Cycloheximide Sensitivity in Regulation of Acyl Coenzyme A:Cholesterol Acyltransferase Activity in Chinese Hamster Ovary Cells. 1. Effect of Exogenous Sterols†

Catherine C. Y. Chang, Gary M. Doolittle, and T. Y. Chang*

Department of Biochemistry, Dartmouth Medical School, Hanover, New Hampshire 03756

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ABSTRACT: Chinese hamster ovary cells grown in medium containing low-density lipoprotein (LDL) express high acyl coenzyme A:cholesterol acyltransferase (ACAT) activity as measured by an [^{3}H]oleate pulse. Removal of LDL from the medium causes rapid inactivation of ACAT activity; the $t^{1/2}$ for the initial inactivation rate is 0.8 h. Preincubation with protein synthesis inhibitors (cycloheximide or emetine) for 2 h or longer lengthens the $t^{1/2}$ for the initial inactivation rate to approximately 2.1 h. When LDL is removed for more than 10 h, the cells contain only 3% of the original ACAT activity. Cycloheximide under this condition causes an 8-fold increase in ACAT activity; the increase approaches a maximum in 6–8 h. The extent of ACAT activation by cycloheximide inversely depends on exogenous sterol present in the medium; LDL diminishes the activation, while cationized LDL or 25-hydroxycholesterol completely abolishes the activation. Adding LDL back to the sterol-free medium causes a 40–70-fold increase in ACAT activity; however, the activation of LDL is *not* further augmented if the cells are pretreated with cycloheximide. The above observations are qualitatively confirmed by ACAT assays in vitro with cell homogenates. LDL or cycloheximide has no effect on the rates of ^{3}H-labeled triglyceride and ^{3}H-labeled polar lipid synthesis. Efflux of prelabeled cholesterol from cells is cycloheximide-insensitive. Rates of degradation of [^{3}H]-leucine-pulse-labeled total protein in cells grown with or without LDL are identical. The above results imply the existence of at least one specific short-lived factor that directly or indirectly inhibits ACAT activity. Halting cellular protein synthesis by adding cycloheximide depletes the short-lived factor, resulting in recovery of ACAT activity. In intact cells, the inhibitory effect is abolished by exogenous sterol. In crude cell homogenates, the inhibitory effect is shown to be reversible, since it is abolished after ACAT activity has been reconstituted into cholesterol-containing liposomes.

Acyl coenzyme A:cholesterol acyltransferase (ACAT)[1] is believed to play an important role in intracellular cholesterol storage and cholesterol homeostasis in various cells [for reviews, see Spector et al. (1979), Chang (1983), Brown & Goldstein (1983), Erickson (1984), and Suckling & Stange (1985)]. Cholesterol ester synthesis and ACAT activity in the arteries of experimental animals are augmented after cholesterol feeding to induce atherosclerosis [for a review, see Goldstein & Brown (1977)]. In tissue culture cells, ACAT activity is highly activated by low-density lipoprotein (LDL), oxygenated cholesterol analogues such as 25-hydroxycholesterol (25-OH-chol) (Goldstein et al., 1974; Brown et al., 1975a), or cationized LDL (Basu et al., 1976). The entry of the latter two agents into the cell interior is not dependent on LDL receptors on the cell surface [reviewed by Goldstein & Brown (1977)]. Studies utilizing reconstituted LDL (Krieger et al., 1978) have shown that the cholesterol ester moiety of the LDL particle, after hydrolytic cleavage by lysosomal cholesterol esterase to form free cholesterol, causes the ACAT activation. The activation process is shown to be insensitive to protein synthesis inhibitors in various cell types (Drevon et al., 1980; Doolittle & Chang, 1982a), implying that the short-term activation of ACAT by sterol is not due to a change in enzyme content but rather a modulation in the activity of preexisting enzyme. Recently, results from several laboratories (Basheeruddin et al., 1982; Gavey et al., 1983; Suckling et al., 1983) suggest that ACAT activity in crude microsomes may be modulated in vitro by a reversible, enzymatic phosphorylation–dephosphorylation mechanism. It is unclear at the present time whether this mechanism operates in intact cells and if it mediates the sterol activation on ACAT activity.

This laboratory previously reported solubilization of ACAT activity from pig liver microsomes and from Chinese hamster ovary (CHO) cells by deoxycholate (Doolittle & Chang, 1982a,b). The solubilized activity was reconstituted into liposomes of known cholesterol and phospholipid content by employing the dialysis procedure. In the reconstituted vesicles, the enzyme activity was shown to be completely dependent on exogenous cholesterol in a concentration-dependent manner; the endogenous cholesterol was diluted to a negligible concentration by adding a large excess of phospholipid during the reconstitution procedure. This method allows enzyme activity to be assayed independent of its original lipid environment. The CHO cell ACAT activity as measured in cell homogenates by a conventional assay (using cellular cholesterol as the substrate) was activated approximately 10–20-fold when LDL

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[1] Abbreviations: ACAT, acyl coenzyme A:cholesterol acyltransferase; BSA, bovine serum albumin; CHO, Chinese hamster ovary; EDTA, ethylenediaminetetraacetic acid; fcs, fetal calf serum; LDL, low-density lipoprotein; LDL$^-$ medium, F-12 + 10% delipidated fcs + 35 μM oleate; LDL$^+$ medium, LDL$^-$ medium + 75 μg of protein/mL of LDL; medium A, F-12 + 10% fcs; PBS, phosphate-buffered saline; TG, triglyceride; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; 25-OH-chol, 25-hydroxycholesterol.

