Pathway of cholesterol biosynthesis in the brain of the neonatal rat

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ABSTRACT Suckling rats were killed at various intervals after intraperitoneal injection of acetate-1-^{14}C and their brain sterols were analyzed by column, thin-layer, paper, and gas–liquid chromatography. The crude sterol (to which carrier zymosterol was added) was separated by column chromatography into cholesterol, desmosterol, and zymosterol fractions, and the specific activities of the recovered digitonides were determined.

The zymosterol fraction, mainly carrier, was not uniformly labeled, in that the trailing half of the peak had a higher specific activity than the leading half. Evidence obtained suggests that this carbon activity was present in one or more sterols resembling zymosterol ($\Delta^{8,24}$-cholestadienol), $\Delta^{7,24}$-cholestadienol, and $\Delta^{7,5,24}$-cholestatrienol. The desmosterol and cholesterol were also carbon-labeled. The time course of the distribution of carbon activity among the above fractions indicated that the zymosterol fraction is a precursor of the desmosterol and that the desmosterol is, in turn, a precursor of the cholesterol.

The data suggest that, in the developing brain of the rat, the course of the transformation of cholesterol precursors into cholesterol is influenced by the presence of at least two slow steps, one involving the conversion of Δ^7- and Δ^8-compounds to Δ^5-compounds and the other, the reduction of the Δ^{24}-unsaturation.

KEY WORDS rat · neonatal · brain · sterols · biosynthesis · cholesterol · desmosterol · zymosterol · cholestadienol · cholestatrienol · turnover · chromatography · acetate-1-^{14}C

THE DEVELOPING brain in all homoiothermic vertebrates which have been examined has been found to contain considerable amounts of companion sterols as well as cholesterol. The major companion sterol found is desmosterol (cholesta-5,24-dien-3β-ol), which has been reported to be present in developing brain of the domestic fowl (1, 2), rat (2–5), mouse (4), guinea pig (4), rabbit (2), and human (2,4) and which represents, for example, approximately 30% of the total sterol of the brain of the 3-day old rat (4). A sterol with the gas chromatographic retention time of zymosterol (5α-cholesta-8,24-dien-3β-ol) has also been reported to be present in neonatal rat brain (3). Although these sterols are also present in rodent skin (6–8), no other vertebrate tissue has been found to accumulate desmosterol to the degree observed in developing brain.

Previous work from this laboratory (1) has shown that the accumulation of desmosterol in the brain of the chick embryo can be ascribed to a rate-limiting step in the pathway of cholesterol synthesis and that this rate-limiting step specifically involves the impaired ability to reduce the Δ^{24}-unsaturation of precursor sterols.

In the present investigation the time course of the carbon-labeling of selected brain sterols was studied after intraperitoneal injection of sodium acetate-1-^{14}C into rats 5 days old, an age at which the desmosterol content of the brain sterols is high (4).

MATERIALS AND METHODS

Compounds

Zymosterol (5α-cholesta-8,24-dien-3β-ol, $\Delta^{8,24}$-cholestadienol), mp 107–108.5°C (corr), was isolated from dried baker's yeast by a modification of the method of Schwenk, Alexander, Stoudt, and Fish (9). Final purification of the zymosterol was effected either by repeated recrystallization of the digitonide from methanol, or by chromatography by Frantz's method (10), or by regeneration from the recrystallized dibromide. Although the first of these methods was most advantageous, all three methods gave

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material 95% homogeneous by gas–liquid chromatography on a modular instrument (F & M Scientific Corporation, Avondale, Pa.) with an SE-30 (methylpolysiloxy gum, General Electric) column. 5α-Cholest-8-en-3β-ol (Δ^8-cholestenol), mp 129–130.5°C (corr), was prepared by hydrogenation of zymosterol over Raney nickel (11). 5α-Cholest-7-en-3β-ol (Δ^7-cholestenol), mp 120°C (corr, unsharp), was purchased from Calbiochem, Los Angeles, Calif. 5α-Cholesta-7,9(11)-dien-3β-ol ($\Delta^{7,9(11)}$-cholestadienol), mp 108°C (corr, unsharp), was prepared by the mercuric acetate oxidation (12) of 5α-cholest-7-en-3β-ol. Desmosterol (cholesta-5,24-dien-3β-ol, $\Delta^{5,24}$-cholestadienol), mp 120–121°C (corr), was purchased from Organon Inc., West Orange, N.J. Cholesterol (cholest-5-en-3β-ol), mp 148–149°C (corr), was prepared from commercial cholesterol by Fieser's method (13). The homogeneity of all the above sterols was checked by gas chromatography. Sodium acetate-1-^{14}C was obtained from Tracerlab Div., Laboratory For Electronics, Inc., Waltham, Mass.

