• TECHNICAL

SYMPOSIUM: BRAIN STEROL METABOLISM conducted by The American Oil Chemists' Society at its 56th Annual Meeting, Houston, Texas April 25-28, 1965

DAVID KRITCHEVSKY, Program Chairman

Brain Cholesterol. XI. A Review of Biosynthesis in Adult Mice

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Abstract

During the past decade, our laboratory has been involved in studying the biosynthesis of brain cholesterol under various conditions. As a result of these studies, we feel that the hypothesis of metabolic stability of cholesterol in adult life is untenable. Our data suggest that there are several compartments of sterol metabolism ranging from extremely fast to metabolically very slow. It is our hypothesis that (a) brain function is more nearly associated with fast turnover compartments than with metabolically slow ones; (b) these compartments can be altered by a variety of stress conditions.

Current experimental work being done with monkeys and baboons, and in human fetii suggest that conclusions formed about the mouse generally are applicable to these higher evolutionary forms.

What remains unsolved is the relationship between cholesterol metabolism and brain function. We continue to search for answers to physiological psychology.

Introduction

THE FOLLOWING IS NOT INTENDED to be a complete survey of the subject of brain cholesterol. I would like to emphasize those aspects of the problem studied by our laboratory. We have attempted to clarify the biosynthesis of cholesterol during development and aging. Our efforts have been directed toward study, with various precursors, of the alteration of brain cholesterol metabolism during various stress conditions. These stress factors include fasting, transitional changes that occur during development and aging, muscular dystrophy, cancer, and drugs. Primarily, these processes have been studied in mice. This report is a summary of our findings accumulated over the past decade.

Material and Methods

Radioactive Precursors

All radioactive nutrients were dissolved in physiological saline to which benzol alcohol (0.9%) was added as a preservative. The resulting solutions were injected intraperitoneally. In all experiments, we made simultaneous use of H^3 and C^{14} precursors (1,2).

Experimental Design

Mice were killed 15 min after isotope injection by decapitation and exsanguination. In all cases, organs were rapidly removed; washed in distilled water; and quick-frozen in a mixture of dry ice and acetone. Tissue samples were kept in the frozen state until required for analysis.

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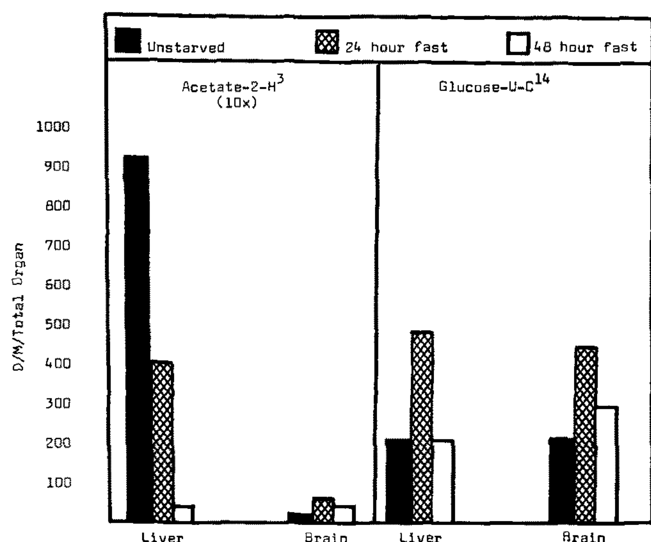

FIG. 1. Incorporation of labeled nutrients into tissue free cholesterol 15 min after isotope injection.

Isolation and Assay of Radioactive Cholesterol

Colorimetric determination of cholesterol content and radioactive assay of isotope incorporation were made on the same tissue extracts. Procedure for the isolation and assay of C^{14} and H^3 labeled cholesterol was a modification of earlier methods (3). Plasma, red blood cell, and tissue cholesterol were extracted with acetone: alcohol (1:1). Digitonin, in 50% alcohol, was added to precipitate the free sterol. The resulting filtrates were then assayed for esterified cholesterol. Digitonide, resulting from the precipitation of free cholesterol, was dissolved in dioxane. A portion was taken for quantitative colorimetric determination; the remainder was assayed simultaneously for C^{14} and H^3 in a liquid scintillation counter (1,2). The resulting data were reported either in terms of specific activity, activity per gram tissue, or activity per total organ.