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was added before harvesting. When the cell homogenates were assayed after optimal reconstitution, the activation produced by LDL was essentially abolished. Also, when cells were switched to grow from sterol-containing medium to sterol-free medium, there was no difference in enzyme activity after the reconstitution in contrast to more than a 7-fold drop in enzyme activity when assayed without reconstitution. These results suggest that the enzyme activity in intact cells is controlled by the content and composition of cellular modulators associated with the enzyme molecule. The effect(s) of the cellular modulators on ACAT activity is (are) reversible, since it is abolished during reconstitution. These results are in agreement with works from other laboratories (Tavani et al., 1982; Stange et al., 1982; Mitropoulos & Venkatesan, 1984), which suggest that cholesterol availability may constitute an important factor in ACAT regulation. However, these studies cannot deduce how cholesterol may act at the molecular level in controlling ACAT activity.

In this work we report our findings on the effect of the protein synthesis inhibitors such as cycloheximide and emetine on sterol-dependent regulation of ACAT in CHO cells. Preliminary reports of this work have been presented (Chang & Chang, 1984, 1985). During our investigation, other laboratories have reported ACAT activation by cycloheximide (Tavani et al., 1982; Field et al., 1982); however, the relation between the cycloheximide effect and the sterol effect on ACAT activity was not reported.

Materials and Methods

Materials. [9,10-^{3}H]Oleic acid (13.3 Ci/mmol) was from New England Nuclear. Sodium [1-^{14}C]acetate (49 mCi/mmol) was from Amersham. [^{3}H]Oleate–bovine serum albumin (BSA, fatty acid free; from Sigma) was prepared as described (Goldstein et al., 1974). [^{3}H]Oleyl coenzyme A was synthesized as described (Doolittle & Chang, 1982b). Nonradioactive oleyl coenzyme A was synthesized according to a published procedure (Stadtman, 1957) with slight modification: Dissolve 250 mg of coenzyme A (sodium salt, from Sigma) in 11.5 mL of H_2O, 33.5 mL of tetrahydrofuran (freshly redistilled in $LiAlH_4$), and 5.2 mL of 1 M $KHCO_3$. Dissolve 1 g of oleyl anhydride (from Sigma) in 5 mL of tetrahydrofuran. Four or five 0.3-mL aliquots of the oleyl anhydride solution are added to the coenzyme A solution at 0 °C with stirring in a span of approximately 1 h until the free sulfhydryl moiety of coenzyme A disappears (Ellman, 1959). Purification and estimation of oleyl coenzyme A were according to a published procedure (Al-Arif & Blecher, 1969). The purified ^{3}H-labeled and the nonradioactive oleyl coenzyme A contained less than 1% impurity of coenzyme A or oleate. Cycloheximide (used at 890 μM in the medium) and emetine (used at 5 μM in the medium) (Chang et al., 1981b) were from Sigma. Deoxycholic acid were from Calbiochem. Human LDL and delipidated fetal calf serum (fcs) were prepared as described (Chin & Chang, 1981). Cationized LDL was prepared according to a published procedure; the protein concentration was determined by the value from the Lowry method divided by 1.5 (Basu et al., 1977). The cholesterol content, as determined by the gas–liquid chromatography described earlier (Chang & Chang, 1982), was 1.58 mg/mg of protein for LDL (with 82% as esterified cholesterol) and 1.05 mg/mg of protein (with 80% as esterified cholesterol) for cationized LDL, consistent with the values reported by Basu et al. (1977). Fetal calf serum was from K. C. Biological. 25-Hydroxycholesterol was from Steraloids, Inc.; it was added to culture medium as previously described (Chang & Chang, 1982).

Continuous cultures of CHO cells previously employed (Chin & Chang, 1981; Chang et al., 1981a) were maintained in F-12 (linoleic acid deleted) plus 10% fcs (medium A) in 80-cm^2 Nunc flasks. To plate cells, confluent monolayers of cells from stock flasks were dissociated with 0.002% trypsin (Sigma; catalog no. T-1005) and were seeded at 0.5×10^6 cells per 25-cm^2 flask in 4 mL of medium A or 1.5×10^6 cells per 80-cm^2 flask in 12 mL of medium A for 24 h. Cells were then switched to grow in 4 or 12 mL of LDL$^+$ medium (F-12 + 10% delipidated fcs + 35 μM oleate + 75 μg of protein/mL of LDL) for 24 h. Cells at this stage were designated as the starting culture. To initiate various experiments in this paper, 2 h before the zero time cells were rinsed twice with prewarmed PBS, and prewarmed media (specified in each experiment) were added; each flask was purged with a smooth stream of a sterilized gas mixture consisting of 6% CO_2 and 94% air for a few seconds. The flasks were tightly sealed with caps and laid on top of a platform partially immersed in a 37 °C H_2O bath. The temperature of the medium in each flask was found to equilibrate at 37 °C within 5 min. A much longer time (2–3 h) was needed for the medium to reach 37 °C if the flasks were returned to the water-jacketed CO_2 incubator. It is important that cells be maintained at constant temperature in order to achieve quantitatively reproducible results in the various kinetic experiments reported here. The pH of the sealed flasks remained constant (7.3 ± 0.1) for more than 8 h.

