Development of Hydrolytic Cholesterol Esterase Activity in Rat Brain*

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ABSTRACT: Hydrolytic cholesterol esterase activity was measured by incubating [4-^{14}C]cholesteryl linoleate with homogenates prepared from the brains of rats of various ages. A method was developed by which the contribution of endogenous cholesterol esters to the substrate pool for the hydrolytic enzyme could be derived from measured initial rates of hydrolysis of the labeled substrate. The net amounts of cholesterol esters hydrolyzed during the incubations could thus be established. Brains of newborn rats showed hydrolytic activity. Additional activity was acquired chiefly between 7 and 20 days of age, a period when brain myelination is known to be most active. The esterase activity remained maximal from about 3 weeks to at least 3 months of age. Hydrolytic cholesterol esterase activity was shared about equally by various particulate subcellular fractions; cell sap was practically devoid of activity.

The brain rapidly acquires cholesterol during the brief period of myelination (Mendel and Leavenworth, 1908; Waelsch *et al.*, 1941). Only free cholesterol is present in the mature brain, but the esterified form has been found during early stages of development (Mandel *et al.*, 1949; Brante, 1949; Adams and Davison, 1959; Clarenburg *et al.*, 1963; Grafnetter *et al.*, 1965). These findings, in conjunction with the histochemical demonstration of the presence of cholesterol esters in developing brain myelin (Adams and Davison, 1959, 1960), have led to the view that esterified cholesterol is a precursor of free cholesterol in myelin. This implies, since virtually all cholesterol in brain is of *in situ* origin (Davison *et al.*, 1958; Morris and Chaikoff, 1961; Clarenburg *et al.*, 1963), that esterified cholesterol is synthesized in the brain and then hydrolyzed in the course of deposition of free cholesterol in myelin.

It should be noted that, even at its peak, the cholesterol ester concentration in brain is quite small, whereas large amounts of free cholesterol are rapidly deposited in myelin (Clarenburg *et al.*, 1963; Grafnetter *et al.*, 1965). This would be compatible with a quantitatively important precursor role for cholesterol esters only if those esters are turned over at an extremely rapid rate. In that event, hydrolytic cholesterol esterase activity should be readily demonstrable in the brain. The literature regarding the presence of this enzyme activity in brain is divided between workers who find activity (Shope, 1928; Pritchard and Nichol, 1964) and workers who do not (Sperry and Brand, 1941). We therefore decided to study the presence and development of hydrolytic cholesterol esterase activity in rat brain. Our experiments show that most of this enzyme activity arises rapidly between the first and fourth weeks of age, a period that includes the time when myelination is most active in rat brain. This activity is maintained in the adult rat.[1]

Procedures for Determination of Enzyme Activity

Treatment of Rats. Long–Evans rats were used. Sex of the rats was found to have no influence on hydrolysis of esterified cholesterol by the brain. Each rat was anesthetized with ether; its abdominal aorta and jugular veins were cut, and its brain was perfused with about 20 ml of a 0.9% (w/v) NaCl solution *via* the innominate artery. The brain above the foramen magnum was rapidly excised and weighed.

Brain Homogenization. Brains were transferred to 25-ml Potter–Elvehjem homogenizing tubes containing 8 volumes (v/w) of ice cold: (a) 0.9% (w/v) NaCl, (b) 0.25 M or 0.32 M sucrose, (c) 0.15 M citrate–sodium phosphate buffer of pH 7.5, or (d) a 1:1 (v/v) mixture of the buffer and NaCl solutions. Hydrolytic cholesterol esterase activity of a brain homogenate was found not to depend on which of these solutions was used. Brains were homogenized for 30 sec with a loose-fitting Teflon pestle rotating at 1000 rpm. Varying homogenization time from 15 to 90 sec had no influence on the hydrolytic activity of a brain homogenate.

Incubation Procedure. An aliquot of a brain homogenate (or fraction of the homogenate, obtained by centrifugation) was mixed with a known volume of a 0.15 M citrate–sodium phosphate buffer (McIlwaine, 1921)

* From the Department of Physiology, University of California, Berkeley, California. *Received August 2, 1965; revised March 18, 1966.* This investigation was supported by a grant from the U. S. Public Health Service.

† U. S. Public Health Service predoctoral fellow of the National Institute of General Medical Sciences (1 F1 GM-21, 716-01A1).

‡ This manuscript was completed before Dr. Chaikoff's untimely death on Jan 25, 1966.

[1] The term *adult rat* refers to rats of about 3 months of age (200 g).

of pH 7.5 and 0.05 ml of acetone containing [4-^{14}C]-cholesterol in the form of cholesteryl linoleate ([^{14}C]-CE).[2] The mixture was incubated, at 37°, with shaking for 1 hr, after which 25 volumes of a chloroform–methanol solution were (2:1 ,v/v) added. The mixture, which contained a single liquid phase, was left overnight at room temperature.

