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ACYL-CoA CHOLESTEROL ACYLTRANSFERASE IN CULTURED GLIOBLASTOMA CELLS

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We investigated the incorporation of radioactive precursors into cholesteryl ester in cultured glioblastoma cells. It was found that polar cholesterol derivatives and exogenous cholesterol contained in lipoprotein complexes greatly enhanced intracellular cholesteryl ester formation. The direct transfer of the acyl moiety from acyl-CoA to free cholesterol was demonstrated in broken cell preparations. Further evidence of the existence of the acyl-CoA:cholesterol acyltransferase (ACAT) in glioblastoma cells came from the conversion of radioactive cholesterol to cholesteryl ester by glial cell homogenates. The characteristics of the enzymic assay were studied in detail. This enzymic activity was greatly enhanced in homogenates prepared from 7-ketocholesterol-treated cells. Thus, cells more active in cholesterol esterification possessed a higher ACAT activity. Progesterone inhibited cholesterol esterification in cell-free preparations. The marked inhibition of intracellular cholesteryl ester formation in intact cells by progesterone is a strong argument for the exclusive role of ACAT in glioblastoma cells. Similar properties of cholesteryl ester biosynthesis have been observed in neuroblastoma cells and primary brain cell cultures. In conclusion, the same enzyme is involved in cholesteryl ester biosynthesis in all neural cells. Neural and nonneural cells share many fundamental characteristics of cholesteryl ester formation.

INTRODUCTION

Cholesterol, a major lipid component of the brain (1), exists mainly in a nonesterified form. A significant amount of an esterified form was found

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in the developing brain. Almost all cholesteryl esters disappear as the brain matures (2–4). The physiological significance and the biochemical basis of this transient existence of cholesteryl esters in development is not known. Certain pathological conditions are known to induce an abnormal accumulation of cholesteryl esters in the mature brain (5–7). The appearance of cholesteryl esters is not an inevitable consequence of nerve degeneration in the adult specimen; cholesteryl esters are conspicuously absent in some demyelination diseases. Temporal changes in cholesteryl esters during brain development and the selective accumulation of cholesteryl esters in certain diseases suggest that the formation of cholesteryl esters in the brain serves an important, yet unidentified, physiological role that requires stringent control of its metabolism.

The control of cholesteryl ester formation in the brain has not been subjected to appropriate biochemical studies nor has the process or enzyme responsible for cholesteryl ester formation in the brain been unambiguously defined. Enzymic formation of cholesteryl ester by brain homogenates directly from cholesterol and free fatty acid has been reported (8–10). The reaction was independent of CoA and ATP; oleoyl-CoA was not utilized as a substrate. It was proposed that cholesteryl ester in the brain was formed by the reversed hydrolytic reaction catalyzed by a cholesterol esterase at pH 5.0. However, the physiological significance of the reaction must remain in doubt. The free energy change which occurs in hydrolysis is such that the equilibrium favors the hydrolysis. On the other hand, acyl-CoA:cholesterol acyltransferase (ACAT) (EC 2.3.1.26), which utilizes acyl-CoA as a substrate for cholesterol esterification, has been found to play an exclusive role in cholesteryl ester formation in all nonneural tissues studied so far.

It was recently reported (11) that CoA and ATP could enhance the incorporation of a free fatty acid into cholesteryl ester, raising the possibility that ACAT might also exist in the developing rat brain. However, it is not prudent to draw this fundamental conclusion based on indirect evidence alone. In order to resolve the question of the enzyme responsible for cholesteryl ester formation in the nervous system, we used cultured glioblastoma cells as a model system for our studies. This system offered a unique opportunity to compare intracellular cholesterol esterification in intact cells with ACAT activity in cell homogenates. In this communication, we report the assay of ACAT activity using radioactive oleoyl-CoA as the substrate and the characteristics of the enzyme. The results have been confirmed in neuroblastoma cells and primary cultured brain cells.

EXPERIMENTAL PROCEDURE

[^{3}H]Oleic acid and [1-^{14}C]Oleoyl-CoA were purchased from New England Nuclear Co., Boston, Massachusetts, cholesterol, cholesteryl oleate, and oleoyl-CoA were obtained from Sigma Chemical Co., St. Louis, Missouri. Glioblastoma C-6 cells and neuroblastoma NB-2a cells were obtained from American Type Tissue Culture Co., Rockville, Maryland, and fetal calf serum (FCS) and Dulbecco's modified Eagle medium (DME) were obtained from Grand Island Biological Co., New York. Lipoprotein fractions were prepared by density centrifugation (12). Acetylation of LDL was prepared and analyzed according to established procedures (13).

Primary cultured brain cells were prepared from 1-day-old rat brain (14). Briefly, cerebra were dissected and placed in DME containing 10% FCS and grown for one week before experimentation. The culture contained a mixed population of nerve cells (≈10%) and glial cells (≈90%) as determined by immunological and morphological criteria (see reference 14 for details).