ACAT Assays. (*A*) *In Intact Cells.* Each 25-cm^2 flask was pulse-exposed to 20 μl of [^{3}H]oleate–BSA (10 mM oleate in 10% BSA in PBS, 7.5×10^6 dpm) at 37 °C for 20 min. To harvest, cells were rinsed in 5 × 10 mL of PBS at 4 °C and dissolved in 1.0 mL of 0.1 M NaOH at room temperature for approximately 30 min. Aliquots (usually 2 × 20 μL) were withdrawn for protein measurement, and the remaining extract was neutralized to pH 7.0 ± 0.7 by adding 3 M HCl. [^{14}C]Cholesteryl oleate (New England Nuclear, 2000 dpm/sample) was added to serve as internal standard for recovery purposes. Amounts of 20 μg each of cholesteryl oleate, triolein, and egg phosphatidylcholine (all from Sigma) were also added for identification purposes. Control experiments showed that hydrolysis of triolein (10 μg/mL) in 0.1 M NaOH without any organic solvent at room temperature for 60 min was negligible (less than 5%). The cellular lipid was extracted with $CHCl_3$–CH_3OH (1:2) as described (Chang & Chang, 1982) and analyzed by thin-layer chromatography using Whatman LK5D plates and petroleum ether–ether–acetic acid (90:10:1) as the solvent system. The lipid bands were identified with iodine vapor (cholesterol esters, R_f 0.9; triglyceride (TG), R_f 0.4; polar lipid (mostly phospholipid), R_f 0); the bands were scraped and counted in 4 mL of Econofluor (New England Nuclear). The recovery averaged approximately 75%.

(*B*) *Cell Homogenates.* Monolayers of CHO cells grown in 80-cm^2 flasks were harvested in buffer K (1 mM Tris-HCl, 2 mM EDTA, pH 7.8) by the rapid scraping method previously described (Chang et al., 1981b). Usually, 240-μL aliquots of cell homogenate at approximately 2 mg/mL of protein were immediately used for assaying. To start the assay, disposable glass tubes (13 × 100 mm) containing the cell extracts were preincubated at 37 °C for 2 min, followed by the addition of 40 μL of a reaction mixture consisting of 50 mM Tris-HCl, 10 mM EDTA, 10 nmol of [^{3}H]oleyl coenzyme A (usually 100 000 dpm), and 0.5 mg of fatty acid free BSA at pH 7.8. The reaction was stopped after 5 min and analyzed for ACAT activity as previously described (Doolittle & Chang, 1982a). We found the ACAT activity gradually deviates from

linearity (concaving down) after 10 min. The shorter reaction time and higher incubation temperature resulted in higher ACAT specific activities than previously reported (Doolittle & Chang, 1982a). To assay ACAT activity after reconstitution [see Figure 7 for reconstitution procedure, which was modified slightly from the procedure described by Doolittle & Chang (1982a)], 100-μL aliquots of dialyzed reconstituted mixture containing approximately 50 μg of protein were used at 37 °C; the assay time was 10 min. After reconstitution, the ACAT activity was linear with time for at least 20 min.

Sterol Synthesis and Fatty Acid Synthesis in Intact Cells. [1-^{14}C]Acetate pulse experiments were performed in intact cells incubated in a 37 °C H_2O bath for 20 min. After saponification, the lipids were processed and analyzed as described previously (Chang et al., 1979). The sum of ^{14}C counts in the C_{27} sterol band and the C_{29} and C_{30} sterol bands (identified by iodine vapor staining of cholesterol and lanosterol standards added internally) was assumed to be the total ^{14}C-labeled sterols synthesized during the pulse.

Measurement of Sterol Efflux. Cells were plated in 25-cm^2 flasks in 4 mL of medium A containing 0.642 μCi/flask of [1α,2α[^{3}H]cholesterol (58 Ci/mmol), grown for 36 h, rinsed 5 times with 5 mL of prewarmed PBS, and fed 4 mL/flask LDL$^+$ medium without labeled cholesterol for 12 h. The medium was replaced, and cells were incubated in a 37 °C H_2O bath for 2 h. At time zero, cells were rinsed 3 times with 5 mL of prewarmed PBS and fed 4 mL/flask LDL$^+$ medium or LDL$^-$ medium. Cycloheximide for PBS as control was added for 3 h. Media were then removed; the cells were rinsed 5 times with 2-mL portions of PBS at 4 °C. The washes were pooled together. The cells were dissolved in 1.0 mL of 0.1 M NaOH. Aliquots (usually 100 μL/flask) from the medium or from the pooled PBS washes were withdrawn, mixed with 3.5 mL of Biofluor (New England Nuclear), and counted. The sum of these two values was assumed to be the [^{3}H]cholesterol released into the medium from the cells. The percent [^{3}H]-cholesterol in the medium was determined as the dpm found in the medium divided by the sum of dpms found in the cells and in the medium. The cell culture at time zero was found to contain 11.9 × 10^3 dpm and 0.5 mg of protein/flask. Thin-layer chromatographic analysis showed that 83% of the ^{3}H counts in the zero-time culture was cholesterol with the rest found as cholesterol ester. Results were obtained from the average of duplicate flasks. Deviation between duplicates was within 5% of the mean. Protein was determined by the modified Lowry method (Peterson, 1977) with BSA standards.

Results

Cells grown in LDL$^+$ medium expressed high ACAT activity. Upon removal of LDL from the medium, ACAT activity decreased rapidly with time (Figure 1A). In seven different experiments, the initial inactivation rate (within 2 h after the LDL removal) was found to exhibit apparent first-order kinetics, with the half time ($t^{1/2}$) of inactivation being 0.81 ± 0.05 (SD) h. For comparison, the increase in rate of sterol synthesis from [^{14}C]acetate was studied in a parallel set of cultures. A slow, gradual increase was found; the increase was approximately 1.5-fold at the eighth hour (Figure 1D). Control experiments showed that the rate of triglyceride synthesis (Figure 1B), the rate of polar lipid synthesis from [^{3}H]oleate (Figure 1C), and the rate of fatty acid synthesis from [^{14}C]acetate were not affected by LDL removal (results not shown).