Analytical Procedures. The chloroform–methanol extract of the incubation mixture was transferred to a separatory funnel along with 26.5 ml of distilled water to provide an 8:4:3 volume ratio of chloroform–methanol–water (Folch *et al.*, 1957). The lipid-containing chloroform layer was collected and evaporated to dryness under reduced pressure. The lipid residue dissolved in a benzene–hexane mixture (1:1, v/v) was chromatographed on a Unisil column (Creech and Sewell, 1962) to separate esterified from free cholesterol. The solvents were evaporated from the eluted fractions, and the residues, dissolved in 13 ml of toluene containing 39 mg of 2,5-diphenyloxazole and 1.3 mg of 1,4-bis-2-(5-phenyloxazolyl)benzene, were assayed for ^{14}C content with a Packard Tri-Carb liquid scintillation spectrometer. Hydrolytic activities have been corrected for the presence of free [^{14}C]cholesterol in control samples incubated without brain.

Experimental Basis for Enzyme Assay Procedure

pH Requirement. A pH range from 3.0 to 8.5 was studied a 0.5-unit intervals. With the citrate–phosphate buffer, maximal hydrolytic activity was observed when the pH of the incubation mixture was 7.5.

Incubation Time. A 1-hr incubation period was chosen after we found that extending the period of incubation to as much as 18 hr led to no further hydrolysis.

Choice of Substrate. Gas chromatographic analysis of the fatty acid moiety of cholesterol esters isolated from young rat brains showed that linoleic acid is present in greatest abundance. Hence, [4-^{14}C]cholesteryl linoleate was used as the substrate for determinations of hydrolytic cholesterol esterase activity. We found that [4-^{14}C]cholesteryl oleate could be used with similar results.

Preparation of Substrate. [4-^{14}C]Cholesterol (0.63 μc/mg) was esterified with linoleic acid, either enzymatically (Swell and Treadwell, 1962) with a pancreas powder (Nutritional Biochemicals Corp., Cleveland, Ohio) or chemically *via* linoleyl chloride (Pinter *et al.*, 1964). The labeled cholesterol ester was purified by column chromatography on Unisil (Creech and Sewell, 1962), and stored at −15° in a benzene–hexane solution (1:1, v/v, 35 μg/ml). This stock solution had to be rechromatographed at least once every 6 weeks; otherwise the concentration of free [^{14}C]cholesterol in the labeled cholesterol ester solution rose above 5% of the ^{14}C present. For each experiment, a known amount of the stock solution was evaporated to dryness under nitrogen, and the residue was dissolved in acetone.

Substrate Solubilization. Solubilization of the [^{14}C]CE with either Tween-20 or Triton X-100 was not always found to be complete. Chylomicrons (Lossow *et al.*, 1962) and plasma β-lipoproteins (Lossow *et al.*, 1963) were used to solubilize the [^{14}C]CE, but in these cases unlabeled cholesterol esters prohibitively diluted the labeled substrate. Therefore organic lipid solvents miscible with water were tested. Acetone solubilization was found to be most satisfactory; the [^{14}C]CE was added in 0.05 ml of acetone/5 ml of incubation medium (Table I).

TABLE I: Effect of Acetone on the Hydrolytic Cholesterol Esterase Activity in Adult Rat Brain.[a]

Acetone Added (ml)	% of [^{14}C]CE Hydrolyzed
0.05	80
	81
0.10	71
	69
0.15	57
	60
0.20	31
	39

[a] An 11% (w/v) homogenate of adult rat brain prepared in a 0.9% (w/v) NaCl solution was spun for 10 min at 800*g*. Duplicate 2-ml portions of the supernatant were each incubated for 1 hr at 37° with 3 ml of a citrate–sodium phosphate buffer (pH 7.5) and about 0.25 μg of [^{14}C]CE contained in various amounts of acetone.

Subcellular Fractionation

A 10% (w/v) homogenate of adult rat brain was prepared in a 0.32 M sucrose solution (Wolfe and McIlwain, 1961), and portions were incubated with [^{14}C]CE (Table II). The various subcellular fractions listed in Table II were prepared from the brain homogenate by differential centrifugation (Wolfe and McIlwain, 1961), in a room maintained at about 2°. Before incubation, the particulate fractions were washed four times by resuspension in 0.32 M sucrose and recentrifugation; the washed pellets were then resuspended in the volumes of 0.32 M sucrose from which they were originally obtained, and portions of the suspensions were incubated with [^{14}C]CE. To assess the effect of the time required to prepare the subcellular fractions upon hydrolytic enzyme activity, samples of the homogenate and its subcellular fractions were incubated after storage at about 2° for periods of up

[2] The abbreviation [^{14}C]CE stands for [4-^{14}C]cholesteryl linoleate; weights of [^{14}C]CE are expressed as micrograms of [4-^{14}C]cholesterol that were esterified; ATP, adenosine triphosphate; CoA, coenzyme A.