The procedures for measuring the incorporation of radioactive oleic acid into cholesteryl oleate in cells and the ACAT assay for broken cell preparation were adopted from established procedures (15–18). The slight modifications are described in the following paragraphs.

Glioblastoma cells were grown in DME containing 10% FCS (heat inactivated). Two million cells were seeded in each 100 × 20 mm culture dish on the first day. On the second day, the medium was changed to DME + 2 mg/ml lipoprotein deficient medium (LPDS) for some of the experiments. On day four the cells were ready for experimentation.

The medium was removed from each dish and the cells were washed twice with DME. Four milliliters of media containing FCS or LPDS (containing less than 5% of the original total cholesterol) were added to each dish along with the various compounds. They were incubated for three hours at 37°C in a humidity controlled incubator. Forty microliters of a solution containing 8 × 10^6 cpm of [^{3}H]Oleic acid in 0.4 μmol oleate-albumin (19) were added to each dish and the dishes were returned to the incubator for two more hours. The cells were then washed twice with 2 ml of ice cold phosphate-buffered saline (PBS) and the dishes were frozen immediately.

The cells were processed by scraping each dish with a cell scraper and putting the suspension in a screw cap 13 × 100 mm test tube. The dishes were rinsed twice with ice cold PBS to ensure the transfer of all the cells. Six hundred microliters from the total of 6 ml were removed for protein determination. The remaining solutions (containing 5.4 ml) were then centrifuged at 900 *g* for 10 minutes at 4°C after which the supernatant was discarded. The pellet was resuspended in 0.1 ml of PBS. Four milliliters of chloroform-methanol (v/v, 2:1) were added and the tubes were rigorously vortexed. The tubes were then centrifuged at 900 *g* for 10 minutes. The supernatant was then transferred to test tubes containing 0.15 mg of cholesteryl oleate and 20,000 cpm of [^{14}C]cholesteryl oleate which was used to determine the recovery (normally between 80% and 85%) and facilitate visualization of the spot on thin layer chromatography. After vortexing, 0.9 ml of distilled water were added to each tube and they were vortexed again. The layers were then separated by centrifuging for 10 minutes. Fifty microliters of each phase were counted in a beta counter to check the radioactivity. Eight hundred microliters of the lower phase were removed to a conical centrifuge tube and the solvent was removed by evaporation in a rotary evaporator. Sixty microliters (3 × 20 μl) of hexane were added to the sample and the solution was streaked on a Brinkman Sil-25 thin layer chromatography plate. The plates were developed in a tank containing heptane-ether-glacial acetic acid (85:15:2, v/v/v). The spots were visualized by iodine crystals. The spots corresponding to the cholesteryl oleate were scraped off and were

counted in scintillation vials containing 6 ml of 3a70B scintillation fluid (Research Products International).

The ACAT assay for broken cell preparation was done according to Brown et al. (15) except for the following modifications. Glioblastoma cells (2×10^6 cells/dish) were seeded on day 1 in 100 × 20 mm culture dishes in DME + 10% FCS. On day 2, the media in some of the dishes was changed to DME + 2 mg/ml LPDS. On day 4, the cells were washed twice with PBS, scraped off in 6 ml of ice cold PBS and centrifuged at 1,450 *g* for 10 min. The pellet was then resuspended in buffer (0.3 ml) containing 100 mM potassium phosphate (pH 7.4), 2 mM dithiothreitol, and 2 mM EDTA and sonicated for 15 sec using an Artek sonic dismembrator (Model 300). Fifty microliters of sonicated membrane (0.1 mg protein) was incubated with a mixture (0.15 ml) containing 50 mM potassium phosphate (pH 7.4), 2 mM dithiothreitol, 1.2 mg bovine serum albumin (fatty acid free), 0.02 mg cholesterol (in 4 μl of acetone), and 40 μM [^{14}C]oleoyl coenzyme A (2×10^5 cpm/tube), 100 cpm/pmol at 37° for 1 hour after which the reaction was stopped with the addition of 4 ml of chloroform-methanol (2:1). The radioactive cholesteryl ester was quantitated by the same procedure as previously described for experiments involving whole cells.

The cholesteryl ester spot in the above TLC system was positively identified in the following manner. The radioactive spot localized by the adjacent standard was scraped off and eluted off with chloroform. After reduction in volume, the samples were analyzed by two additional TLC systems. (i) Petroleum ether (bp 38–47°)-ethyl ether-glacial acetic acid = 80:20:1 (v/v/v), Rf = 0.77, and (ii) cyclohexane-chloroform = 50:50 (v/v), Rf = 0.61. It was found that more than 98% of the radioactivity isolated as cholesteryl ester in both intact cell and cell-free experiments was associated with cholesteryl ester.

RESULTS

Effects of Exogenous Lipids on Cellular Cholesteryl Ester Formation in Glioblastoma Cells. Cellular cholesteryl ester formation in glioblastoma cells could be conveniently measured by the incorporation of radioactive oleic acid into cholesteryl ester. Time course and dependency on oleate concentration are shown in Figures 1 and 2. Cholesteryl ester formation was linear with time for at least 6 hours (Figure 1). The rate of cholesteryl ester formation reached a maximal level at an oleate (bound to albumin) concentration of 50 μM. About 25 μM oleate was needed to reach half of the maximal rate of incorporation (Figure 2). We routinely used 100 μM oleate in our study.