Incubation of cells with the protein synthesis inhibitor cycloheximide partially blocked the ACAT inactivation caused by removal of LDL (Figure 2A). After cells have been

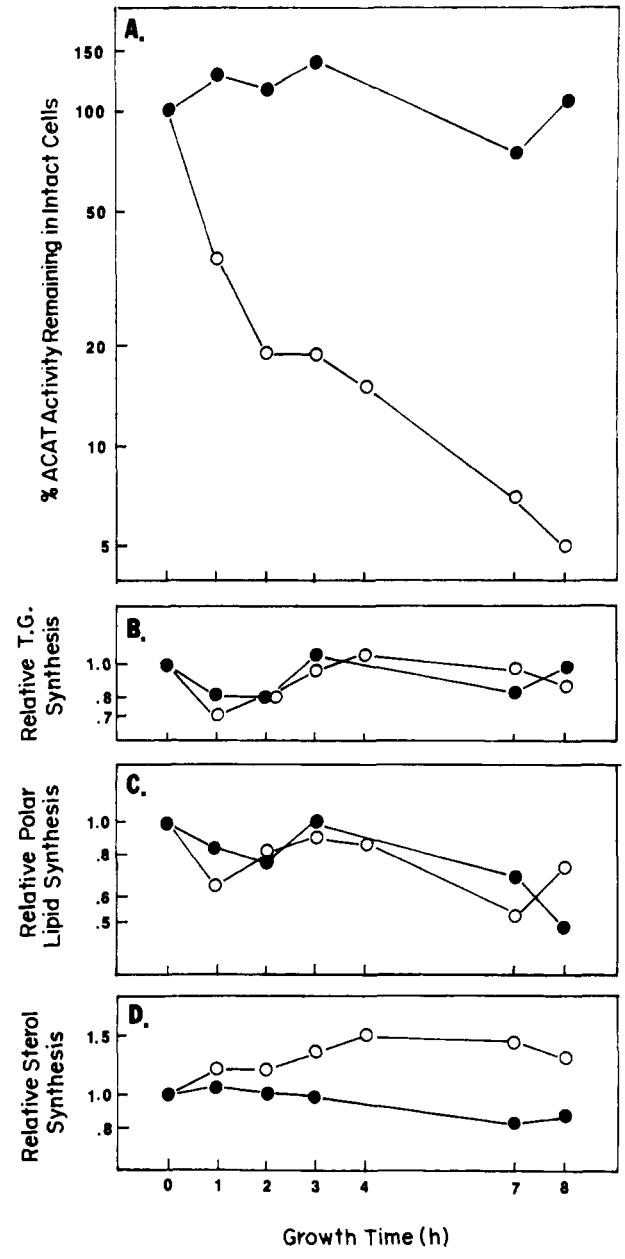

FIGURE 1: Effect of LDL removal on (A) ACAT activity, (B) rate of TG synthesis, (C) rate of polar lipid synthesis, and (D) rate of sterol synthesis in intact cells. Two parallel sets of starting culture seeded in 25-cm^2 flasks were refed once with LDL$^+$ medium and incubated in a 37 °C H_2O bath for 2 h. At time zero, the monolayers were rapidly rinsed 3 times by 5 mL/flask prewarmed PBS; 4 mL of LDL$^+$ medium or LDL$^-$ medium was then added. At the time indicated, each flask was pulse-labeled with [^{3}H]oleate or with [^{14}C]acetate to measure ACAT activity, rate of TG synthesis, rate of polar lipid synthesis, and rate of sterol synthesis in intact cells as described under Materials and Methods. Each value represents the average of results from duplicate flasks. Variation between duplicates was within 7% from the mean. Zero-time culture contained 61 pmol min^{-1} mg^{-1} (1.38 × 10^3 dpm min^{-1} mg^{-1}) for ACAT activity, 20.0 × 10^3 dpm min^{-1} mg^{-1} for [^{3}H]TG synthesis, 10.5 × 10^3 dpm min^{-1} mg^{-1} for ^{3}H-labeled polar lipid synthesis, and 67.5 dpm min^{-1} mg^{-1} for ^{14}C-labeled sterol synthesis. The amount of protein in the zero-time cultures was approximately 0.4 mg/25-cm^2 flask. Symbols: (●) LDL$^+$ medium; (○) LDL$^-$ medium.

preincubated with cycloheximide for 2 h or longer, removal of LDL caused a slower but still measurable rate of ACAT inactivation (Figure 2B). In three experiments, the average $t^{1/2}$ for the initial inactivation rate was found to increase from 0.8 ± 0.05 to 2.1 ± 0.2 h after the cycloheximide treatment.