During the past ten years, a series of evolutionary

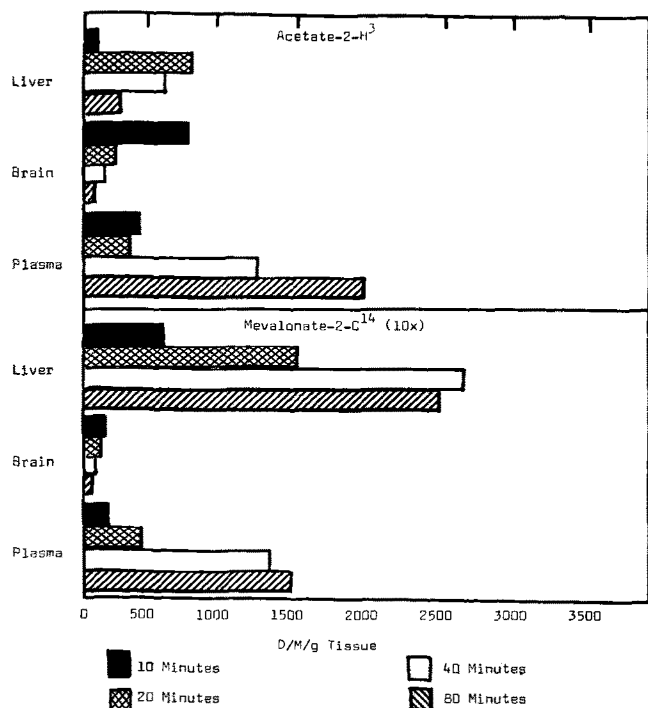

FIG. 2. Labeled nutrients incorporated into mouse tissue sterol at various intervals.

TABLE I
Incorporation of Various Precursors into Free Cholesterol of Brain

Precursor (40 μc/kg)	CF-1 Female Mice		
	Expt. 1.	Expt. 2.	Expt. 3.
	(disintegrations per min per g wet tissue)		
[1-^{14}C]acetate	198[a]	770	1319
[2-^{3}H]acetate	124	310	453
DL-[2-^{14}C]leucine	217	453	596
[2-^{3}H]acetate	120	710	293
D-[^{14}C]glucose[b]	2581	2797	3367
[2-^{3}H]acetate	354	288	503

[a] Each value represents weighted mean of five animals.
[b] Uniformly labelled.

changes were made in the analytical procedure. The introduction of tomatine proved an effective and efficient replacement for digitonin as a more specific agent for the precipitation of cholesterol. These improvements have been reported in the following papers (4,5).

Radiochemical Purity

Purification of cholesterol, isolated either by digitonin or tomatine, was achieved through the dibromide method (3–5). After a variety of precursors, samples isolated even 15 min after injection, were radiochemically pure (4).

Results

The Effect of Starvation

In all of our reported studies, solid food was taken from the mice 24 hr prior to isotope injection. It was of interest to study the effect of fasting on cerebral metabolism.

Cholesterol synthesis, as measured by the incorporation of labeled precursors, is markedly influenced by the physiological state of the animal. Dietary cholesterol decreases the rate of isotope incorporation (6). Hutchens et al. (7) showed that the rate of biosynthesis is decreased during starvation. These observations have subsequently been confimed in a number of laboratories (8,9). Although brain lipid levels have been reported as not being affected by starvation (10), our tracer experiments were at variance with this conclusion.