Preparation of Brain Sterol Samples

Rats from our colony, derived from the Wistar strain, were fed on Purina Chow. Pregnant females were isolated and observed daily. Litters were recorded as born on the midnight preceding first observation. On the 5th day after recorded birth, i.e. after more than 5 full days and less than 6 full days of postnatal life, suckling rats were injected intraperitoneally with 0.15 ml of saline containing 0.10 mg of sodium acetate-1-^{14}C (10.3 μc) and decapitated at various time intervals thereafter. If the rats were allowed to live more than 2 hr after injection, they were returned to their mothers; otherwise they were kept warm until killed.

The brains of three to seven rats were removed and pooled for each time interval, and carrier zymosterol was added. The pooled brains were hydrolyzed in ethanolic potassium hydroxide for 2 hr under nitrogen. The unsaponifiable fraction was extracted with ethyl ether, and the ethereal solution was washed with sodium acetate solution and water, and passed through a short column of anhydrous sodium sulfate. The ether was removed under nitrogen and the residue dissolved in 90% ethanol and converted to the digitonide by the addition of an excess of a 1% solution of digitonin in 90% ethanol. Digitonides were filtered out, washed with 90% ethanol and with ethyl ether–acetone 1:1, and counted under a thin-window β-counter (14).

Fractionation of Brain Sterols

The sterol digitonide, comprising a portion of the brain sterol from rats injected with acetate-1-^{14}C, was cleaved with pyridine and ethyl ether (14); and an aliquot of the sterol was reconverted to digitonide as a reference sample. The remainder, about 20 mg of sterol, was chromatographed according to the procedure of Frantz (10), on a column of 1:2 Hyflo Super-Cel (Johns-Manville)–silicic acid (Mallinckrodt), eluted with benzene. One hour fractions were collected for at least 100 hr; and the cholesterol, desmosterol, and zymosterol zones were located and estimated by carrying out the Zak reaction (15) on the residues from aliquots of the effluent fractions and reading the optical densities at 560 mμ on a Coleman Junior Spectrophotometer. It was confirmed that the chromogens derived from cholesterol and desmosterol had practically identical molar extinction coefficients at 560 mμ (16), but it was found that zymosterol gave a chromogen with a weaker absorbance. The composition of the mixture of brain sterols and carrier zymosterol was therefore determined by gas–liquid chromatography on an Epon 1001 resin column (F & M Scientific Corporation) (17). This separated the sterols into a cholesterol peak and a mixed desmosterol–zymosterol peak. Since the relative amounts of cholesterol and desmosterol present were already known, the amount of zymosterol present could be determined by findings the areas under each of the two peaks and deducting the calculated contribution of desmosterol from the area of the desmosterol–zymosterol peak.

In a typical separation by Frantz's method, the peak of the cholesterol zone emerged at tube 64, that of desmosterol at tube 72, and that of zymosterol at tube 86. There was a slight overlap of the cholesterol and desmosterol zones, but the zymosterol zone was well separated from desmosterol. The fractions in the valley between cholesterol and desmosterol and those between desmosterol and zymosterol were discarded. Digitonides were formed from the material in the tubes corresponding to the three sterol peaks, and the specific and total ^{14}C activity of each zone were then determined. The homogeneity of each of the three zones was then tested in the following manner:

Cholesterol Zone. The cholesterol fraction from the 20 hr sample was diluted with carrier cholesterol, the mixture brominated, the dibromide recrystallized from ethyl acetate, and the sterol regenerated from the dibromide by treatment with zinc dust. Digitonides of samples of the sterol before and after bromination were made and freed from traces of excess digitonin by dissolving them in small amounts of pyridine and reprecipitating them with 90% ethanol. The digitonide of the cholesterol purified through the dibromide had a specific activity which was 97.3% (SD, 3.6%) that of the starting material.