The amount of cholesteryl ester formed was determined by TLC. The compound that migrated with nonradioactive cholesteryl ester in the standard TLC system was characterized. The two additional thin layer chromatographic systems did not detect any impurities (details described in Experimental Procedure). In addition, other known lipids, phospholipids and triglycerides, etc., were tested in the standard TLC system and found to move differently from cholesteryl ester. Thus, this thin layer chromatographic system was suitable for studying intracellular cholesteryl ester formation in glioblastoma cells.

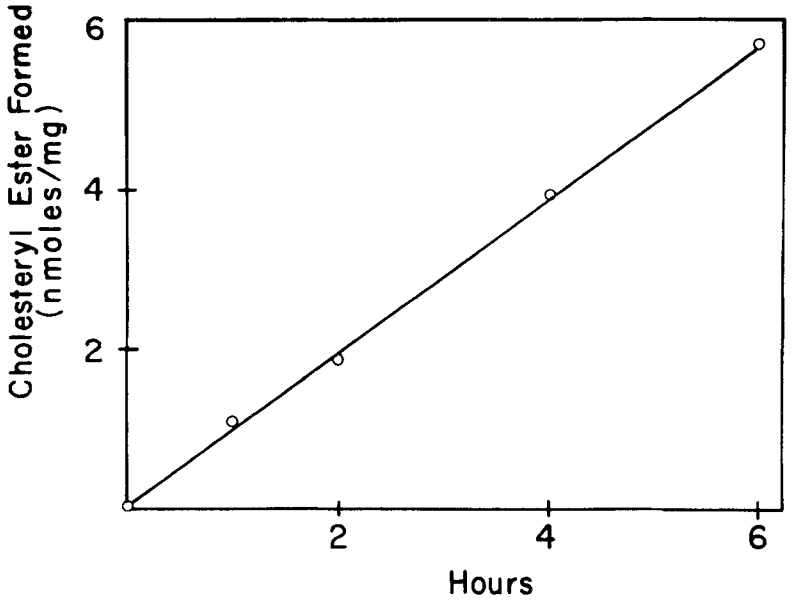

FIG. 1. Incorporation of oleic acid into cholesteryl ester as a function of time: Procedure was identical to that described in Experimental Procedure. Cells were grown in FCS and times of labelling are indicated in the Figure.

To evaluate the regulatory effects of exogenous lipids in the medium on cellular cholesteryl ester formation in glioblastoma C-6 cells, we compared the incorporation of radioactive oleic acid into cholesteryl ester in a lipoprotein-deficient medium with that in lipoprotein-containing medium (Table I). Absence of lipoproteins in the medium resulted in a marked decrease in cholesteryl ester formation. Only about one-fourth of the cholesterol esterification capacity remained when the medium was devoid of lipoproteins. This result was similar to that reported in fibroblasts and macrophages (15–18). The total radioactivity incorporated into the nonpolar lipid phase was not significantly altered by exogenous lipids. This suggested that exogenous lipids mainly affected the metabolic pathway for cholesteryl ester biosynthesis and not the influx of fatty acid into cells or the activation of fatty acid to a more active form such as CoA thioester. The supplement of lipoprotein deficient medium with lipoprotein fractions restored the cells' ability to form cholesteryl esters (Table I). Similar results were reported by Volpe et al. (20).

In order to determine which lipoprotein fractions caused the activation of cholesteryl ester formation, we analyzed the influence of the individual

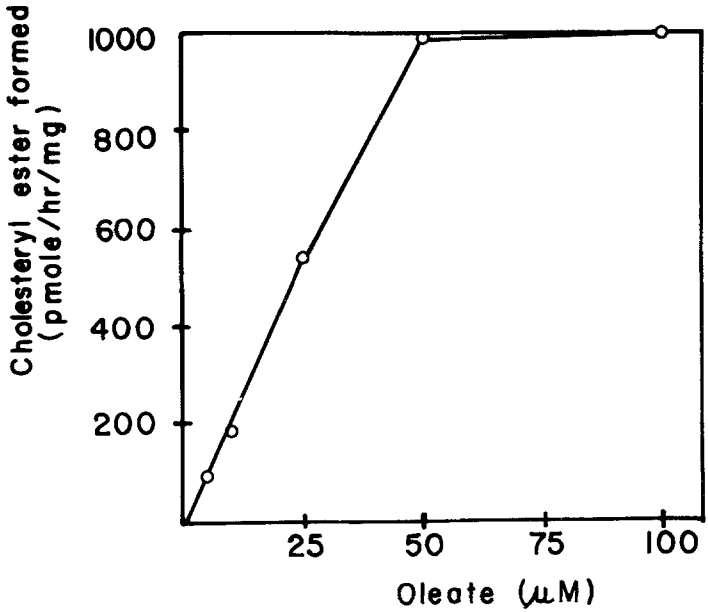

FIG. 2. Cholesterol esterification as a function of oleate concentration: Cells were grown in FCS and oleate concentrations varied according to the figure. Radioactivity added to each dish remained the same and specific activity changed. The remainder of the procedure was identical to that described in Experimental Procedure.