As can be seen from Figure 1, starvation decreases the amount of incorporation of acetate-2-H^3 in the liver by almost 50% in 24 hr and 90% in 48 hr. Contrary to the findings in the liver, incorporation of methyl-labeled acetate into brain cholesterol was significantly increased after 24 and 48 hr, the greater increase being noted at 24 hr. The use of glucose-U-C^{14} produced different results in these same animals. Under experimental starvation conditions, glucose was first increased in liver cholesterol and then decreased after 48 hr. Results in the brain, however, were similar to results indicated with acetate; i.e., increases at both time intervals but the greater increase at 24 hr.

Independently of our earlier report (11), Smith demonstrated the effect of fasting on lipid metabolism of the rat brain. In Smith's experiments, only a decrease of acetate-1-C^{14} and glucose-C^{14} incorporation was measured under in vitro conditions at 48 and 72 hr fasting periods.

While the two studies (11,41) are not wholly in agreement, both do indicate that cerebral lipid metabolism can be altered during the fasting state.

Biosynthesis with Selected Precursors

While the majority of studies on cholesterol metabolism involve the use of acetate, it was of interest

TABLE II
Incorporation of Various Precursors into Free Cholesterol of Brain

Precursor (40 µc/kg)	DBA/2 Female mice		
	Expt. 1.	Expt. 2.	Expt. 3.
	(disintegrations per min per g wet tissue)		
[1-^{14}C]acetate	644[a]	685	828
[2-^{3}H]acetate	371	341	426
DL-[2-^{14}C]leucine	69	84	223
[2-^{3}H]acetate	247	582	-
D-[^{14}C]glucose[b]	3750	5020	5218
[2-^{3}H]acetate	528	1136	1238

[a] Each value represents weighted mean of five animals.
[b] Uniformly labelled.

to compare other labeled nutrients. Labeled acetate, leucine, and glucose were used in two different strains of female mice.

The normal adult mice were killed 15 min after intraperitoneal injection of labeled precursors. This was at or before the time of maximal incorporation for lipid labeling for the precursor being studied (12). Both normal CF/1 mice and normal DBA/2 animals incorporated the same amount of acetate-1-C^{14} into brain cholesterol (Tables I and II). The amount of radioactivity after acetate-2-H^3 administration was one third of 1-C^{14} acetate incorporation. The amino acid leucine, labeled in position-2-, also can act as a precursor to cholesterol. Leucine is metabolized to acetate, labeled in position-1-. With this proposed metabolic scheme as the basis for comparison, the amount of radioactivity that appeared in brain cholesterol from leucine-2-C^{14} represented only about 20% of the expected radioactivity derived as an acetate fragment. In the DBA/2 animals, even greater deviation from anticipated leucine incorporation values was measured.

Activity from 2-C^{14} mevalonate reached its highest value between 40 and 80 min (Fig. 2). Maximum labeling of tissue sterol from 2-H^3 acetate occurred between 10 and 20 min. The exact opposite was observed in the brain: radioactivity was higher with acetate as a precursor to brain sterol than with mevalonate as the precursor.

The failure of mevalonic acid to act as an efficient precursor for sterol metabolism was emphasized by Garattini et al. (14) and Nicholas and Thomas (15). These results reflect either a difference in permeability of the precursor into brain cells or the inability to utilize (activate?) mevalonic acid.

Incorporation of radioactive glucose into brain cholesterol was greater than that of acetate, leucine, or even mevalonate. This contrasted with the more efficient incorporation of mevalonic acid into liver cholesterol. Incorporation of C^{14}-glucose into brain cholesterol was four to ten times greater than that of acetate.

These results are in accord with those of Moser and Karnovsky (13) who also advocated the use of glucose as an indicator of brain cholesterol, rather than acetate.