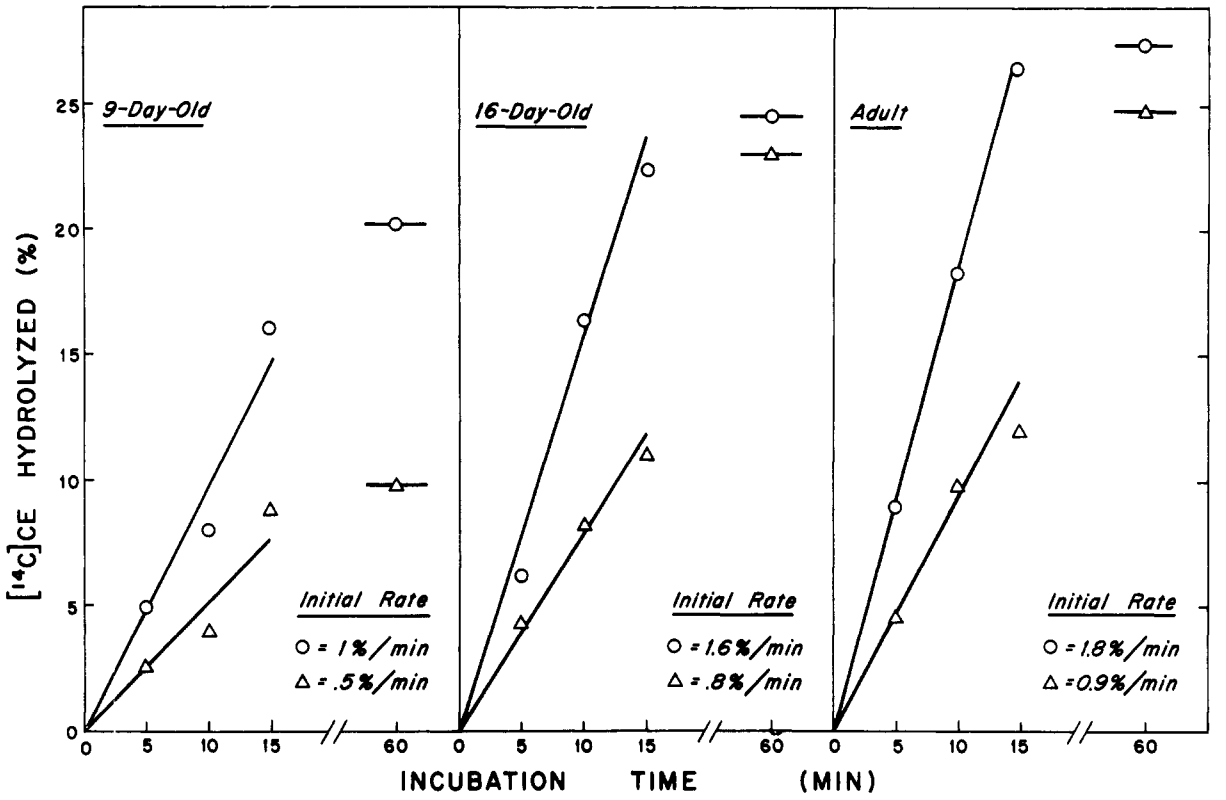

FIGURE 1: Initial rates of hydrolytic cholesterol esterase activities in brains of 9-day-old and adult rats. Portions (2 ml) of 10% (w/v) brain homogenates were incubated for various times at 37° with 3 ml of a citrate–sodium phosphate buffer (pH 7.5) and either 0.25 or 0.50 μg of [^{14}C]CE contained in 0.05 ml of acetone. Mean values of duplicate determinations obtained with 0.25 μg of the labeled substrate are presented as ○—○; those with 0.50 μg as △—△.

TABLE II: Hydrolytic Cholesterol Esterase Activity in Subcellular Fractions of Adult Rat Brain.[a]

Sample	% of [^{14}C]CE Hydrolyzed
Whole homogenate	51 (49–53)
800*g* pellet	27 (23–34)
800*g* supernatant	37 (33–45)
15,000*g* pellet	33 (30–41)
15,000*g* supernatant	25 (21–27)
105,000*g* pellet	33 (31–38)
105,000*g* supernatant	1 (0–3)

[a] By conventional centrifugation methods, subcellular fractions were obtained from a 10% (w/v) homogenate of adult rat brain prepared in a 0.32 M sucrose solution (Wolfe and McIlwain, 1961). Particulate fractions were washed, and each was resuspended in a volume of 0.32 M sucrose solution equal to that from which it had been isolated. Hydrolytic activity of each fraction was determined for at least four 2-ml samples incubated for 1 hr at 37° with 3 ml of a citrate–sodium phosphate buffer (pH 7.5) and about 0.25 μg of [^{14}C]CE contained in 0.05 ml of acetone. Mean values and ranges are given.

to 3 hr. No effect of storage time upon the enzyme activity was detected.

Since all particulate fractions were resuspended in the original volumes from which they had been isolated, the percentages of [^{14}C]CE hydrolyzed (Table II) represent relative measures of the total hydrolytic activities of the various subcellular brain fractions. Practically all hydrolytic activity was associated with the particulate fractions, cell sap being almost devoid of hydrolytic activity. All particulate fractions were hydrolytically active to about the same extent.