TABLE I

EFFECT OF EXOGENOUS LIPIDS ON THE CHOLESTERYL ESTER FORMATION

Medium	Supplement	Concentration of cholesterol (μg/ml) in lipoprotein fraction	Cholesterol ester formed pmole hr^{-1}, mg $protein^{-1}$
LPDS	None	—	163 (100%)
LPDS	LDL	5	298 (183%)
LPDS	LDL	50	700 (429%)
LPDS	HDL	5	154 (94%)
LPDS	HDL	50	185 (113%)
FCS	None	—	772 (474%)

Cells were grown in 10% FCS for 48 hours. The medium was changed to 2 mg/ml LPDS for 2 days before the experiment. On the day of the experiment, the cells were incubated with lipoprotein fraction for 8 hours before being labeled with radioactive oleate for 2 hours.

TABLE II

EFFECT OF STEROIDS ON CHOLESTERYL ESTER FORMATION IN GLIOBLASTOMA C-6 CELLS

Steroid added	Cholesteryl oleate formed pmole hr^{-1} mg $protein^{-1}$	
	LPDS	FCS
None	180 (100%)	818 (454%)
Cholesterol	419 (232%)	914 (507%)
7-Ketocholesterol	796 (442%)	1209 (617%)
25-Hydroxycholesterol	855 (475%)	1682 (934%)

Cells were grown in either FCS or LPDS for three days. The medium was replaced with fresh medium with the same compositions containing the following supplements: [^{3}H]oleate (1000 cpm/nmole) and the steroids in 1.5 μl of ethanol. The final concentration of oleate and steroids were 0.1 mM and 10 μg/ml, respectively. Each monolayer was labeled for three hours at 37°, after which the formation of [^{3}H]cholesteryl ester was measured.

lipoprotein fractions on cholesteryl ester formation in cells previously grown in lipoprotein-poor medium for three days. Low density lipoproteins were found to have a strong effect on the cellular cholesteryl ester biosynthesis (Table I). Acetylated LDL, a representative of modified LDL, was reported to be recognized by a receptor different from LDL receptors (13). In a separate experiment, we found that acetylation of LDL did not enhance the ability of LDL to stimulate cholesterol esterification (data not shown).

In a separate experiment, the cholesteryl ester formation from endogenous radioactive cholesterol was analyzed in a manner similar to procedures reported in Table III. At first, [1-^{14}C]acetate was used as a precursor for the synthesis of unesterified cholesterol in cells grown in LPDS so that all cholesterol was unesterified (16), nonradioactive oleic acid was then added. The condensation of radioactive cellular cholesterol with newly-added nonradioactive oleic acid to form radioactive cholesteryl ester was determined. Exogenous lipoprotein showed strong stimulatory effects (280% increase) on cholesteryl ester formation. Thus, the formation of cholesteryl ester from two separate portions of cholesteryl ester was similarly stimulated. This reinforced the notion that exogenous lipoprotein activated cholesteryl ester formation by altering a metabolic step unique to cholesteryl ester formation.

We evaluated the effect of several cholesterol derivatives on the rate of cholesterol esterification. Among these, 25-hydroxycholesterol had the strongest stimulatory effect (Table II). 7-Ketocholesterol was almost as

TABLE III

THE EFFECT OF 7-KETOCHOLESTEROL ON ESTERIFICATION OF ^{14}C-LABELED CHOLESTEROL IN INTACT CELLS

Medium	7-Ketocholesterol	Incorporation of acetate into cholesteryl ester nmole hr^{-1}, mg $protein^{-1}$
LPDS	−	4.46
	+	10.47
FCS	−	9.85
	+	31.74

The experiment was similar to that in Experiment II except for the following modifications. Cells were incubated with [1-^{14}C]acetate (1.6×10^3 cpm/nmole) for 24 hours. Cells were then washed with medium containing no acetate before adding 7-ketocholesterol (10 μg/ml).

effective as 25-hydroxycholesterol. A similar regulatory pattern was observed in the fibroblast (15, 21, 22) which was attributed to the stimulation of ACAT. When endogenous cholesterol prepared from [1-^{14}C)acetate was used as a precursor similar drastic effects were also observed (Table III). These observations argued strongly that polar steroids also acted directly on the immediate condensation step between these two portions of cholesteryl ester.

Fatty acid specificy of cholesterol esterification in glioblastoma cells was examined (Table IV). Both oleic acid and palmitic acid were effective precursors Arachidonic acid was a poor one. 7-Ketocholesterol stimulated the utilization of all three fatty acids tested.