The Effect of Development on Incorporation

Earlier studies on the subject all agree that the ability of the brain to synthesize cholesterol is lost in adult life (16). At the International Symposium on the Developing Brain (17) I showed that this interpretation of existing data was not warranted, under certain conditions. The calculation of glucose (1µc, C^{14}) and acetate (20µc, H^3) incorporation data on specific activity basis showed that there was a decreased rate with aging. However, where differ-

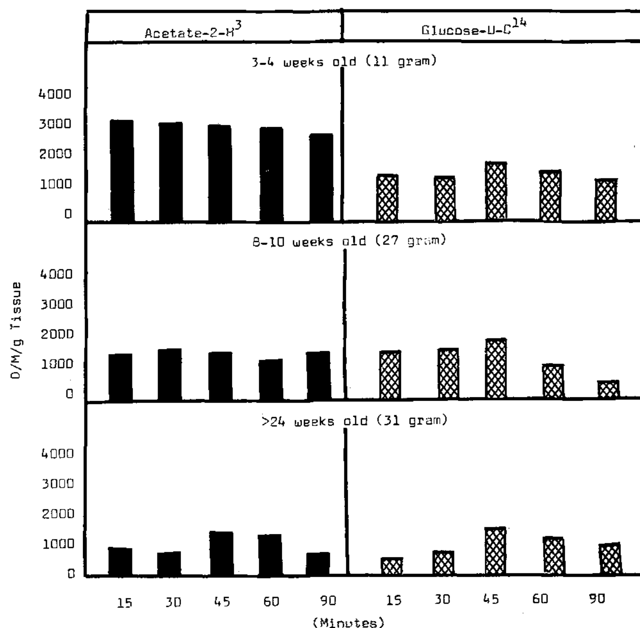

FIG. 3. Effect of development and aging on the incorporation of Acetate-2-H^3 (2.0 µc) and Glucose-U-C^{14} (1.0 µc) into mouse brain sterol.

ences in sterol concentration were taken into account as a diluent of newly synthesized cholesterol, a closer correlation was noted between liver and brain synthesizing ability (Fig. 3). Further, by considering differences in total body weight of animals in various age groups as another factor, there was little if any difference observed in the neural metabolism of younger and older animals. Any difference which may exist suggests greater rather than lower capacity for adult animals. A fourth corrective factor could also be introduced. If cell density of tissues was examined, the total number of cells (neurons, glia, and vascular cells) in brain was calculated to be ten times less per unit weight than liver. Using such a qualifying factor, the brain compares even more favorably with liver as an active site for cholesterol synthesis. Experiments reported here indicated that the brain of adult mice seems to have the same or greater synthesizing capacity as that of younger animals but only when proper qualifying corrective factors are applied to the data.

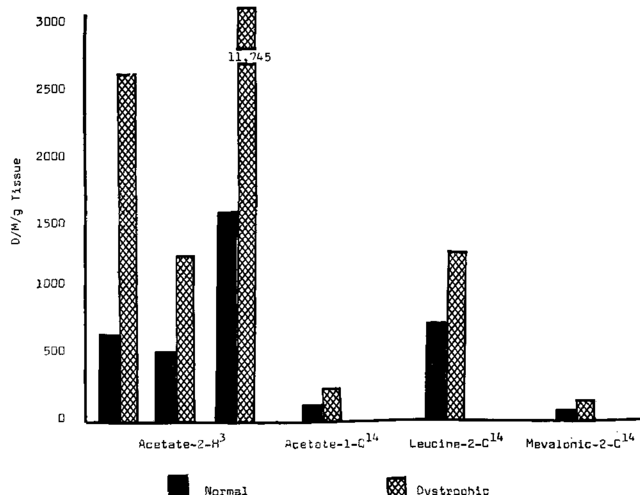

FIG. 4. The effect of muscularis dystrophia on incorporation of labeled nutrients into brain sterols.