Determination of Endogenous Substrate Concentration

When the hydrolysis of [^{14}C]CE is to be used as a measure of total enzyme activity, the possibility of dilution of [^{14}C]CE by endogenous (unlabeled) esterified cholesterol of the brain must be considered. About 17 μg of esterified cholesterol/g of brain were found at 1 and 5 days of age; at 11 days the concentration increased to 28 μg, declining thereafter to 18, 10, and 7 μg/g of brain at 16, 19, and 90 days of age, respectively. Hence, the [^{14}C]CE could be diluted to varying extents, depending on age of the rats. But knowledge of the total concentration of cholesterol esters in brain does not tell us about their formation

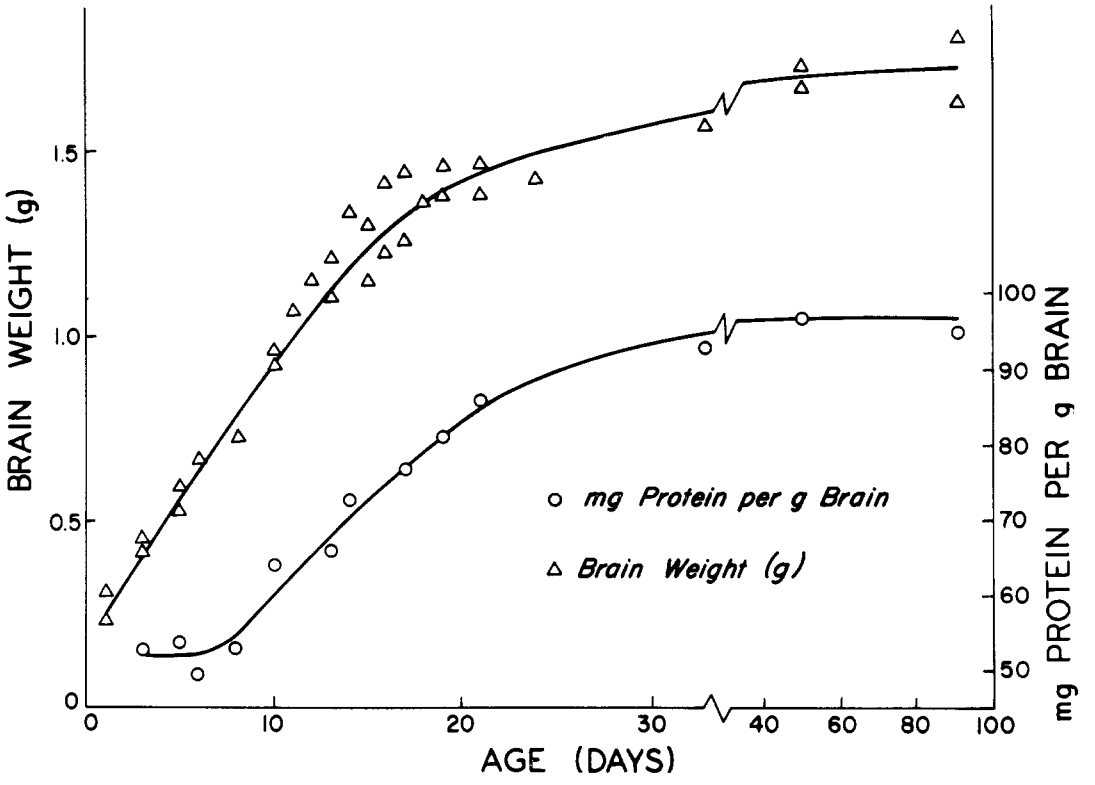

FIGURE 2: Weights and protein concentrations of brains in rats of various ages. Brain weights were determined for 1–12 rats at each of the ages studied. Ranges of the observations are plotted. Protein contents per gram of brain were determined for the rats presented in Figure 4.

or turnover, nor whether the age of the rat affects that turnover. Moreover, we must know what proportion of endogenous cholesterol esters is available as substrate for the hydrolytic cholesterol esterase enzyme, and the influence, if any, of the rat's age on that proportion.

Information about the extent of dilution of [^{14}C]CE by endogenous cholesterol esters in our enzyme assay can be obtained from a study of initial rates of hydrolysis in brain homogenates of rats at different ages, on the basis of the following rationale. Assume that, in a given brain homogenate, an amount (S_e μg) of endogenous cholesterol esters contributes to the pool of substrate for our hydrolytic enzyme. When portions of this homogenate are incubated with different amounts of [^{14}C]CE, of which the smallest amount (S_1) is already large enough to produce enzyme saturation, the same (maximal) initial rate of hydrolysis will obtain in all cases, X μg of cholesterol esters is hydrolyzed/min. Hence, $[X/(S_e + S_1)] \times 100$ represents the percentage of the total amount of cholesterol esters present (*i.e.*, labeled plus unlabeled) that is initially hydrolyzed per minute when S_1 μg of [^{14}C]CE is added. Likewise, with a larger amount (S_2 μg) of the labeled substrate, $[X/(S_e + S_2)] \times 100\%$/min is initially hydrolyzed. The ratio (R) of these two expressions for the initial rate of hydrolysis with S_1 and S_2 μg of [^{14}C]CE, respectively, equals $(S_e + S_2)/(S_e + S_1)$. Hence, S_e can be derived, after a value for R is obtained by expressing the initial rate of hydrolysis in terms of percentages of added amounts of [^{14}C]CE converted per minute. Knowing, then, the endogenous contribution to the substrate pool for our hydrolytic enzyme, we find the total amount of cholesterol esters hydrolyzed per minute (X) from our equation for the maximal initial rate of hydrolysis.

