AgNO_3$ thin-layer plates as described in Materials and Methods. Areas were scraped according to reference compounds. R_f values of these reference sterols were given in Table 1.

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

Sterol	Treatment: Zuclomiphene (% of Total radioactivity)	t_R	Control (% of Total radioactivity)	t_R
Lanosterol	40.9	4.00	29.8	3.98
Unknown 4,4-dimethyl sterol	21.3	4.08	†	†
4α-Methyl-5α-Cholesta-8,24-dien-3β-ol	5.7	3.36	6.4	3.35
5α-Cholesta-8,24-dien-3β-ol	27.1	3.01	5.0	2.99
Desmosterol	3.4	2.88	17.6	2.86
Cholesterol	1.6	2.38	41.2	2.38

* The remainder of free sterol fractions were separated preparatively on 7% $AgNO_3$ thin-layer plates as was described in Table 4. Regions were scraped and eluted with anhydrous ethyl ether, and common regions were pooled in each type of experiment. This was done so that adequate radioactive sterol would be available for the radioactivity-monitored gas–liquid chromatography. Sterol retention times are given relative 5α-cholestane (t_R). The retention time of 5α-cholestane was 4.1 min.

† No radioactivity present.

In addition, considerable labeled sterol was present in the 7-dehydrocholesterol thin-layer region of the samples derived from zuclomiphene-treated rats.

The labeled sterols present in the individual regions described for silver nitrate t.l.c. were further characterized by radioactivity-monitored g.l.c. (Table 5). The lanosterol region contained a mixture of radioactive lanosterol and 4α-methyl-5α-cholesta-8,24-dien-3β-ol. The cholesterol region contained [^{14}C]cholesterol and [^{14}C]-5α-cholesta-8,24-dien-3β-ol. Only labeled desmosterol was found in the thin-layer region so designated. In the 7-dehydrocholesterol region there was one [^{14}C]sterol with a retention time relative to 5α-cholestane of 4.08. Its mobility on silver nitrate t.l.c. suggests the presence of a $\Delta^{5,7}$ or $\Delta^{8,14}$ double bond system. No naturally occurring methyl sterols are known that have a $\Delta^{5,7}$ double bond system. Its retention time would rule out C_{27} and C_{28} sterols of that type, and a $C_{29}^{\Delta^{8,14}}$ sterol would have a greater R_f. The addition of a double bond at C-24 would result in a much longer retention time on g.l.c.

All of the ^{14}C-sterols recovered from the silver nitrate t.l.c. were also examined in the other sterol solvent system described in Materials and Methods. This system separates well 4,4-dimethyl, 4α-methyl and 4-demethyl sterols. Using this system, it was found that the labeled distribution agreed with the previous radioactivity-monitored g.l.c. findings; namely, that the lanosterol region from the initial silver nitrate t.l.c. contained only labeled 4,4-dimethyl and 4α-methyl sterol, the cholesterol region only labeled 4-demethyl sterol, etc. When the 7-dehydrocholesterol region was examined in the same manner, all the radioactivity was localized in the 4,4-dimethyl region, indicating a C_{30} or C_{29} sterol.

Total lipid labeling of neural and non-neural tissues with [3-^{14}C]-D-(−)-β-hydroxybutyrate yielded a better overall picture of the effect of zuclomiphene on developing rats (Table 6). Generally there was more [^{14}C]lipid/g wet wt tissue in the treated animals than in controls. This observation was particularly apparent in liver and heart, but not evident in the spinal cord. Distribution of radioactivity among lipid classes of the neural tissues indicated a ralative decrease in neutral lipid labeling and an increase in [^{14}C]phospholipids. The liver [^{14}C]lipid distribution exhibited no significant difference between control and zuclomiphene-treated animals.

The composition of each [^{14}C]lipid class was examined further by t.l.c. The neutral lipid fractions derived from the brain and spinal cord of drug-treated animals had proportionately more labeled triglyceride and less [^{14}C]sterol than controls (Table 7). The drug-treated livers demonstrated a slight increase in ^{14}C-free fatty acid relative to control livers. Determination of labeled sterol content by digitonide and [^{14}C]cholesterol by dibromide demonstrated that neural [^{14}C]sterol formation in the drug-treated animals was depressed markedly in contrast to digitonide–sterol labeling when mevalonate was the precursor. The [^{14}C]cholesterol formation by the zuclomiphene-treated animals was also depressed more when β-hydroxybutyrate was precursor than with mevalonate. The galactolipid fractions of the neural tissues contained only labeled cerebroside in all instances. The phospholipids highly labeled by [3-^{14}C]-D-(−)-hydroxybutyrate were ethanolamine and choline phospholipid and sphingomyelin + phosphatidyl serine.