Desmosterol Zone. The desmosterol fraction from the 20 hr sample was diluted with carrier desmosterol, and a portion was converted to the tetrabromide and chromatographed on paper according to Fabro's procedure (18). Longitudinal strips, 1 cm in width, were cut from the

paper and heated to make visible the desmosterol–tetrabromide zone. The paper containing the rest of the zone was then cut out and the desmosterol was regenerated by refluxing this paper with activated zinc (19) in 95% ethanol for 1 hr. Most of the ethanol was removed under a stream of nitrogen, the residue was extracted with ethyl ether, the zinc salts were washed out with sodium hydroxide solution and water, and the desmosterol was recovered from the ethereal solution. Digitonides of the sterol before and after passage through the tetrabromide were prepared as before. The purified desmosterol had a specific activity 91.3% (SD, 1.9%) that of the starting material.

Zymosterol Zone. The zymosterol zone was predominantly composed of carrier zymosterol. The leading and trailing halves were recovered as separate digitonides. The total activities in each half at 0.25 hr were 69 cpm in the leading, and 101 cpm in the trailing half. The corresponding values for the other time intervals were: 2 hr, 173 cpm (leading), 228 cpm (trailing); 20 hr, 43 cpm (leading), 82 cpm (trailing); 96 hr, 4.8 cpm (leading), 4.8 cpm (trailing).

The observation that the specific activity of the leading half was always less than that of the following half suggested the presence of a labeled compound that moved more slowly than zymosterol. Two other sterols with the structures of cholesterol precursors are reported to travel close to zymosterol on the Frantz column. It is not clear whether one of these, cholesta-5,7,24-trien-3β-ol, precedes or follows zymosterol; but the other, 5α-cholesta-7,24-dien-3β-ol, is known to follow it. Attempts were made to separate the Δ^7- and Δ^8-compounds of the zymosterol fraction by reducing the mixture to Δ^7- and Δ^8-cholestenol over Raney nickel (11, 20) and oxidizing the Δ^7-cholestenol to $\Delta^{7,9(11)}$-cholestadienol with mercuric acetate, a reaction reported not to alter Δ^8-compounds (21). $\Delta^{7,8(11)}$-Cholestadienol and Δ^8-cholestenol were then separable by thin-layer chromatography on silicic acid impregnated with silver nitrate (22). The results were inconclusive since some isomerization may have occurred either during the steps associated with Raney nickel reduction or during mercuric acetate oxidation. The study was completed before there was an opportunity to use Dempsey's elegant method of separating $\Delta^{5,7}$-sterols as epiperoxides (23).

RESULTS AND DISCUSSION

In this investigation, acetate-1-^{14}C was injected intraperitoneally into rats 5 days old, and the brains were removed 0.25, 2.0, 24, 48, and 92 hr after injection. After zymosterol was added as carrier, cholesterol, desmosterol, and zymosterol fractions were separated by column chromatography, as indicated in Table 1. Two hours after injection 84% of the crude sterol ^{14}C activity was present in these three fractions, and attention was therefore confined to these. From the data presented by Frantz (8, 10), who devised the form of chromatography employed, it was anticipated that these three sterols would be separated from most of the other known cholesterol precursors and that any $\Delta^{7,24}$-cholestadienol present would travel with the carrier zymosterol.

TABLE 1 INCORPORATION OF ^{14}C INTO STEROL FRACTIONS OF BRAINS OF RATS KILLED AT VARIOUS INTERVALS AFTER INTRAPERITONEAL INJECTION WITH ACETATE-1-^{14}C

Fraction	Interval between Injection and Death, Hr				
	0.25	2.0	20	96	192
	cpm per brain				
Total sterol digitonide	363	935	1330	1220	1070
Cholesterol	10.3	146	535	1100	919
Desmosterol	35.4	242	471	192	44
Zymosterol (front half of zone)	69.3	173	42.9	4.8	*
Zymosterol (rear half of zone)	101	228	82.1	4.8	*

Injection was at 5 days of age.
* Not determined.