Initial rates of hydrolysis were established by incubating two different amounts of labeled cholesterol esters, 0.25 and 0.50 μg, for various time intervals with brain homogenates of 9-day-old, 16-day-old, and adult rats (Figure 1). For each of these ages, the percentages of [^{14}C]CE hydrolyzed per minute, during the first 15 min, were directly proportional to the amounts of added substrate (Figure 1), which means that the smaller amount, 0.25 μg, of [^{14}C]CE was already large enough to initially produce enzyme saturation. For each of the three ages studied, a value of 2 is obtained for the ratio (R) of the initial rates of hydrolysis using 0.25 and 0.50 μg of [^{14}C]CE (Figure 1). In this case $R = 2 = (S_e + 0.50)/(S_e + 0.25)$, from which it follows that $S_e = 0$. Therefore, during this period of brain development, in which cholesterol esters accumulate, it is shown that, with our enzyme assay, no endogenous cholesterol esters are available as substrate for the hydrolytic enzyme.

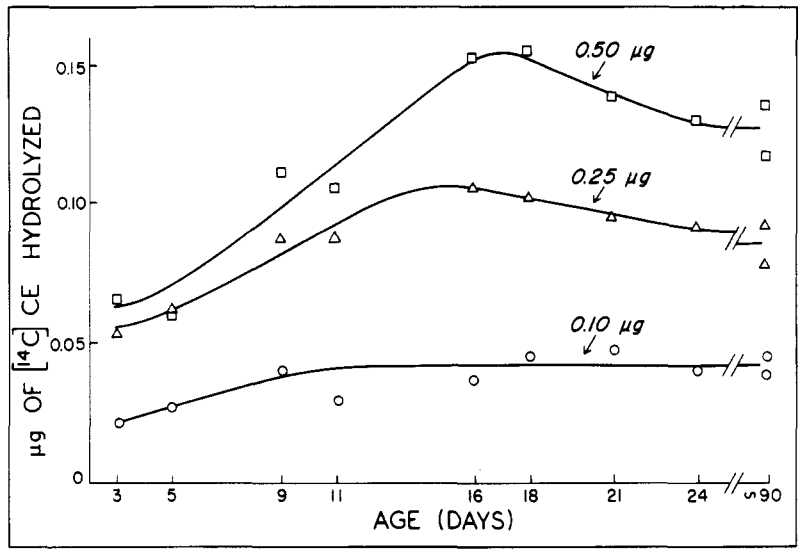

FIGURE 3: Hydrolytic cholesterol esterase activity/7 mg of brain protein in rats of various ages. A 16% (w/v) brain homogenate was prepared from rats at each of the ages studied. The homogenates were spun at 800*g* for 10 min, and the volumes of supernatants were adjusted to contain about 7 mg of protein/ml. Portions (1 ml) of the brain preparations were incubated with 0.10, 0.25, or 0.50 μg of [^{14}C]CE/2 ml of incubation mixture.

Age Effect

Since endogenous cholesterol esters are not available as substrate in our enzyme assay, we may use hydrolysis of [^{14}C]CE to compare the levels of hydrolytic cholesterol esterase activity in rat brains during various stages of development. Figure 1 shows that the initial rate of hydrolysis greatly increases between the ages of 9 and 16 days, from 2.5 to 4.0 mμg of [^{14}C]CE hydrolyzed/min. Only slightly more, 4.5 mμg of [^{14}C]CE, was hydrolyzed per minute in the case of the adult rat.