Examination of the ^{14}C-free sterols derived from labeled β-hydroxyl butyrate by silver nitrate t.l.c. yielded a distribution of radioactive sterols similar to that already seen when [2-^{14}C]mevalonic acid was the precursor (Table 8). There was increased lanosterol-4α-methyl labeled sterol in the drug-treated brain and spinal cord and decreased radioactivity in the cholesterol and desmosterol regions, when compared to control values. The zuclomiphene-treated tissues again exhibited labeling of sterol which migrated to the 7-dehydrocholesterol region on silver nitrate t.l.c. Insufficient material was available to examine this fraction further by radioactivity-monitored g.l.c. or re-chromatography in a different thin-layer solvent system.

Table 6. Incorporation *in vivo* of [3-^{14}C]-D-(−)-β-hydroxybutyrate into tissue lipids of zuclomiphene-treated and control rats*

Tissue	Total lipid Extract (dis./min × 10^{-3}/g wet wt)	Distribution of label among Major lipid classes (% of total [^{14}C]lipid) Neutral lipids	Galactolipids	Phospholipids
Spinal cord				
Treated 1	58.2	40.2	17.0	42.8
Treated 2	74.6	46.2	17.1	36.7
Control 1	58.2	59.1	14.9	25.9
Control 2	62.8	54.6	15.0	30.4
Brain				
Treated 1	27.8	34.4	14.5	51.1
Treated 2	33.0	39.1	14.8	46.1
Control 1	20.2	48.7	14.2	37.1
Control 2	24.1	40.4	14.1	45.5
Liver				
Treated 1	15.9	28.2	†	71.8
Treated 2	14.3	20.9	†	79.1
Control 1	4.43	23.1	†	76.9
Control 2	4.62	19.9	†	80.1
Heart				
Treated 1	6.46	14.7	†	85.3
Treated 2	6.20	‡	‡	‡
Control 1	0.72	‡	‡	‡
Control 2	1.08	‡	‡	‡

* Each 20-day-old animal was injected intraperitoneally with 2 μCi [3-^{14}C]-D(−)-β-hydroxybutyrate and sacrificed 5 hr later. Lipid extraction was with chloroform–methanol (2:1). Lipid classes were separated by means of silicic acid column chromatography.

† No radioactivity present.

‡ Not analyzed.

DISCUSSION

Previous work has shown that zuclomiphene results in the accumulation of two sterols in the central nervous system of the developing rat, desmosterol and zymosterol (5α-cholesta-8,24-dien-3β-ol) [1]. Although endogenous sterol composition changes were evident 24 hr after treatment, the full effect of the drug on desmosterol–zymosterol content was not seen for several days. By using tracers such as [2-^{14}C]mevalonic acid and [3-^{14}C]-D-(−)-β-hydroxybutyrate, it has been possible to look at metabolic blocks brought about by zuclomiphene, which are not

Table 7. Incorporation *in vivo* of [3-^{14}C]-D-(−)-β-hydroxybutyrate into tissue neutral lipids of zuclomiphene-treated and control rats*

Tissue	Total neutral lipid (dis./min × 10^{-3}/g wet wt tissue)	Sterol digitonide	Cholesterol dibromide	Distribution of label among neutral lipids (% of total [^{14}C]neutral lipid) Triglyceride	Free fatty acid	Sterol
Spinal cord						
Treated	29.0	13.4	‡	14.4	†	85.6
Control	34.4	23.3	‡	7.6	†	92.4
Brain						
Treated	11.2	2.11	0.048	16.5	†	83.5
Control	9.81	3.66	2.40	7.7	†	92.3
Liver						
Treated	3.73	‡	‡	19.1	40.6	40.3
Control	0.97	‡	‡	22.3	29.0	48.7

* Aliquots of neutral lipid recovered from silicic acid column chromatography were taken for sterol digitonide and cholesterol dibromide formation as well as for further fractionation by thin-layer chromatography. The results above are the average of two determinations on each of the individual neutral lipid fractions.

† No radioactivity present.

‡ Not analyzed.

Table 8. Silver nitrate thin-layer chromatographic distribution of labeled free sterols after intraperitoneal injection of [3-^{14}C]-D-(−)-β-hydroxybutyrate into zuclomiphene-treated and control animals*

Reference sterols	R_f of area scraped	Brain				Spinal cord			
		Zuclomiphene-Treated		Control		Zuclomiphene-Treated		Control	
		1	2	1	2	1	2	1	2
		(% of total ^{14}C-free sterol)							
Lanosterol	0.55–0.38	15.4	19.2	5.5	9.5	22.4	19.3	14.8	14.9
Cholesterol	0.38–0.28	55.0	59.6	66.9	63.4	57.1	59.2	69.0	65.7
Desmosterol	0.28–0.23	†	†	23.8	19.6	†	†	15.2	19.4
7-Dehydro-cholesterol	0.23–0.03	29.0	21.3	3.7	7.5	20.5	21.4	†	†
Total dis./min $\times 10^{-3}$/g wet wt tissue		7.88	10.9	8.89	9.15	20.5	28.8	32.0	31.5

* After aliquots for other assays were taken from the chloroform fraction from the silicic acid column chromatography, the remaining material was fractionated on alumina columns to obtain the labeled free sterol fractions. Duplicate aliquots of each individual free sterol fractions were then further chromatographed on 7% $AgNO_3$ thin-layer plates and appropriate regions scraped and counted.