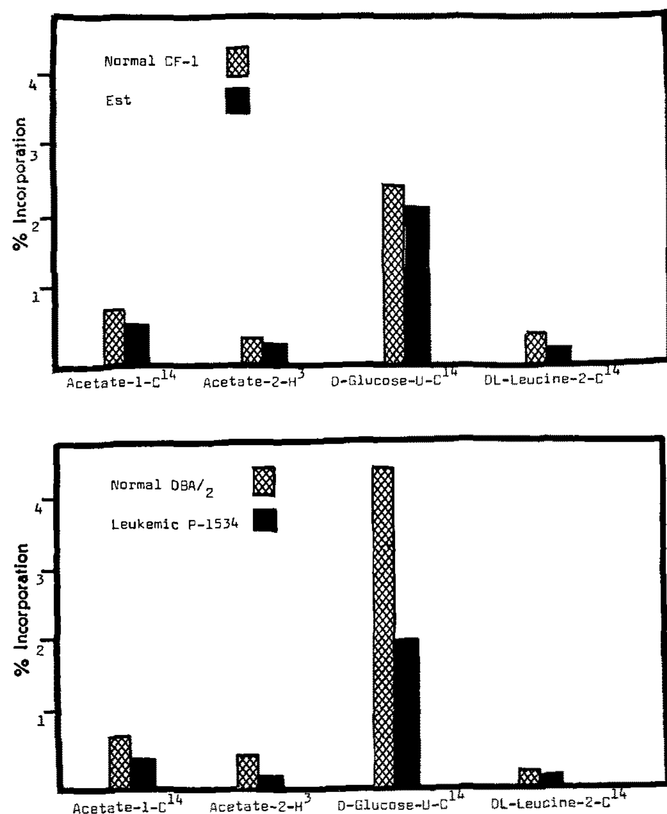

FIG. 5. Effect of Ehrlich Solid Tumor (EST) and a lymphatic leukemia (P-1534) on brain sterol metabolism.

Dystrophia Muscularis and Brain Cholesterol Metabolism

Most reports dealing with lipids in muscular dystrophy show an increase during the dystrophic process (18). I have attempted to study this process by the use of labeled nutrients in a mutant mouse strain with inherited muscular dystrophy, Dystrophia Muscularis (19–21). In general, our results with acetate-1-C^{14} and acetate-2-H^3 showed an increased incorporation of methyl labeled acetate into tissue of dystrophic animals. The greatest and most consistent increase in incorporation took place into cholesterol of the brain. Similar but less marked changes were noted when acetate-1-C^{14} was used as the precursor (Fig. 4).

DL-2-C^{14} leucine and 2-H^3 acetate were injected simultaneously into normal and dystrophic mice. Simultaneous use of acetate and leucine in these experiments suggested that dystrophic mice incorporated the same or more isotope in tissue cholesterol than do normal animals. The most significant changes, measured by either isotope, were the increased incorporation of acetate and leucine into brain and muscle cholesterol of dystrophic animals. The increased radioactivity measured in tissues of dystrophic animals was not quite as significant, in terms of difference, as those found for brain sterol.

Further studies with 2-H^3 acetate and 2-C^{14} mevalonate also indicated that muscle and brain cholesterol metabolism were altered. The most pronounced effect was the increase in isotope concentration in brain, free cholesterol. Although poorly utilized by brain tissue, mevalonate is a more direct precursor to squalene and cholesterol in mammalian tissue than acetate. The increased incorporation of mevalonic acid and other precursors in diseased animals suggests either (a) alteration in cell permeability between normal and dystrophic tissues or (b) a change in brain metabolism as cause or effect of specific changes occurring in the dystrophic muscle.

These results are in agreement with Rabinowitz's (22) in vitro findings, using acetate-2-C^{14} as a precursor. Using brain homogenates, Rabinowitz demonstrated a marked ability of tissue samples from dystrophic mice to incorporate methyl labeled acetate into sterol.

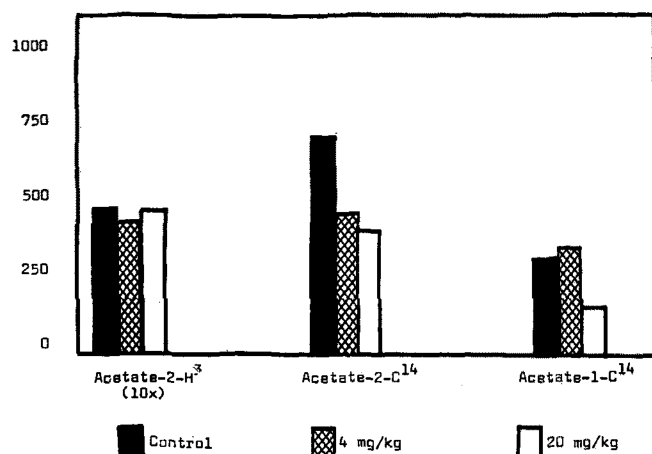

FIG. 6. Methylphenidate produces changes in incorporation of acetate (D/m/g tissue) into mouse brain sterols.