With all ages studied, hydrolysis of [^{14}C]CE stops within 1 hr of incubation. The final levels of hydrolysis obtained after 1 hr are largely determined by the periods over which the maximal rates of hydrolysis prevail. Among the factors that influence the duration of maximal enzyme activity are inactivation of the enzyme and depletion of substrate—both susceptible to an age effect. In the 9-day-old (Figure 1), both the initial rate (2.5 mμg of [^{14}C]CE hydrolyzed/min) and the final level of hydrolysis (0.05 μg of [^{14}C]CE hydrolyzed) were independent of whether 0.25 μg or 0.50 μg of substrate was used. Evidently, for this age, the enzyme was saturated with substrate during the full period of its activity, even with the lower substrate concentration. In the 16-day-old and adult, however, even though the amounts of [^{14}C]CE initially hydrolyzed per minute did not depend on the substrate concentrations used, the final levels of hydrolysis did (Figure 1). In the adult, our most active enzyme preparation as judged by the initial rate, the total amount of [^{14}C]CE hydrolyzed in 1 hr was the same when either 0.50 μg or 1.0 μg of substrate was used, which indicates that 0.50 μg of [^{14}C]CE allows enzyme saturation during the entire period of enzyme activity. The finding, then, that in the 16-day-old and the adult rat brains the final amounts of [^{14}C]CE hydrolyzed with 0.25 μg of substrate were smaller than those observed with 0.50 μg (Figure 1) suggests that for these ages 0.25 μg of [^{14}C]CE did not saturate the enzyme during the later part of its active period. However, the final levels of hydrolysis obtained with 0.25 μg of substrate (Figure 1) are still useful for qualitative comparison of the enzyme activities in brains of rats of various ages.

In young rats, both the weights and protein concentrations of the brains increase with age (Figure 2); hence, the effect of age on the brain's hydrolytic cholesterol esterase activity must be related to weights as well as to protein contents of the brains. Two experimental approaches were chosen: (A) for each age studied, the same amount of brain protein was incubated in order to compare hydrolytic activities for a given weight of protein (Figure 3); and (B) the same brain weight was incubated for all ages in order to compare hydrolytic activities for a given weight of brain (Figure 4).

A. Hydrolytic Cholesterol Esterase Activity per Given Weight of Brain Protein in Rats of Various Ages. A brain homogenate prepared in 5 volumes of buffer–saline solution (1:1, v/w) was spun at 800*g* for 10 min. The protein concentration of the supernatant was determined (Gornall *et al.*, 1949) and then adjusted with the buffer–saline solution to 7 mg/ml. Portions (1 ml) of this brain preparation were mixed with 1-ml amounts of 0.15 M citrate–sodium phosphate buffer and incubated at pH 7.5 for 1 hr at 37°, each with 0.05, 0.10, 0.25, or 0.50 μg of [^{14}C]CE.

When 0.05 μg of [^{14}C]CE was used, no effect of age on the hydrolytic activity was found (not shown

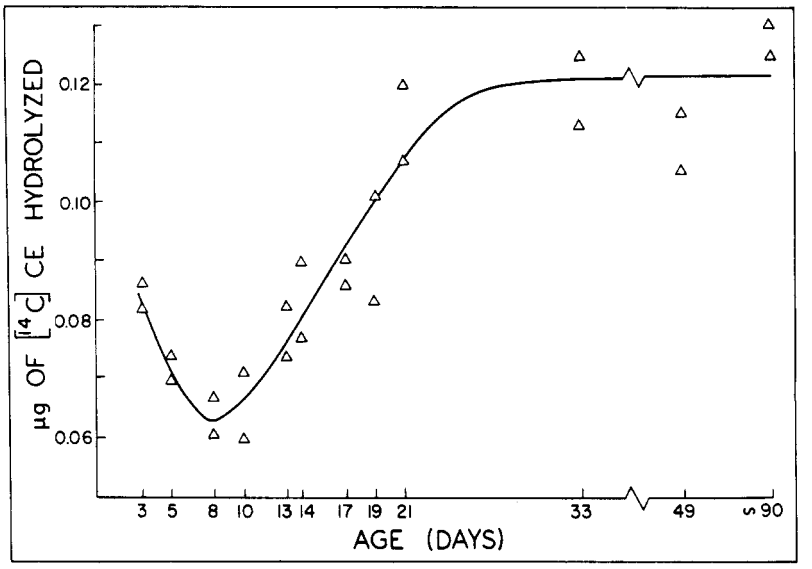

FIGURE 4: Hydrolytic cholesterol esterase activity per 0.33 g of brain in rats of various ages. Duplicate 2-ml portions of a 16% (w/v) brain homogenate prepared from rats at each of the ages studied were incubated with about 0.25 μg of [^{14}C]CE.

in Figure 3). In this case the low substrate concentration, rather than the enzyme activities, seemed to limit the extent of hydrolysis. With 0.1 μg of [^{14}C]CE, a small effect of age became noticeable (Figure 3); limited enzyme activity was indicated for the early ages. With 0.25 or 0.50 μg of [^{14}C]CE, a marked increase in hydrolytic activity between the fifth and sixteenth day of age was found (Figure 3); the hydrolytic activity decreased slightly between about 18 and 24 days of age, and leveled off thereafter.

To further evaluate the effect of age, the enzyme activity per 7 mg of protein was related to the increase of total brain protein with age. Between the first and fourth weeks of age, both brain weight and protein concentration increased (Figure 2), so that protein accumulation for the whole brain was particularly rapid. During most of this period of very active protein acquisition, enzyme activity per given weight of protein increased rapidly (Figure 3), indicating that the rate of formation of the hydrolytically active enzyme protein was faster than that of other proteins in the brain until the sixteenth day of age. The slight decrease in the activity per 7 mg of protein observed between about 18 and 24 days of age (Figure 3) suggests that during this period of continued rapid formation of total brain protein (Figure 2) less of the enzyme protein is produced than of other proteins. After 24 days of age the slow formation of total brain protein (Figure 2) is apparently matched by a constant and equally slow production of hydrolytic activity, so that the activity for a given weight of total brain protein remains constant (Figure 3).