As already seen in Figure 2, cycloheximide added to cells

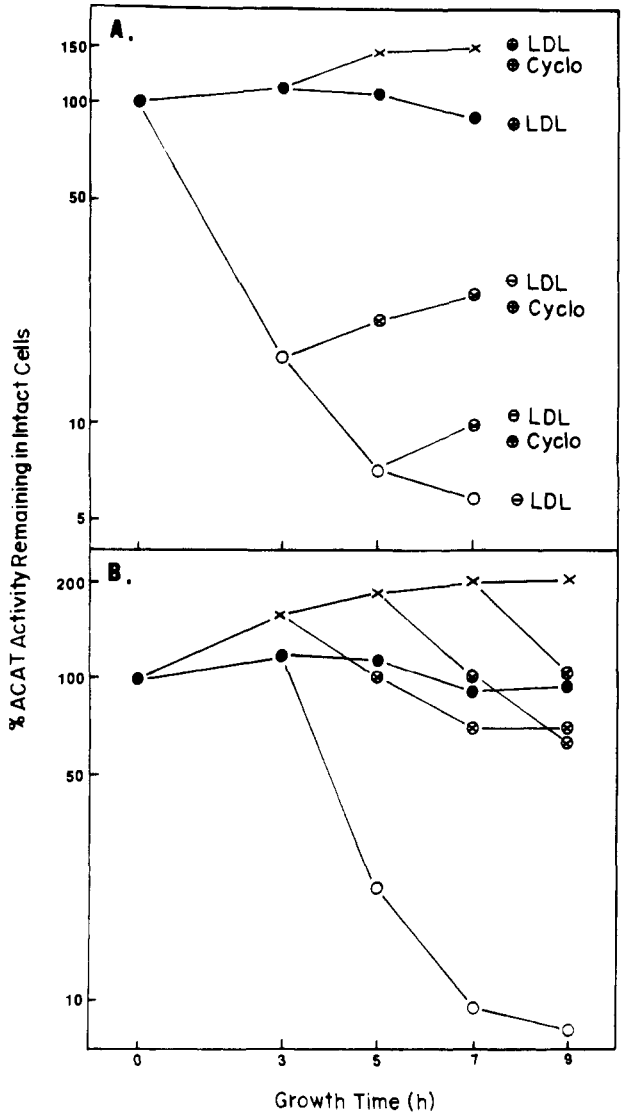

FIGURE 2: (A) Incubation of cells with cycloheximide partially blocks ACAT inactivation. (B) Preincubation of cells with cycloheximide slows down the apparent rate of ACAT inactivation. Monolayers of starting culture were incubated with 4 mL of LDL^+ medium for 8 h. The medium was replaced, and cells were incubated in a 37 °C H_2O bath for 2 h. In (A), at time zero, each flask was rapidly rinsed 3 times by 5 mL/flask prewarmed PBS and then fed LDL^+ or LDL^- medium. At the time indicated, cycloheximide or PBS as control was added. In (B), at time zero, either cycloheximide or PBS as control was added in cells grown in LDL^+ medium. At the time indicated, the monolayers were rinsed 3 times by 5 mL/flask of prewarmed PBS before being switched to grow in LDL^+ or LDL^- medium; cycloheximide was included only in medium for cells preincubated with cycloheximide. ACAT activity was measured by an [^{3}H]oleate pulse as described under Materials and Methods. Each value represents the mean of duplicate flasks; deviation from the mean was within 7%. Symbols: (●) LDL^+ medium; (○) LDL^- medium; (×) cycloheximide in LDL^+ medium; (⊗), cycloheximide in LDL^- medium. A circled plus sign used in the figures of this and the accompanying paper (Chang & Chang, 1986) represents "plus".

incubated in LDL^+ medium caused an activation of ACAT. This observation was substantiated in the experiment shown in Figure 3A, top curves. The activation process was time-dependent, reaching a maximum of 2-fold in 2.5 ± 0.5 h (average of four experiments). A parallel experiment showed cycloheximide added to cells incubated in LDL^- medium (F-12 + 10% delipidated fcs + 35 μM oleate) also caused an activation of ACAT (Figure 3A, bottom curves). However, the maximal extent of this activation was much larger [(8 ± 2)-fold]; the time required to reach the maximal activation

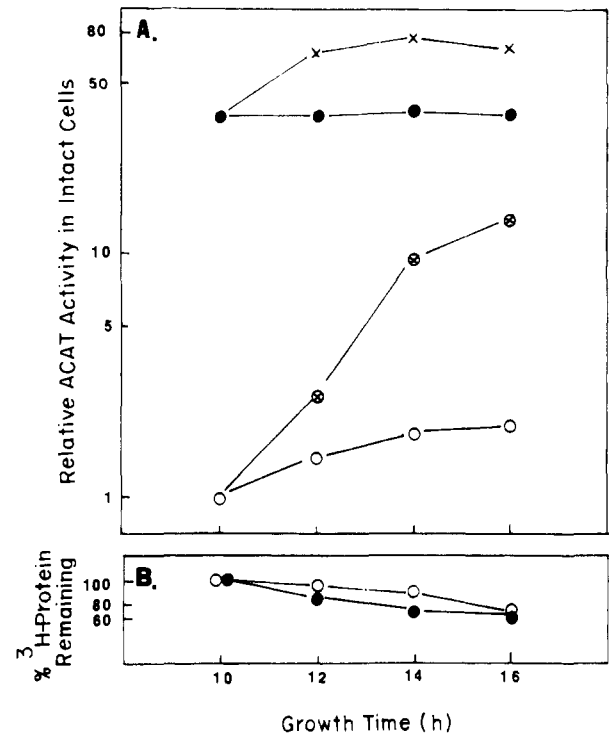

FIGURE 3: (A) Kinetics of ACAT activation by cycloheximide. (B) Effect of LDL on rate of disappearance of [^{3}H]leucine-pulse-labeled proteins in intact CHO cells. Starting culture was grown in LDL^+ or LDL^- medium for 8 h. The medium was replaced, and cells were incubated in a 37 °C H_2O bath for 2 h to start zero time. In (A), at time zero, cycloheximide or PBS was added. At the time indicated, cells were pulsed with [^{3}H]oleate to measure ACAT activity and rates of TG and polar lipid synthesis as described under Materials and Methods. In (B), in a separate experiment, after cells were incubated in a 37 °C H_2O bath for 1 h, 6 μL/flask L-[4,5-^{3}H]leucine (Amersham, 50 Ci/mmol, 0.28 μCi/μL) was added, and the cultures were incubated for an additional 1 h. At time zero, cycloheximide was added. At the time indicated, cultures were washed 5 times with PBS and 5 times with 5% trichloroacetic acid (TCA). The precipitates were dissolved in 0.2 M NaOH, and aliquots were counted in Biofluor (New England Nuclear) to determine ^{3}H dpm remaining per flask. Data are expressed as percentages of the value obtained immediately after the 1-h pulse, which was 77.7 × 10^2 dpm/flask. Values shown in (A) and (B) represent the average of results from duplicate flasks. Symbols: (●) LDL^+ medium; (○) LDL^- medium; (×) cycloheximide in LDL^+ medium; (⊗) cycloheximide in LDL^- medium.