Cancer and Brain Cholesterol Metabolism

Studies on cholesterol biosynthesis in tumor-bearing animals showed an altered lipid metabolism (23,24). It was of interest to measure what metabolic changes in the brain would be associated with this disease process. Mice, representing normal as well as two different transplantable mouse neoplasms, were investigated. Female CF-1 mice were used as controls, and recipients of Ehrlich carcinoma cells were injected subcutaneously. The other group consisted of normal DBA/2 female mice and those having a lymphatic leukemia (P-1534).

The results of these experiments are summarized in Figure 5. The finding of a general decrease in the incorporation of isotopes into brain cholesterol follows the trend noted in other tissues (24). These results suggest that while the brain is protected by a blood-brain-barrier, general systemic changes can influence cerebral data.

Effect of Methylphenidate

This drug was chosen for study because its structure is similar to substituted acetic acid derivatives, compounds previously shown to inhibit cholesterol biosynthesis. Methylphenidate was studied at two dose levels, 4 mg/kg and 20 mg/kg, representing respectively 1/35 and 1/7 of the LD_{50}. Our studies indicated that while methlphenidate lowers both concentration and total amount of cholesterol in the liver, the greatest deviation was noted in brain lipid values.

Decreases in brain cholesterol represented a significant loss (10 to 20%) of sterol during six days of drug administration. To account for the kinetics involved in such a decrease in cholesterol level, half life times of 17 to 35 days would be necessary. These calculations were based on the assumption that sterol formation, but not degradation, was inhibited by the drug. This is improbable. The over-all half life would then be even shorter. Incorporation studies were made with specifically labeled acetate in order to differentiate between (a) loss of synthetic ability

and (b) and/or increased utilization of sterol as causative factors for the lowering of cholesterol in organ tissue.

The decrease in sterol in the liver can be explained by assuming that decrease in incorporation of methyl-labeled acetate reflects decrease sterol synthesis (Fig. 6).

Acetate-2-H^3 incorporation into brain sterol was not affected by methylphenidate treatment. Changes with acetate-2-C^{14} and acetate-1-C^{14} showed lowered rates of incorporation in animals receiving the larger drug dose. Animals receiving smaller amounts of drug showed a decreased incorporation with acetate-2-C^{14} but not with acetate-1-C^{14}. Tentatively, these data indicated that the lowering of brain cholesterol could best be explained on the basis that less methyl-labeled carbon enters biosynthesis in the drug-induced state as compared to untreated animals. Because of the differential effect between methyl- and carboxyl-labeled acetate incorporation, the effect of the drug (ritalin) seems to be on the Kreb's cycle. While other explanations are possible, this conclusion seems the most plausible.

Since the main carbon source for brain metabolism is glucose, it was of further interest to determine whether any metabolic block was produced by the drug, before acetate conversion into sterol. For this purpose, specifically labeled glucose were used (Fig. 7). The C-1/C-6 ratio (1.70) found for liver cholesterol of untreated animals was lowered by methylphenidate. This decrease ratio, with the lower drug dose, would seem indicative of the direct effect on the hexose monophosphate shunt or on a fixation reaction, involving released carbon dioxide into the cholesterol moiety. The ratio in brain sterol indicates the same effect but in the opposite direction, an increase incorporation of the C-1 into the sterol nucleus.