B. Hydrolytic Cholesterol Esterase Activity per Given Weight of Brain in Rats of Various Ages. Brains were homogenized in 5 volumes (v/w) of 0.9% (w/v) NaCl solution. Duplicate 2-ml portions of an homogenate, corresponding to about 0.33 g of brain, were incubated for 1 hr at 37° with 3 ml of citrate–phosphate buffer (pH 7.5) and about 0.25 μg of [^{14}C]CE.

Brains of rats 1 day before and 1 day after birth were found to hydrolyze about 0.09 μg of [^{14}C]CE in an experiment carried out on a different day. The hydrolytic activity decreased during the first days after birth (Figure 4), then increased rapidly between the first and fourth weeks, and finally leveled off.

Apparently, the rat is born with hydrolytic cholesterol esterase activity in its brain. During the first 8 days after birth, because the weight of the brain increases faster than does its enzyme activity, a drop in the activity per unit of brain weight occurs. Between the first and fourth weeks of age the brain acquires additional hydrolytic activity faster than its weight increases. Thereafter the small increases in brain weight and enzyme activity parallel each other, so that there is no further increase in the activity for a given weight of brain.

Discussion

Development of hydrolytic cholesterol esterase activity in the rat brain may be divided into three phases. (a) During the first days after birth the brain grows rapidly (Figure 2). Acquisition of the enzyme activity does not keep pace with the formation of hydrolytically inactive material, as judged from the decline in hydrolytic activity per gram of brain (Figure 4). Since the protein concentration did not change (Figure 2) during the period of decreasing enzyme activity per gram of brain, a decrease in hydrolytic activity per unit weight of total brain protein during this early phase

of development would be expected. But no such decrease was observed in the 800*g* supernatant fraction prepared from brain homogenates (Figure 3). Apparently, more hydrolytically inactive than active protein had been removed by centrifugation at 800*g*.

(b) Between the first and fourth weeks of age, the increase in brain weight (Figure 2) is surpassed by the rate of appearance of hydrolytic activity, so that the activity per gram of brain rises (Figure 4). Similarly, the increase in protein concentration during this phase of development (Figure 2) is exceeded by the rate at which hydrolytic cholesterol esterase activity appears, and a gain in hydrolytic activity per milligram of protein results (Figure 3).

(c) After the third week, the growth rate of the brain gradually levels off (Figure 2). The rates of acquisition of total protein (Figure 2) and of hydrolytic activity both become proportional to the weight increase of the brain, so that the enzyme activity expressed either per gram of brain (Figure 4) or per milligram of brain protein (Figure 3) levels off.

In 1928 Shope demonstrated hydrolysis of cholesterol esters by guinea pig brain preparations. However, Sperry and Brand (1941) reported no such hydrolytic activity in rat brains. The transient occurrence of esterified cholesterol in the developing brain (Mandel *et al.*, 1949; Brante, 1949; Adams and Davison, 1959), in addition to histochemical evidence for the presence of esterified cholesterol in developing, but not in mature, myelin (Adams and Davison, 1959, 1960), led the latter authors to suggest that esterified cholesterol is a precursor of free cholesterol in brain myelin. A precursor role of esterified cholesterol implies hydrolysis of the ester at some stage of myelination. This led us to search for the appearance in brain of a hydrolytic cholesterol esterase enzyme. Pritchard and Nichol (1964), reasoning similarly, reported preliminary evidence for the presence of hydrolytic cholesterol esterase activity in rat brains; these authors found no change in the hydrolytic activity in brains of 1- to 40-day-old rats. It is shown here, however, that rat brain acquires this enzyme activity predominantly between the first and fourth weeks of age. The transient occurrence of sterol esters in the brain during early development may thus be related to a paucity of hydrolytic esterase activity during this period.

An alternative explanation for the occurrence of sterol esters in the brain could be the presence of esterifying enzyme activity. However, esterification of [4-^{14}C]-cholesterol in slices, homogenates, and acetone powders of brains of rats between 1 and 90 days of age escaped our detection. Of course, the possibility cannot be ruled out that a preponderance of hydrolytic activity masked any esterifying activity present in the same assay mixture. There was, however, no sign of esterifying cholesterol esterase activity at any pH, even with addition of combinations of the most likely cofactors and cosubstrates (fatty acids, lecithin, ATP, CoA, Mg, F, bile salts, and nicotinamide), and even though these same conditions are suitable for esterification of cholesterol in numerous other tissues that also contain hydrolytic cholesterol esterase activity (such as liver, plasma, adrenal, pancreas, and small intestine). These findings could well mean that rat brain is devoid of esterifying cholesterol esterase activity.