Our experiments indicated that cellular cholesteryl ester formation in cultured glioblastoma cells was regulated in a manner similar to nonneural

TABLE IV

FATTY ACID SPECIFICITY OF INTRACELLULAR CHOLESTEROL ESTERIFICATION

Fatty acid used	Chlesteryl ester formed pmol hr^{-1} mg $protein^{-1}$	
	+ 7-Ketocholesterol	− 7-Ketocholesterol
Oleic acid	807	157
Palmitic acid	740	124
Arachidonic acid	154	58

Cells were grown in LPDS. The experiment was the same as described in *Experimental Procedure* except for a different radioactive fatty acid used.

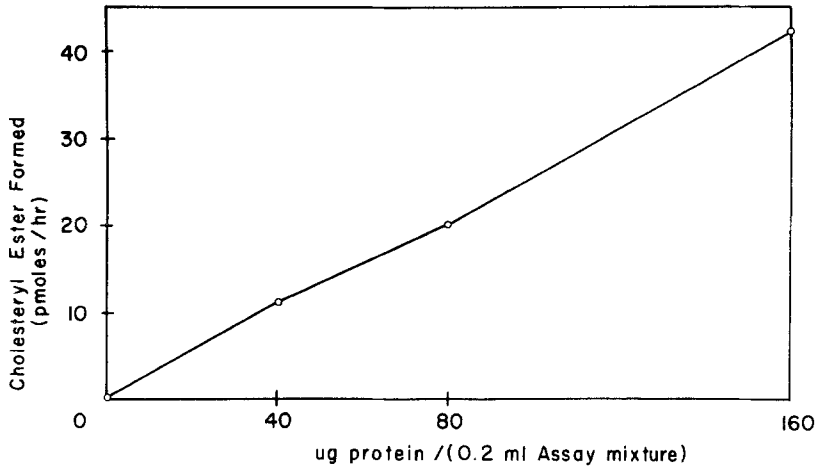

FIG. 3. Dependency of ACAT activity on protein concentration: The procedures were carried out as described in Experimental Procedure except that the protein concentration varied.

tissues such as fibroblasts and macrophages. In these cells, ACAT is solely responsible for cholesteryl ester formation.

Involvement of ACAT in Cholesteryl Ester Formation in Glioblastoma Cells. To determine the involvement of ACAT in the cholesteryl ester metabolism in glioblastoma cells, an attempt was made to directly assay the ACAT enzymic activity in a broken glioblastoma cell preparation.

The enzymic activities as the functions of time of assay, protein concentration, and substrate concentrations were determined. The assay was linear, up to 0.8 mg protein per ml (0.2 mg protein/assay mixture) (Figure 3). The transfer of radioactive oleoyl moiety from oleoyl-CoA to cholesterol to form cholesteryl ester continued for up to 120 minutes (Figure 4).

The ACAT activity was dependent on the oleoyl-CoA concentration (Figure 5). However, even 100 μM oleoyl-CoA failed to saturate the enzyme. This was evidently higher than the reported critical micelle concentration of oleoyl-CoA (23). At this pH (pH 7.4), the direct incorporation of free oleic acid into cholesteryl ester was found to be negligible (results not shown). The possible degradation of cholesteryl ester in the ACAT assay was tested by including radioactive cholesteryl ester (462,000 cpm) in the assay mixture. It was found that less than 1% was decomposed after one hour at 37°C.

The ACAT activity as measured by the incorporation of radioactive oleoyl-CoA into cholesteryl ester was only partially dependent on exo-

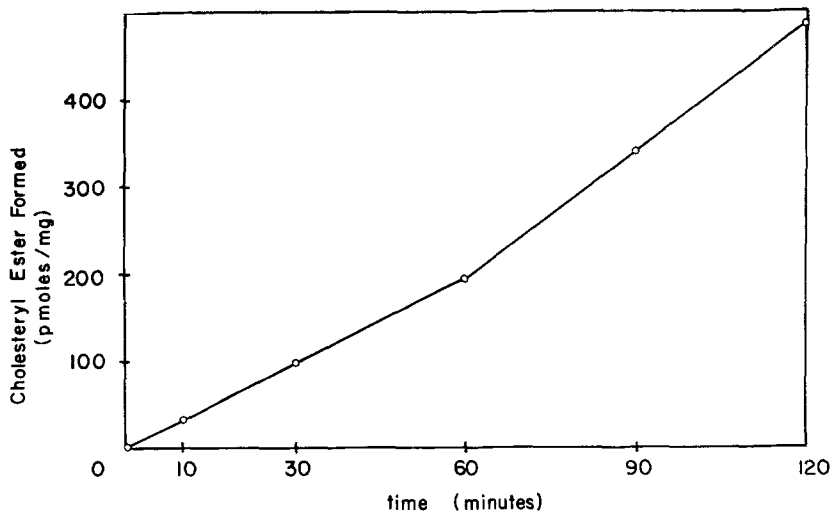

FIG. 4. Dependency of ACAT activity on time of assay: The procedures were the same as Figure 3 except for the different times of incubation.