Tests of the homogeneity of the cholesterol and desmosterol fractions indicated that the bulk of their carbon activity was associated with cholesterol and desmosterol respectively. The specific activity gradients in the zymosterol zones suggested that at least part of the activity was associated with one or more compounds resembling $\Delta^{7,24}$-cholestadienol and $\Delta^{5,7,24}$-cholestatrienol in chromatographic behavior. In Fig. 1 the log of the carbon activity per brain for each of the three fractions obtained by column chromatography of the brain sterols is plotted against the log of time after injection. The curves shown represent the least-square quadratic equations that fit the experimental points. It is evident that the curves for activity in the zymosterol and desmosterol fractions show accumulation and decay characteristic of precursor pools and that the accumulation and decay of activity in the zymosterol fraction precedes that in the desmosterol fraction, while that in the desmosterol fraction precedes the accumulation of activity in the cholesterol fraction. The data thus fit the hypothesis that, in the brain of the neonatal rat, zymosterol or a similar sterol is a precursor of desmosterol, which in turn is an immediate precursor of cholesterol.

A mass of evidence (summarized by Bloch (24)) indicates that cholesterol biosynthesis passes through lanosterol, which is converted to cholesterol by a group of enzymes responsible for demethylation, reduction of the side-chain double bond, and "rearrangement" of the 8,9-double bond to a 5,6-double bond. Either these enzymes show low substrate specificity, or the substrate specificity differs in different tissues, so that a variety of pathways is possible, the predominant pathway in each

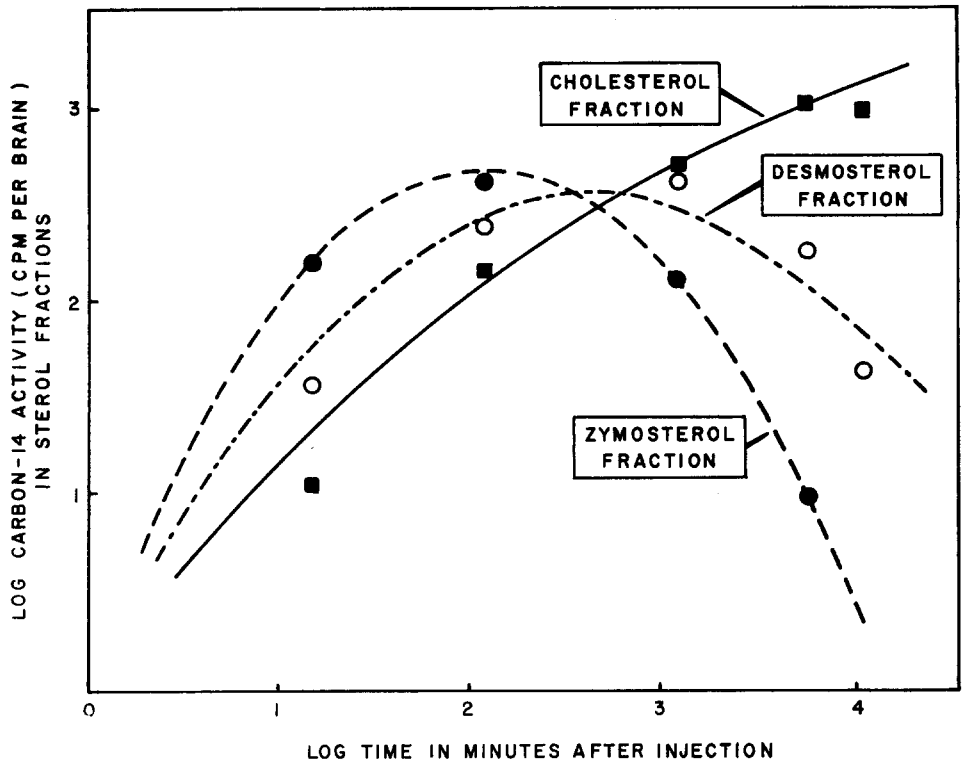

FIG. 1. The time course of ^{14}C labeling in sterol fractions from the brains of rats injected intraperitoneally with acetate-1-^{14}C at the age of 5 days and killed at various time intervals thereafter. The three fractions shown were isolated by column chromatography of the crude brain sterol plus carrier zymosterol, the carbon activity in each fraction was determined, and the curves were obtained by fitting least-square quadratic equations to the experimental points.

tissue being determined by the particular pattern of enzymes and precursors present.