was much longer (7 ± 1 h). The results in Figure 3A were confirmed by using emetine, a different protein synthesis inhibitor (Gupta & Siminovitch, 1977; Chang et al., 1981b) (results not shown), ruling out the possibility that the effect seen was due to side effect(s) of cycloheximide other than inhibiting protein synthesis in intact cells. Control experiments showed that the rate of triglyceride synthesis and the rate of polar lipid synthesis in cells grown in LDL^- medium were not significantly affected by cycloheximide (data not shown). Furthermore, the rates of degradation of [^{3}H]leucine-pulse-labeled total cellular proteins in cells grown with or without LDL were identical (Figure 3B), ruling out the possibility that the difference in kinetics and extent of ACAT activation by cycloheximide in cells grown with or without LDL might be due to the presence of LDL in affecting the efficiency of the cellular protein degradation machinery. Results in Figure 4A-a showed that the extent of ACAT activation by cycloheximide progressively diminished as the concentration of LDL in the medium increased. This relationship became more apparent when the extent of ACAT activation by cycloheximide was plotted as the fold increase in ACAT activity, as shown in Figure 4A-b.[2] The result in Figure 3 was quanti-

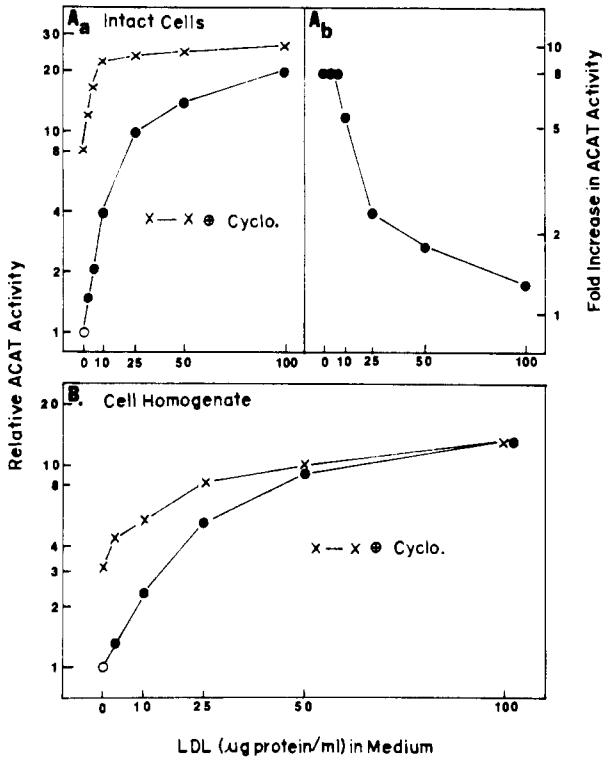

FIGURE 4: Extent of ACAT activation by cycloheximide diminishes as LDL concentration increases, as measured in (A) intact cells and (B) cell homogenates. Starting cultures seeded in 25- or 80-cm^2 flasks were switched to media containing increasing amounts of LDL for 8 h. The media were replaced, and cells were incubated in a 37 °C H_2O bath for 2 h. Cycloheximide (×) or PBS (●) was added, and cells were incubated for 6 h before ACAT activity was measured. In (A), the ACAT activity in intact cells was measured by an [^{3}H]oleate pulse. Each value represents the mean of results from duplicate flasks. Cell cultures grown without LDL or cycloheximide expressed an ACAT activity of 2.4 pmol min^{-1} (mg of protein)$^{-1}$. In (B), the ACAT activity in cell homogenate was measured by an ACAT assay in vitro as described under Materials and Methods. Each value represents the mean of duplicate assays from a single flask of cells. Cell cultures grown without LDL or cycloheximide expressed an ACAT activity of 4.1 pmol min^{-1} mg^{-1}. In (b), the fold increase in ACAT activity was defined as the ACAT activity of cycloheximide-treated cells divided by that of untreated cells at a given LDL concentration.