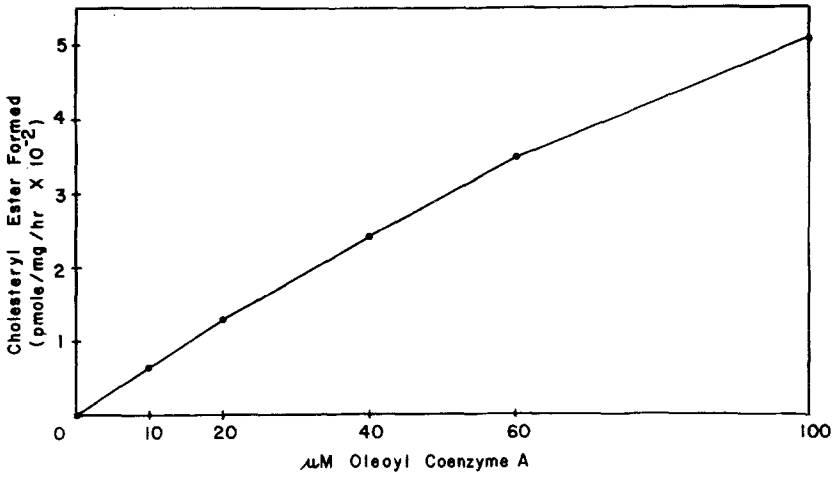

FIG. 5. Dependency of ACAT activity on oleoyl-CoA concentration: The procedures were carried out as described in Experimental Procedure except for varied oleoyl-CoA concentrations. Radioactive oleoyl-CoA used was the same in all assays and nonradioactive oleoyl-CoA was the variable. Crude extracts were prepared from glioblastoma cells grown in FCS.

TABLE V
ACAT ACTIVITY IN CRUDE EXTRACTS PREPARED FROM CELLS GROWN IN DIFFERENT CONDITIONS

	Cholesteryl ester formed pmol hr^{-1} mg $protein^{-1}$	
LPDS	610	(100%)
LPDS + 7-ketocholesterol (in medium)	3,200	(529%)
FCS	1,900	(312%)
FCS + lysophosphatidylcholine (in assay)	570	(93%)

Cells were grown in FCS and LPDS for three days. 7-Ketocholesterol (final concentration 10 μg/ml) was added to cells grown in LPDS. After 5 hours incubation, cells were harvested and ACAT activities were analyzed. In one assay, lysophosphatidyl choline (final concentration 10 μM) was added to the assay solution.

genously-added cholesterol. Apparently, endogenous membrane-associated cholesterol was sufficient for near maximal activity. We found that the addition of 20 μg or 30 μg of cholesterol did slightly increase ACAT activity (maximal stimulation was about 20%)(results not shown). Although the endogenous cholesterol sufficiently supplied enzyme for near optimal activity, the enzyme was capable of utilizing exogenously-added cholesterol as evident from the ability of enzyme to convert exogenously-added radioactive cholesterol to cholesteryl ester.

The esterification of [^{14}C]cholesterol to cholesteryl ester was completely dependent on oleoyl-CoA. Oleic acid was not able to substitute for oleoyl-CoA. The addition of ATP and CoA was necessary for oleic acid to serve as acyl donor (data not shown). The enzymic generation of oleoyl-CoA by acyl-CoA ligase in situ may explain this observation.

It was reported that 0.3% (w/v) Triton-1339 enhanced the ability of liver and adrenal ACAT to utilize exogenously-added cholesterol (24, 25). In the presence of 0.3% Triton-1339, glial ACAT activity was more dependent on exogenously-supplied cholesterol. However, Triton X-1339 itself was strongly inhibitory (58% inhibition). Consequently, Triton X-1339 was not used in our ACAT assay.

ACAT activity of cells grown in different conditions was analyzed in order to understand the enzymological basis of cellular cholesterol ester metabolism. ACAT was found to be more active in homogenates prepared from cells grown in the presence of 7-ketocholesterol or lipoprotein (Table V), confirming that oxygenated cholesterols and lipoproteins had a direct effect on the ACAT itself. The corresponding increase in ACAT activity in these treated cells served as evidence for the proposed role of ACAT in glial cellular cholesteryl ester formation.

In addition, we also evaluated the effect of 5,5'-dithiobis(2-nitrobenzoic acid) (1 mM) on cholesterol esterification in broken cell preparation. Lecithin-cholesterol acyltransferase was reported to be completely inhibited by this compound (26). ACAT activity of human and rat liver was inhibited by 20% (29). We found that 20% to 30% of glial ACAT activity was reduced by this thiol reagent. Therefore, cholesteryl ester in glioblastoma cells was probably not produced via lecithin-cholesterol acyltransferase reaction.

Lysophospholipid severely diminished the apparent activity (Table V). This observation was also inconsistent with the existence of lecithin-cholesterol acyltransferase in glial cells. We detected active acyl-CoA-lysophospholipid acyltransferase in glial extract. If both lecithin-cholesterol acyltransferase and acyl-CoA-lysophospholipid acyltransferase were present, lysophospholipid would mediate and probably stimulate the formation of cholesteryl ester from oleoyl-CoA. The inhibitory effect of lysophospholipid suggested that lecithin-cholesterol acyltransferase did not exist in glioblastoma cells.