The developing brain in the homoiothermic vertebrates examined appears to synthesize cholesterol by similar pathways. This is evidenced by the composition of their brain sterol mixtures, which are characterized by the presence of large amounts of desmosterol. The functional maturation of the brain is accompanied by a striking decline in the content of desmosterol, which is virtually absent from adult brain (4).

The course of sterol synthesis in the brain of the chick embryo was further studied by Fish, Boyd, and Stokes (1), who injected sodium acetate-1-^{14}C into embryonated eggs and isolated and fractionated the crude brain sterol fraction at intervals thereafter. When the brain sterol mixture of 14-day embryos was analyzed 16 hr after injection, most of the carbon activity was present in desmosterol and cholesterol. The time course of the accumulation and decay of carbon activity in the desmosterol fraction and of its accumulation in the cholesterol fraction was consistent with the hypothesis that a rate-limiting step exists for the enzymatic reduction of the 24,25-double bond of cholesterol precursors that results in the channeling of sterol biosynthesis in the direction of desmosterol, which accumulates and is slowly converted to cholesterol.

Trace amounts of other sterols have been reported in developing brain. Kritchevsky and Holmes (3) have found a compound with the gas–liquid chromatographic retention time of $\Delta^{8,24}$-cholestadienol (zymosterol) in rat brain, while Scallen, Condie, and Schroepfer (25) report the isolation of a "fast acting" sterol with the column chromatographic behavior of $\Delta^{7,24}$-cholestadienol from the brains of young mice treated with a drug which inhibits cholesterol biosynthesis. $\Delta^{7,24}$-Cholestadienol would be expected to show a gas chromatographic retention time similar to that of zymosterol. These reports are consistent with our observation that, after acetate-1-^{14}C labeling, brain sterol fractions with chemical and physical properties resembling those of $\Delta^{7,24}$-, $\Delta^{8,24}$-cholestadienol, and $\Delta^{5,7,24}$-cholestatrienol become highly labeled.

The pattern of sterol synthesis in developing rat brain indicates the presence of two slow steps. The first of these occasions the slow turnover of carbon activity in the zymosterol fraction, the turnover time of which is about 27 hr. This suggests that the first slow step is located somewhere along the pathway that leads from Δ^{8}- to Δ^{5}-compounds. The second slow step resembles that ob-

served in chick embryo brain and involves impaired ability to reduce the 24,25-double bond of cholesterol precursors. It leads to the accumulation of desmosterol and the slow turnover of the desmosterol pool, the turnover time of which is about 72 hr.

It is interesting that somewhat similar patterns of sterol synthesis were observed by Dempsey (23) in some of her experiments. She found, for example, that a system prepared from rat liver homogenate would, in the absence of oxygen, convert $\Delta^{8,24}$-cholestadienol to $\Delta^{7,24}$-cholestadienol. She also demonstrated that, in the presence of oxygen, this enzyme system, supplemented with reduced NADP and MER-29 (an inhibitor of cholesterol biosynthesis), would convert $\Delta^{7,24}$-cholestadienol to $\Delta^{5,7,24}$-cholestatrienol. When the concentration of MER-29 was reduced tenfold, the same system converted $\Delta^{7,24}$-cholestadienol to a mixture of $\Delta^{5,24}$-cholestadienol and cholesterol. In another experiment $\Delta^{7,24}$-cholestadienol was converted to $\Delta^{5,7}$-cholestadienol by an enzyme system to which an inhibitor of Δ^{7}-reductase had been added. These experiments by Dempsey show that cholesterol biosynthesis involves the conversion of a Δ^{8}-compound to a Δ^{5}-compound by way of Δ^{7}- and $\Delta^{5,7}$-intermediates, with prior or concomitant reduction of Δ^{24}-unsaturation, and that selective inhibition of most of these transformations is possible in vitro. Work by other investigators (26, 27) has shown that the same relatively specific drug-induced inhibitions can be demonstrated in vivo.

Thus, the developing rat brain synthesizes cholesterol by a distinctive pathway that differs from those suggested by the experimental evidence obtained from the study of rat liver (28) and mouse preputial gland tumor (29), and resembles, at least superficially, pathways that result from the drug-induced enzyme inhibitions cited above. The factors influencing the course of biosynthesis and the rate of accumulation of sterols in the developing brain before and during myelination are at present largely unknown (30).

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