In view of (a) the apparent inability of the brain to esterify cholesterol, and (b) the accumulation of sterol esters in developing brain, it may be proposed that not cholesterol itself but rather its sterol precursors are esterified, and that these esterified precursors then accumulate. Such esterification could serve to protect the 3β-OH group of the sterol precursors during their conversion to cholesterol. Further evidence for this view may be our finding that endogenous sterol ester is not available as substrate for the hydrolytic cholesterol esterase: the esterified sterols may consist of noncholesterol, digitonin-precipitable compounds for which the hydrolytic enzyme studied is not specific.

Myelination of the rat brain extends from about 10 to 40 days of age, a period that includes the time of maximal development of hydrolytic cholesterol esterase activity. This coincidence in time suggests that hydrolysis of esterified cholesterol plays a role in the process of myelination, although additional functions for this enzyme in the brain may exist. It is interesting to note that maximal cholesterol esterase activity is maintained in the brain long after myelination is completed, at least through the 90th day of age (Figures 3 and 4). Possible functions for the enzyme might include hydrolysis of plasma cholesterol esters to provide either essential fatty acids or sterols required for growth and sustenance of the brain.

Acknowledgment

The technical assistance of Miss Sandra K. Stoddard is gratefully acknowledged.

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Polymerization–Depolymerization of Tobacco Mosaic Virus Protein. VII. A Model*

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ABSTRACT: A model is presented which imitates the major aspects of the endothermic polymerization of tobacco mosaic virus protein. The model involves the assumption that the increase in entropy necessary to drive the polymerization comes from the liberation of water from different kinds of water binding centers located at different positions on the surface of the monomeric unit. Each center is assumed to have its own $\Delta H°$ and $\Delta S°$ for water "binding" and its own "melting temperature," defined as the temperature at which $\Delta H° - T\Delta S°$ is 0. Below its "melting temperature," contact with water will be a thermodynamically favorable environment for a center, above, isolation from water. Change from an aqueous to a nonaqueous environment can in some cases be achieved by rotation of monomeric centers and in others by polymerization.

The following facts which bear on the mechanism of polymerization of tobacco mosaic virus (TMV) protein, first described in detail by Schramm (1943), have been established: (1) Polymerization is endothermic (Lauffer *et al.*, 1958). (2) The starting material, commonly known as TMV "A" protein, because it was first obtained by alkaline degradation of the virus, is a stable trimer of the ultimate protein subunit (Banerjee and Lauffer, 1966). (3) The ultimate protein subunit with a molecular weight of 17,500 is obtained at pH 6.5 at very low concentration (Ansevin and Lauffer, 1959), in 67% acetic acid (Banerjee and Lauffer, 1966; Anderer *et al.*, 1964), and in solution at pH 13 (Anderer, 1959; Wittmann, 1959). (4) At temperatures between 4 and 14°, in 0.1 ionic strength buffer at pH 6.5, the reaction follows the mathematics of condensation polymerization with values of $\Delta H°$ and $\Delta S°$ of 30,000 cal/mole and 124 eu, respectively (Banerjee and Lauffer, 1966). (5) In the temperature range 14–*ca.* 20°, under the same conditions, the reaction also follows the pattern of condensation polymerization, but with much higher values of $\Delta H°$ and $\Delta S°$, approximately 190,000 cal/mole and 682 eu, respectively (Smith, 1961; Lauffer, 1962, 1964b). (6) A double disk consisting of 32 ultimate protein subunits is a stable intermediate, certainly in the dry state (Markham *et al.*, 1963) and possibly in the wet state, because a sedimenting boundary with approximately the right sedimentation coefficient, 20–25 S, can be observed (Schramm and Zillig, 1955; Lauffer *et al.*, 1958; Caspar, 1963). (7) Further polymerization leads to stacked disks (Markham *et al.*, 1964) which rearrange to form helical structures resembling the protein in TMV (Franklin, 1955, 1956; Markham *et al.*, 1964). At temperatures of 25–30° in 0.1 ionic strength buffer at pH 6.5, these rodlike polymers can be about the size of TMV particles (Lauffer *et al.*, 1958). (8) The polymerization is accompanied by the release of "bound" water molecules and by an increase in volume (Stevens and Lauffer, 1965; Lauffer, 1964a). (9) Polymerization involves a decrease in negative charge of 2–3 *f*/mole of trimer, but this is accompanied by a

* From the Department of Biophysics, University of Pittsburgh, Pittsburgh, Pennsylvania, and The Max-Planck-Institut für Virusforschung, Tübingen, Germany. *Received February 9, 1966; revised April 29, 1966.* Publications I–VI of this series are Ansevin and Lauffer (1963), Lauffer (1964a), Ansevin *et al.* (1964), Stevens and Lauffer (1965), Lauffer (1966b), and Banerjee and Lauffer (1966), respectively. This is publication No. 122 of the Department of Biophysics, University of Pittsburgh. Work was supported by a U. S. Public Health Service grant (GM 10403).