SQUALENE-2,3-EPOXIDASE ACTIVITY IN RAT BRAIN DURING GROWTH AND MATURATION

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SUMMARY

The in vitro block in cholesterol biosynthesis which causes squalene accumulation in brain preparations has been investigated using ^{3}H-squalene and assaying for labeled squalene-2,3-oxide formation. It has been determined that: 1) squalene epoxidase activities in brain and liver are comparable; 2) there is no change in brain epoxidase activity corresponding to the peak in brain cholesterol synthesis during myelination; 3) the epoxidase is inhibited 3.5-fold by a squalene-2,3-oxide trap. These facts indicate that brain epoxidase activity is not a control point for brain cholesterol biosynthesis during development, in spite of the marked squalene accumulation observed in vitro.

INTRODUCTION

There is mounting evidence that in vitro brain preparations from adult (1) and immature (2) rats, when incubated with labeled cholesterol precursors, do not reflect the full capacity of the tissue to synthesize cholesterol. For example, using cell-free preparations with 2-^{14}C-mevalonate as the precursor, considerable squalene accumulates (3). This does not occur when mevalonate is given intracerebrally (3). Although several "metabolic blocks" may exist under in vitro conditions past the squalene - squalene-2,3-oxide step, (4,5) the conversion of squalene to squalene-2,3-oxide may constitute the major in vitro metabolic block. It seemed of importance, therefore, to determine squalene-2,3-epoxidase activity by a specific in vitro assay throughout the life-span of rats.

MATERIALS AND METHODS

Chemicals - All solvents were distilled before use. Omnifluor (New England Nuclear, Boston, Mass.) in toluene was used as liquid scintillator solution for lipids and TLC scrapings. To check water-soluble radioactivity, 10 ml Instagel (Packard Instruments, Downer's Grove, Ill.) was mixed with 5 ml of aqueous sample and allowed to gel. Nicotinamide, glucose-6-phosphate monosodium salt (G-6-P), glucose-6-phosphate dehydrogenase, nicotinamide adenine dinucleotide phosphate, sodium salt (NADP), and 2,6-di-tert-butyl-p-cresol (BHT) were purchased from Sigma Chemical Co., St. Louis, Mo. 1-Dodecylimidazole (DDI)[1] was generously provided by Dr. B. Morgan of Beecham Research Laboratories, U. K. Squalene-4-^{3}H (4.7 mCi/mmole) and squalene-2,3-oxide (SOX)[2] were synthesized as described in Methods in Enzymology (6). Both were purified by TLC before use.

Preparation of brain extracts - Brains were excised from Wistar-derived rats (National Laboratory Animal, O'Fallon, Mo.), cleaned and washed with cold water, then homogenized with an equal weight of 80 mM potassium phosphate buffer, pH 7.4 containing 4 mM $MgCl_2$. Rats were decapitated and brains cooled within 30 seconds. For liver, the buffer also contained 30 mM nicotinamide (8). An 800 x g, (15 min) supernate of the homogenate was centrifuged 15 min at 15,000 x g, any lipid scum removed by adhesion to a glass rod, and the supernate (S-15)[3] used for incubations.

Benzene (0.2 ml) containing 5 µl Tween 80, 7 x 10^4 disintegrations per minute (dpm) squalene-4-^{3}H (except as noted), 440 µg 2,6-di-ter-butyl-p-cresol (BHT), and 2 µmoles of either DDI (9) or squalene-2,3-oxide (SOX)was dried in vacuo in a scintillation vial, then 0.5 ml of buffer containing 3 µmole G-6-P, 1 µmole NADP, and

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Abbreviations: [1] DDI, 1-Dodecylimidazole; [2] SOX, squalene-2,3-oxide; [3] S-15, 15,000 x g supernate.

2 units of G-6-P dehydrogenase were added and mixed 15 sec on a Vortex mixer to produce a clear micellar solution. S-15 (1.5 ml) was added and the open vial was shaken at 37° for either 1 hr (DDl) or 2 hr (SOX trap). The incubation mixture was extracted with 20 volumes of chloroform-methanol, 1:1, v/v, and the latter removed by evaporation in a N_2 stream. Final drying of the residue was accomplished in vacuo. Lipids in the residue were quantitatively transferred to a TLC plate in a small volume of chloroform-methanol. Controls were "substrate", with substitution of buffer for S-15; and "heat-killed", using S-15 heated on a steam bath for 2 hr in a sealed tube. All results are the means of duplicate incubations.

Thin-layer chromatography (TLC) - All TLC was performed on 20 x 20 cm glass plates coated with silica gel H, 0.75 mm in thickness applied as a water slurry and subsequently activated at 110°C overnight. For preparative TLC, the plates were washed by ascending chromatography with ethyl acetate.

For radio-TLC of the incubation extracts, the extract was spotted in 8 cm wide bands. A standard mixture of cold squalene oxide and squalene (approx. 10 μg each) was spotted at each end and at the center of the band to serve as internal standards. The plate was developed to approximately 15 cm by ascending chromatography with hexane: ethyl acetate, 100:5, v/v. It was then air-dried and exposed to I_2 vapor. The exact band positions were marked, then the plate was heated 2 hr at 70° to remove the I_2. Bands were scraped into scintillation vials, and counted in the toluene-based fluor. Scintillation counting was accomplished on a Packard Model 3320 instrument. Efficiencies were calculated by channels ratios. R_f's in the TLC system were 0.90 for squalene, 0.50 for squalene oxide, and 0.10 for cholesterol. Aliquots of S-15 supernate were analyzed for protein by the Biuret method (7).