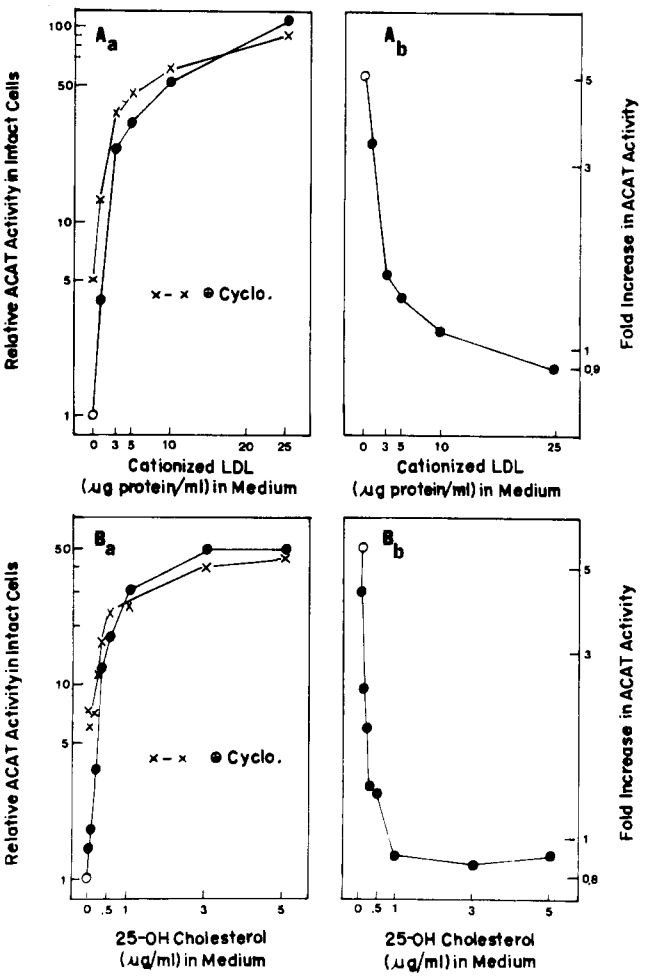

FIGURE 5: Extent of ACAT activation by cycloheximide diminishes as concentration of (A) cationized LDL or (B) 25-OH-chol increases. Monolayers of starting culture were incubated with 4 mL/flask LDL$^-$ medium containing the indicated amounts of cationized LDL or 25-OH-chol for 8 h. The media were replaced, and cells were incubated in a 37 °C H_2O bath for 2 h. Cycloheximide (×) or PBS (●) was added, and cells were incubated for 4 h. Each flask was pulse-labeled with [^{3}H]oleate to measure ACAT activity in intact cells as described under Materials and Methods. In the right panels (A-b and B-b), values for fold increase in ACAT activity due to cycloheximide were obtained in the same manner as described in Figure 4. Cell cultures grown in medium without cationized LDL, 25-OH cholesterol, or cycloheximide expressed an ACAT activity of 4.6 pmol min^{-1} mg^{-1}.

tatively confirmed by assaying ACAT activity in vitro with crude cell homogenates (Figure 4B). Furthermore, it was shown that cationized LDL (at 25 μg of protein/mL, Figure 5A) or 25-hydroxycholesterol (at 1 μg/mL or higher, Figure 5B) could completely abolish the activation by cycloheximide. The results shown in Figure 5 were consistently obtained in three separate experiments.

As shown in Figure 6A when LDL was removed for more than 10 h, the cells were found to contain only a few percent of the original ACAT activity; adding LDL back to the medium caused a 40–70-fold increase in ACAT activity. This activation process was cycloheximide-insensitive, confirming earlier studies (Drevon et al., 1980; Doolittle & Chang, 1982a). However, the activation by LDL (at 75 μg/mL) was *not* further augmented if the cells were pretreated with cycloheximide; i.e., the activation of ACAT by cycloheximide and the activation by LDL were not additive, substantiating the notion that the ACAT-inactivating activity of the cycloheximide-sensitive process is intimately related to the effect of LDL. The results in Figure 5 were qualitatively confirmed by ACAT assays in vitro with cell homogenates (Figure 6B). This experiment showed that the inactivating effect of the cycloheximide-sensitive process on ACAT was at least partially preserved in the cell homogenates.

When ACAT activities in cell homogenates were solubilized by deoxycholate and reconstituted into artificial phospholipid vesicles containing an optimal concentration of exogenous cholesterol by a slight modification of our previously published procedure (Doolittle & Chang, 1982a), the difference in ACAT activity caused by cycloheximide in cells grown in LDL$^-$ medium (Figure 7, open bars) was found to be abolished (closed bars). (The same result was seen in three separate experiments.) The difference in ACAT activities in cells grown with or without a sterol source (LDL, cationized LDL, or 25-hydroxycholesterol) was also found to be abolished.

It was previously shown in various cell types (Oram et al., 1981; Glick et al., 1983) that efflux of sterol from cells into the growth medium caused a decrease in ACAT activity. We

[2] The fold increase in ACAT activity due to cycloheximide is calculated as the ratio of ACAT activity in cycloheximide-treated cells vs. that in untreated cells. This plot is justified since the ACAT content under these conditions stayed constant [as measured after reconstitution in artificial phospholipid vesicles; Doolittle & Chang (1982a) and Figure 7 of this paper].