† No radioactivity present.

as obvious as the inhibition of sterol Δ^{24} reductase (desmosterol accumulation) or of the isomerization of the Δ^8 double bond to Δ^7 (zymosterol accumulation).

The experiments *in vitro* involving brains from animals pretreated with drug suggest that the effects on [^{14}C]neutral lipid formation may not have been purely the result of zuclomiphene stored in the brain. Addition of zuclomiphene to incubations of brains from animals already pretreated with the drug brought about changes that were not apparent with pretreatment only. In fact, the two types of incubations involving addition of zuclomiphene, control and pretreated brain tissue, gave quite similar results. Since the concentration of zuclomiphene that might be present in the brains of the pretreated animals in unknown, the role of endogenous zuclomiphene in the incorporation of [2-^{14}C]mevalonic acid in the incubations *in vitro* is definitely an unknown factor.

The present work *in vivo* suggests that zuclomiphene in the central nervous system blocks the further metabolism of a 4,4-dimethyl sterol, presumably containing a conjugated double bond system. Although the assumption that a $\Delta^{5,7}$ or $\Delta^{8,14}$ double bond system is present is only tentative, no other arrangement of sterol double bonds has yet been shown to have the same mobility on silver nitrate t.l.c. [21, 22]. Also, since $\Delta^{8,14}$ double bond systems occur naturally in methyl sterols but $\Delta^{5,7}$ double bond systems do not [10], the nature of the labeled compound would seem obvious, 4,4-dimethyl-5α-cholesta-8,14,24-trien-3β-ol. The presence of a Δ^{24} double bond would be necessary because without it the sterol would have a much greater R_f on silver nitrate t.l.c. The retention time of the unknown on radioactivity-monitored g.l.c. was entirely inappropriate for this compound. The relative retention time found was 4.08. The calculated retention time for 4,4-dimethyl-5α-8,14,24-trien-3β-ol would be 4.26. This calculated retention time would appear to be correct, for we have found a radioactive sterol in the brains of developing rats treated with AY-9944 that has a g.l.c. retention time of 4.26 [11]. The mobility of this sterol in the same solvent systems and on the same g.l.c. phases described in the present paper was compatible with a C_{29} sterol with a $\Delta^{8,14}$ double bond system. We believe that in the case of AY-9944 inhibition of the brain sterol formation that [^{14}C]4,4-dimethyl-5α-cholesta-8,14,24-trien-3β-ol is present. The nature of the labeled methyl sterol accumulated by zuclomiphene treatment, therefore, remains unidentified and requires further characterization. The high level of lanosterol suggests that C-14 methyl demethylation may also be affected by the drug.

The build-up of [^{14}C]zymosterol in the drug-treated tissue is not surprising in view of the endogenous sterol changes. The reduced labeled demosterol content is doubtless time dependent. Had the animals survived longer, there probably would have been a decrease of labeled lanosterol, unknown 4,4-dimethyl sterol and zymosterol, and an increase of [^{14}C]desmosterol and [^{14}C]cholesterol. A kinetic study involving sacrifice of animals at different time periods after injection of the labeled precursor will, of course, resolve this question.

The use of [3-^{14}C]-D-(−)-β-hydroxybutyrate to label body lipid, and particularly neural lipids, has definite advantages in the developing animal. It has been adequately demonstrated that the developing brain and spinal cord favors ketone bodies, such as β-hydroxybutyrate, for cerebral metabolism [23–29]. It has also been shown that a considerable portion of the ketone bodies that the brain and spinal cord take up is incorporated into sterols [26, 28]. Using this precursor, it was evident that not only was sterol biosynthesis disrupted by zuclomiphene but also phospholipid metabolism. The liver and heart tissues of treated animals acquired much more labeled β-hydroxybutyrate than the controls, suggesting a ketotic or diabetic type state in the animal. Reduction of labeled digitonin-precipitable sterol in the drug-treated animals' brain and spinal cord with β-hydroxybutyrate as precursor and only minor reduction with labeled mevalonate suggest that the zuclomiphene may also affect sterol formation between acetate and

mevalonate. Kinetic studies will undoubtedly provide more specific information ragarding degree and points of lipid metabolism inhibition.

Although considerable effort has been given to the exploration of the sterol changes of the central nervous system upon administration of various hypocholesterolemic agents [7, 8, 22, 30–35], most have been studies involving days, weeks or months. Relatively little radioactive tracer work has been done with regard to sterol formation [36] or with regard to general neural lipid metabolism [37, 38]. Since the data available are only of a limited scope, it is not possible to compare them to the present information.

A more comprehensive biochemical examination of the effects of hypocholesterolemic agents on the metabolism of the developing central nervous system may give more definitive answers as to how these agents work and, in turn, the manner in which the nervous system is assembled and maintained.

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