The differential incorporation of glucose-2-C^{14} and glucose-6-C^{14} into tissue cholesterol follows the pattern indicated by the conversion into carboxyl- and methyl-labeled acetate, respectively. Similarity between data from specifically labeled glucose and acetate suggests that glycolysis of glucose is not affected. With the limited data available at the present time, it is impossible to differentiate the mechanism of drug action. There are two apparent areas for further investigation. The first would involve research into the effect of drug on CO_2 fixation reactions (hexose monophosphate shunt) in cholesterol formation. The second would explore the differential incorporation of acetate-1-C^{14} and acetate-2-C^{14} into the sterol nucleus and the possible implications of the tricarboxylic cycle in this reaction. Further intensive work is necessary before the mechanism of methylphenidate on brain cholesterol can be elucidated.

Discussion

Cholesterol in the brain, the largest repository of sterol in the body, was thought to be metabolically stable in adult animals. Waelsch et al. (28) found little or no labeling of the unsaponifiable lipids of the brain in adult rats whose body fluids had been enriched with deuterium. Under the same conditions, there was marked labeling in very young rats. The loss of synthetic capacity in adult life was supported by the findings of several investigators who found little or no labeling of cholesterol in brains of adult rats (a) after feeding deuterium-labeled acetate (29); (b) after intraperitoneal injection of acetate-1-C^{14} (30–32); in brains of tissue from adult rats (33) and

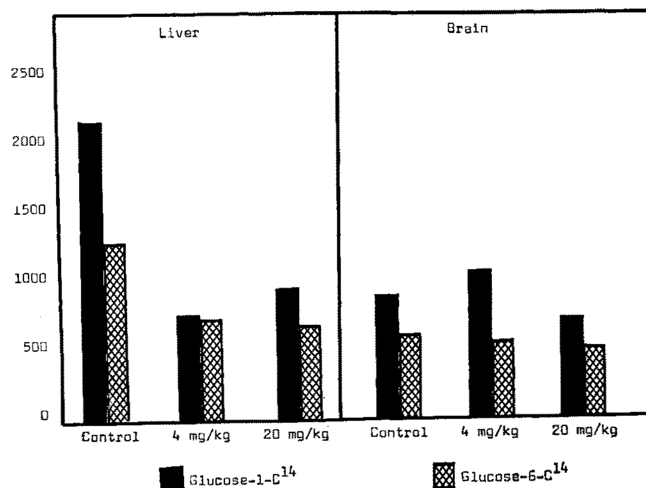

FIG. 7. The effect of methylphenidate on the pattern of labeling cholesterol from either C^{14}-1- or C^{14}-6-glucose (D/m/g tissue).

humans (34), after incubation in vitro with acetate-C^{14}; or (d) in brains of adult cats after perfusion with octonoate-1-C^{14} (35). Early studies also indicated there was little or no exchange between brain cholesterol and plasma lipoproteins (29). Avigan et al. (36) and Morris and Chaikoff (37) have shown that adult rat brain can very slowly absorb circulating cholesterol. In contrast, Davison et al. (38) and Kritchevsky and Defendi (39) observed that chicken brain can absorb cholesterol injected into the yolk sac. There may therefore be a distinct species difference between the chick and rat brain in the absorption of plasma cholesterol into the nervous tissue.

On the other hand, small but significant incorporation of C^{14} into cholesterol of the adult brain occurred after injection of acetate-1-C^{14} into the cisterna magna by McMillan et al. (31) or directly into the cerebrum by Nicholas and Thomas (32). Grossi et al. (40), using several different radiometabolites (acetate-1-C^{14}, n-butyrate-1-C^{14}, glucose-U-C^{14}, and mevalonate-2-C^{14}) demonstrated activity in brain slices for both young (10 day) and older (60 day) rats. In vivo data also has been provided by Moser and Karnosky (13) on brain lipid synthesis from glucose-U-C^{14} in intact mature animals (6 months). Despite these findings, there remains the general feeling that (a) intraperitoneal injection of a precursor leads to little if any brain lipid labeling in the adult animal; and (b) that the negligible incorporation found, after this type of injection, reflects a lack of metabolic penetration into the brain, rather than an inherent decrease in the synthetic capacity of this organ.