The apparent inhibition of ACAT activity by lysophospholipid was perhaps due to the diminishing oleoyl-CoA available for ACAT. It was noted that the amounts of oleoyl-CoA employed were nonsaturating. Since we found that acyl-CoA-lysophospholipid acyltransferase (28) was active in glial cells, this enzyme could consume acyl-CoA which was also a critical substrate of ACAT. (We found that the inclusion of lysophosphatidyl choline increased phospholipid formation by 25 fold.) In consistent with this hypothesis, the inclusion of quinacrine, a phospholipase A2 inhibitor, increased (40–50%) apparent ACAT activity (data not shown). More detailed studies are obviously needed to establish unequivocally this important point, since lysophospholipid could form a complex with cholesterol (28) which is also a substrate for ACAT.

The Inhibition of Cholesteryl Ester Formation by Progesterone. ACAT was found to play a significant role in cholesteryl ester formation in glioblastoma cells, but the existence of more than one route for cholesteryl ester formation was not excluded. To resolve this point, the inhibition of cholesteryl ester formation by progesterone was investigated. It had been reported that progesterone was a potent and specific inhibitor of ACAT in nonneural tissues (17, 27). At first, we established that ACAT activity in the broken cell preparation of glioblastoma cells could be inhibited by progesterone. Progesterone (10 μg/ml) was found to inhibit 87% of the ACAT activity. Cellular cholesteryl ester formation in the glial cell was completely blocked by progesterone (5 μg/ml) (Figure 6). Therefore, the

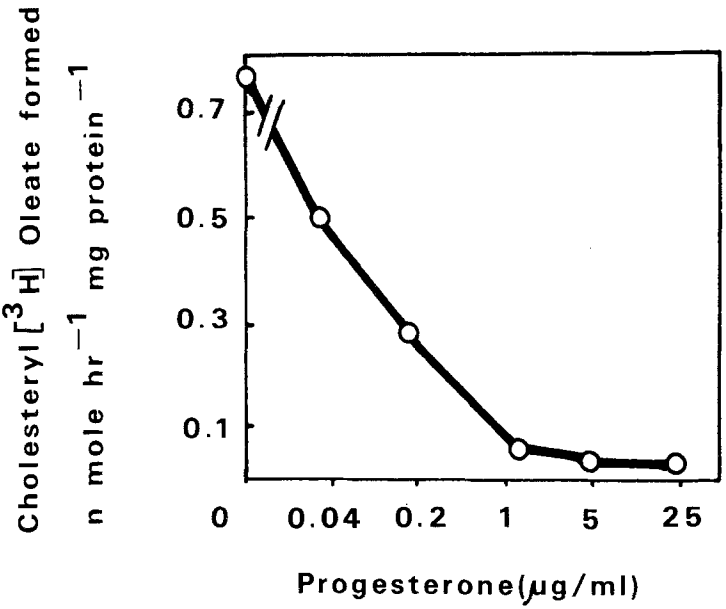

FIG. 6. Inhibition of cellular cholesteryl ester formation by progesterone in glioblastoma cells: After cells were incubated with FCS for three days, the medium was replaced with fresh medium (4 ml) containing 0.1 mM [^{3}H]oleate-albumin (1000 cpm/nmol) and the indicated amount of progesterone. After incubation for three hours at 37°, the cells were harvested for the determination of radioactive cholesteryl oleate.

role ACAT plays in cholesteryl ester formation in glioblastoma cells is an exclusive one.

Cholesteryl Ester Formation in Neuroblastoma Cells and the Primary Culture of Rat Postnatal Brain Cells. To rule out the possibility that the involvement of ACAT in cholesteryl ester formation in glioblastoma cells was a unique property that might not be shared by other cells of neural origin, we studied the regulatory properties of cholesteryl ester formation in neuroblastoma cells and the primary culture of rat postnatal brain cells. It was found that the cholesteryl ester formation in neuroblastoma cells and the primary culture of brain cells prepared from 1-day-old postnatal rats could also be strongly induced by 7-ketocholesterol (Table VI). We also observed that cellular cholesterol esterification in primary cultured brain cells was inhibited by progesterone. This indicated that the mechanism of cholesteryl ester formation in nontumorous brain cells and in neuroblastoma cells were identical to that found in glioblastoma cells. In

TABLE VI
REGULATION OF CHOLESTEROL ESTER FORMATION IN NEUROBLASTOMA CELLS AND PRIMARY CULTURED BRAIN CELLS

	7-Ketocholesterol	Cholesteryl ester formed pmole hr^{-1} mg $protein^{-1}$
Neuroblastoma cells	–	159
	+	1,169
Primary cultured cells	–	741
	+	2,230

The experiment was identical to that in Table II except that cells were grown in LPDS for only one day.

conclusion, the prominent role that ACAT plays in cholesterol esterification is universal for cells of neural origin.