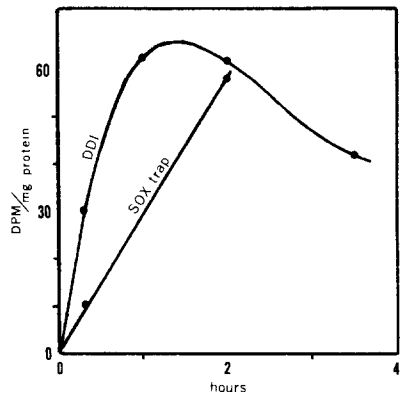

Fig. 1. Synthesis of squalene oxide from 70,000 DPM of 4-^{3}H-squalene during incubations of 20-day-old rat brain S-15 fraction, as a function of incubation time.

RESULTS

Assay characteristics - Both "substrate" and "heat-killed controls" gave complete recovery of squalene ± 2%, and a small (0.2-0.5%) amount of activity at the TLC origin. No other areas of activity were detected (old or impure squalene shows activity also at R_f 0.3 and 0.6). Active preparations show activity at the R_f of squalene oxide, with concomitant decrease in squalene activity. Identity of squalene oxide was confirmed by co-chromatography as the glycol derivative (7).

The rate of formation of squalene oxide (Fig. 1) is linear for approximately 1 hr with DDl, a squalene oxide-lanosterol cyclase inhibitor (9), or for at least 2 hr with the squalene oxide (SOX) trap. In long incubations without trap, the squalene oxide begins to decrease, and more activity appears at the origin.

According to the results in Fig. 1, the squalene oxide trap reduces the initial rate of squalene oxide synthesis by a factor of approximately 3.5.

Age study - Incubations of S-15 supernate from brains of rats

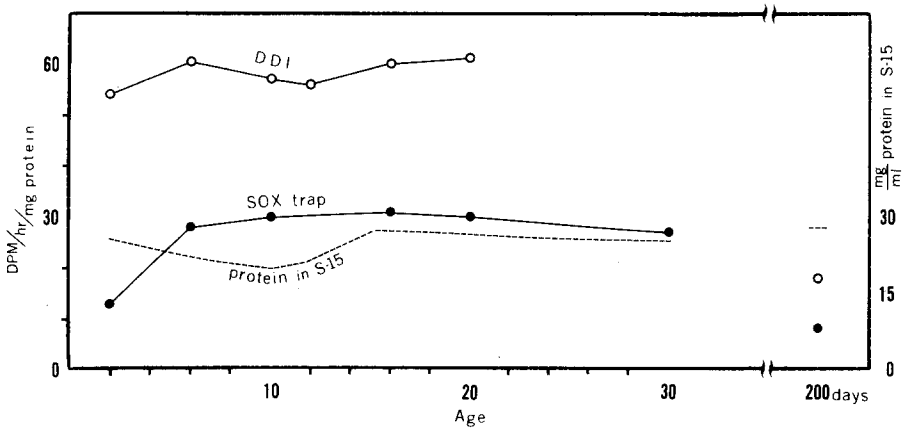

Fig. 2. Rate of squalene oxide synthesis from 70,000 DPM of 4-^{3}H-squalene in incubations of rat brain S-15 fraction, as a function of age.

2 days to 200 days of age containing either DDl (1 hr) or squalene oxide trap (2 hr) gave the results shown in Fig. 2. As expected, the incubations containing squalene oxide (SOX) trap were inhibited relative to the DDl-containing preparations. However, no notable changes in epoxidase activity occurred during the myelination period (ages 11-15 days).

Comparison of brain and liver squalene epoxidase activity - As shown in Table 1, liver and brain preparations of 12-day-old rats show approximately the same epoxidase activity. This is true of other ages as well.

DISCUSSION

The data show: 1) significant squalene epoxidase activity in rat brain from 2 days to 200 days of age. 2) No correspondence of epoxidase activity with squalene accumulation in mevalonate-substrate incubations (2).

These results suggest that the in vitro metabolic block at the squalene epoxidation step during cholesterol synthesis from mevalonate is due to other factors than enzyme activity level, possibly

Table 1: Comparison of epoxidase activity in brain and liver preparations of 12-day-old rats, using 240,000 dpm squalene-4-^{3}H substrate.

Tissue	Squalene-2,3-oxide product of incubation	
	DPM/incubation	DPM/hr/mg protein
Liver (+DDI)	13715	219
Liver (+SOX)	14667	117
Brain (+DDI)	7790	178
Brain (+SOX)	9906	113

to the availability of epoxidase to squalene synthesized *in situ* by the brain preparations. While liver sterol synthesis is an on-going process throughout the life of the animal, brain sterol synthesis is a process which sharply peaks during myelination. In the adult animal, sterol synthesis is extremely slow although the adult brain retains the capacity to synthesize sterol (10). This continued capacity may have a repair function, but the means by which this pathway is kept "turned off" in adult brain is a topic of considerable interest.

ACKNOWLEDGEMENTS

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