In our own studies, we have shown that the effect of starvation, muscular dystrophy, cancer, and a CNS stimulating drug — all can alter normal cholesterol metabolism in the adult animal. Our data emphasize that cholesterol still undergoes active metabolism in the adult animal. It is evident that the efficiency of labeling cholesterol in the adult animal depends, in part, on the precursor used, the tissue under study, the experimental approach employed (in vivo, in vitro), and the lapse of time between administration or addition of precursor and extraction of the tissue. Our results on the effect of fasting on lipogenesis in liver and brain, using glucose and acetate as precursors, agree qualitatively with the results of Smith (41). In both studies, the brain was shown to

be resistant to compositional changes resulting from 24, 48, and 72 hr of fasting. The incorporation of isotopes, however, was shown to vary in proportion to the fasting state, changes in incorporation being significant. Failure to demonstrate greater compositional changes in the rest of the central nervous system, especially with respect to cholesterol, may be due to the fact that in the adult central nervous system, the newly synthesized lipid is a small but important proportion of the total amount of lipid present.

The above data, integrated with the idea that labeled cholesterol persists in the mammalian central nervous system for over a year (31,32,38), seems to suggest a series of sterol compartments in brain tissues, each with individual turnover rates. The idea of several sterol compartments, each with a different metabolic rate, has been given support by Davison and Dobbing (42), Pritchard (43), and Radin (44). The size and relative importance of each compartment would vary with age and physiological condition.

ACKNOWLEDGMENT

For the past ten years, various phases of brain cholesterol biosynthesis have been supported by the following: (1) Public Health Service Research Grant NB-02235 from the National Institute of Neurological Disease and Blindness; (2) Public Health Service Research Grant CA-07318 from the National Cancer Institute; (3) Muscular Dystrophy Association of America, Inc.; (4) National Multiple Sclerosis Society, Michigan Chapter; (5) Michigan Heart Association; (6) The Michigan Cancer Foundation; (7) Ciba Pharmaceutical Company.

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Cholesterol Turnover in the Central Nervous System

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Abstract

Immature rat brains were examined for a metabolite of cholesterol which could conceivably form in the central nervous system as a result of cholesterol turnover. A basic assumption was made that an acidic product would be formed, preferentially by oxidative degradation of the cholesterol side chain. Chemical fractionation of brain tissue and thin-layer chromatography of an appropriate acidic fraction indicated the presence of a monohydroxy steroidal acid(s) which remains to be positively identified. Preliminary tracer experiments did not clarify the origin of the unidentified metabolite.

THE CLASSICAL WORK of Waelsch, Sperry and Stoyanoff (1) twenty-five years ago set the foundation for our present basic knowledge of cholesterol metabolism in the central nervous system (CNS). According to this concept cholesterol biosynthesis occurs extensively in the immature brain and spinal cord, especially during the period of active myelination. The cholesterol content reaches a fixed level as the animal approaches maturity and then remains essentially undisturbed for the rest of the individual's life.

Within the past eight years a new facet has been added to this concept, a facet which we believe gives considerable impetus to further attempts to extend our knowledge of brain and spinal cord cholesterol metabolism. Experiments of varied nature from several laboratories have all supported the fact that the cholesterol concentration in the CNS remains unchanged as maturity is reached, but cholesterol *biosynthesis* can still take place in the adult. In other words the cholesterol already present may be metabolically inert but *the capacity* to synthesize the sterol still exists in the mature CNS. Exactly where in the CNS new sterol can be synthesized and deposited has not been determined, but this lack of knowledge does not influence the basic premise. This new concept has been suggested by in vivo studies in which C^{14}-labeled precursors were given parenterally (2–4), by intracisternal (5) and intracerebral (6,7) injection, and by in vitro studies (8–10). We believe the isolation of squalene from adult brain (11) also supports this concept. Why else should squalene be present in adult nervous tissue? Unfortunately the most simple experiment to demonstrate the ability of adult nervous tissue to synthesize cholesterol, namely: cholesterol biosynthesis in isolated tissue from C^{14}-la-