DISCUSSION

It has been repeatedly demonstrated that fatty acyl-CoA thioesters rather than free fatty acids are the physiological donors for re-esterification of hydrolyzed products. Although the condensation of free fatty acids with alcohols, such as cholesterol, can be catalyzed by an esterase in vitro at low pH, there is no solid evidence that this occurs under appropriate physiological conditions.

The changes in cellular cholesterol esterification were correlated with corresponding alterations of ACAT activity in a variety of conditions. We used (i) lipoprotein, (ii) 7-ketocholesterol, and (iii) progesterone to illustrate this point. We also observed that other manipulations such as sodium fluoride had similar effects on both intracellular esterification and ACAT activity. Therefore, the involvement of acyltransferase in cholesterol esterification in neural tissues was experimentally substantiated.

The presence of a cholesterol-esterifying enzyme utilizing free fatty acid has been reported in the brains of rats (8–10, 30, 31) and humans (32). It was established that the myelin-bound cholesterol-esterifying activity was different from other myelin-specific cholesterol esterase (33). The presence of a similar enzyme in peripheral nerve and in cerebrospinal fluid was also reported (34, 35). All these reactions have pH optimal at about 5.0. The finding was confined to the in vitro reaction which may differ from those that occur in vivo. Jagannatha and Sastry (11) characterized

the developmental pattern of this acidic enzyme in rats and found that the maximal activity was reached at the 6th day after birth and did not show further increase. The peak period of myelination occurred between 10–20 days. Thus, this acidic enzyme may not be important in myelinogenesis. In contrast, a pH 7.4 enzyme, which may represent ACAT, showed a developmental pattern parallel to the process of myelination. This is consistent with the notion that ACAT participated in myelinogenesis.

Yao and Dyck reported that the activity of an acidic enzyme (pH optimum 4.8) using fatty acid as a substrate was increased in the degenerating nerve (34). This enzyme was thought to be associated with cholesterol esterification during Wallerian degeneration. However, it is possible that this "esterifying enzyme" may be responsible for the hydrolysis of increased cholesterol esters during demyelination rather than responsible for the formation of cholesteryl esters. The cholesteryl ester cycle, including esterification and hydrolysis, is a continuous process in macrophages (17, 18). The activity of ACAT and cholesterol esterase may be coordinated. In sum, the mere association of an enzyme induction with nerve degeneration is not sufficient to distinguish whether the role for this enzyme is in cholesteryl ester formation or in its degradation. It may be noted that there are several reports about an association between the activity of cholesterol esterase and a demyelinating disease (32).

In the presence of exogenous lipoproteins, the rate of cholesterol esterification in different cells becomes comparable. The degree of stimulation caused by 7-ketocholesterol was dependent on cell types, (Table VI). It was interesting that ACAT in neuroblastoma cells was more drastically affected by 7-ketocholesterol. In contrast, HMGCoA reductase in neuroblastoma cells was reported to be less sensitive to regulation than that in glioblastoma cells (12).

The initial step in the alteration of cholesterol metabolism in fibroblasts by LDL is a specific, high affinity interaction of LDL with a cell-surface receptor. In contrast, an altered form of LDL rather than native LDL is recognized by a macrophage receptor (13). To determine which form of LDL was recognized by glioblastoma cells, we determined the effect of acetylation of LDL (13) on the ability to stimulate cholesterol esterification in glioblastoma cells. It was found that the acetylation of LDL actually decreased the stimulatory effect of LDL. Therefore, an acetylation of LDL is not necessary for the recognition by glioblastoma cells. It may be noted here that the stimulatory effect of LDL did not necessarily prove that the LDL internalization pathway, which is well established in other tissues, also operates in neural tissues. To prove the existence of the "LDL pathway", it is essential to establish the direct binding, inter-

nalization, and lysosomal digestion of LDL. Our results simply show that exogenous cholesterol in the medium was capable of stimulating ACAT activity.

Although the enzymic generation of acyl-CoA in situ from CoA, ATP, and fatty acids can substitute acyl-CoA for ACAT, this method suffers several critical drawbacks. (i) The formation of acyl-CoA is dependent on fatty acid acyl-CoA ligase. There is no guarantee that the ligase does not become rate-limiting under experimental conditions. (ii) The activity of neural ACAT (36), similar to its nonneural counterpart, is regulated by phosphorylation and dephosphorylation (37, 38). The inclusion of ATP would convert the nonphosphorylated enzyme to more active phosphorylated enzyme. CoA-dependent cholesterol esterification is the sum of two reactions catalyzed by fatty acid:CoA ligase (EC 6.2.1.3.) and ACAT. CoA is the substrate of fatty acid:CoA ligase but is the product of ACAT. CoA would stimulate the first reaction but inhibit the second one. The opposite effects of CoA on these two enzymes in the sequence of reactions makes it hazardous to interpret any effect of CoA. In summary, the superiority of using acyl-CoA as a substrate is such that the substitution of acyl-CoA by CoA, ATP, and fatty acid should be discouraged.

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