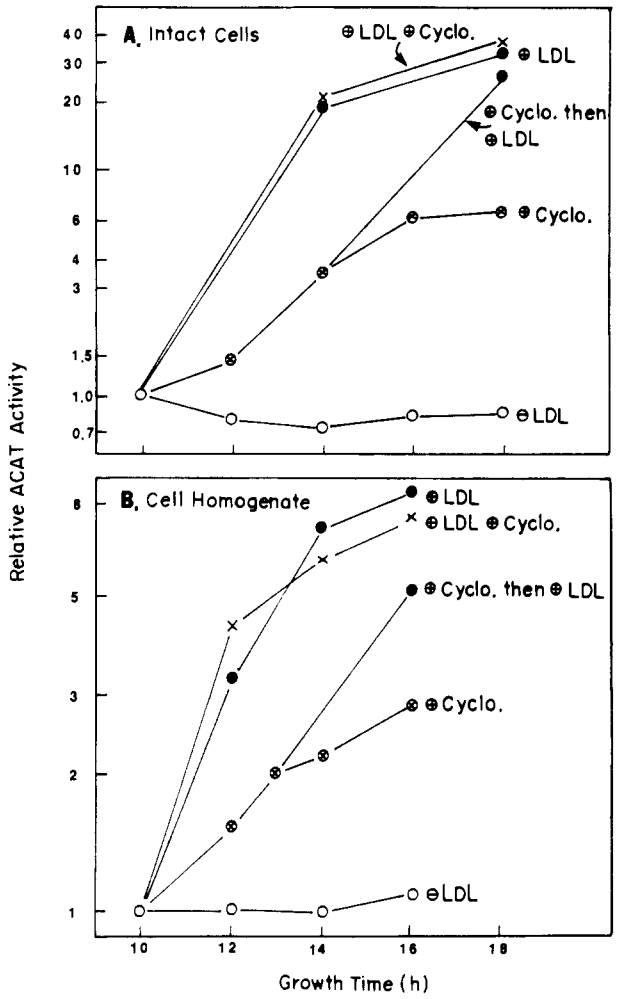

FIGURE 6: Activation of ACAT activity by LDL and/or by cycloheximide, as measured by (A) an [^{3}H]oleate pulse in intact cells and (B) in vitro assay with cell homogenates. Starting cultures in 25- or 80-cm^2 flasks were switched to LDL$^-$ medium for 10 h, with a medium replacement at the eighth hour. At the time indicated, cells were exposed to PBS (○) as the control, 100 μg of protein/mL of LDL (●), cycloheximide (⊗), cycloheximide and LDL (×), or cycloheximide for 3 or 4 h followed by LDL (●). The ACAT activity in intact cells or in cell homogenates was then measured as described under Materials and Methods. Each value represents the average of results of duplicate flasks. Variation between duplicates was within 7% of the mean. ACAT activity in zero-time culture was 6.8 pmol min^{-1} mg^{-1} in the intact cell assay and 7.2 pmol min^{-1} mg^{-1} in cell homogenates.

performed sterol efflux experiments using CHO cells and found that the percent of [^{3}H]cholesterol released into the medium in a 3-h period was 21.1% in LDL$^+$ medium or 11.1% in LDL$^-$ medium. In either case, we found that cycloheximide had no effect on sterol efflux, thus eliminating the possibility that the cycloheximide-sensitive process reported here may play a direct role in mediating cellular sterol efflux.

Discussion

The results in Figures 1 and 2 show that when monolayers of CHO cells are properly maintained at 37 °C, the inactivation of ACAT activity is an early, specific, and rapid event responding to the removal of LDL. This event precedes the induction of endogenous sterol synthesis. Our data employing cycloheximide are consistent with the following hypothesis: the rapid inactivation of ACAT activity requires the presence of at least one specific, short-lived inhibitory factor as well as a decrease in content or activity of a cycloheximide-insensitive factor. The inhibitory activity of the short-lived factor is diminished by LDL and can be completely abolished when

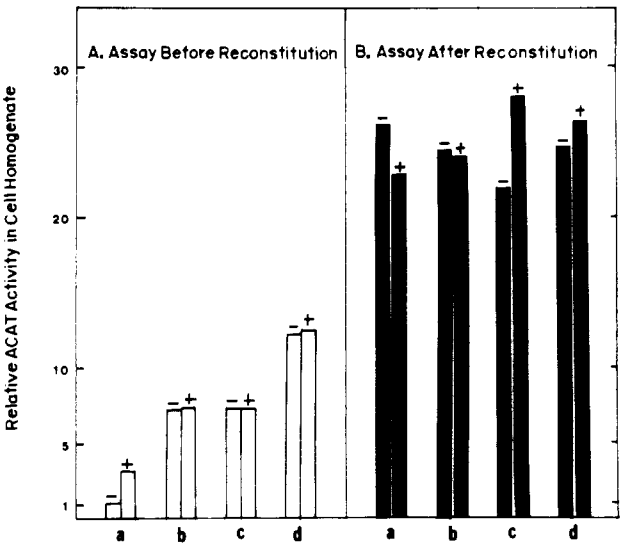

FIGURE 7: Comparison of ACAT activities from cells grown in the absence (−) or presence (+) of cycloheximide by reconstituted (solid bars) or nonreconstituted (open bars) enzyme assays. Monolayers of starting culture were incubated with 12 mL/flask LDL$^-$ media containing (a) none, (b) 100 μg of protein/mL of LDL, (c) 1 μg/mL 25-hydroxycholesterol, or (d) 25 μg of protein/mL of cationized LDL for 8 h. The media were replaced, and cells were incubated in a 37 °C H_2O bath for 2 h. Cycloheximide or PBS as control was added, and cells were incubated for 4 h. Cell homogenates without reconstitution were assayed immediately. For the reconstitution assay, the following procedure, which was a slight modification of the method reported earlier from this laboratory (Doolittle & Chang, 1982a), was employed. Cell homogenates were added to a mixed micelle solution to yield a final concentration of 4 mg/mL phosphatidylcholine, 9.5 mg/mL deoxycholate, 0.6 mg/mL cholesterol, and approximately 0.5 mg/mL cell protein. The mixture was dialyzed at 4 °C for 9 h against a 200-fold excess of buffer (50 mM Tris/1 mM EDTA, pH 7.8) and assayed for enzyme activity. Results are plotted as the fold increase relative to the nonreconstituted ACAT activity from cells without LDL and cycloheximide treatment, which has the enzyme specific activity of 8.9 pmol min^{-1} mg^{-1}. Each point represents the average of duplicate assays from a single flask of cells; deviation from the mean was within 7%.

cationized LDL or 25-hydroxycholesterol is present in the medium. The time required for cellular depletion of this short-lived factor is accelerated from 6–8 h to approximately 2.5 h by LDL present in the medium (Figure 3A). The specificity of this system is supported by the control experiment, which showed that LDL did not alter the rate of degradation of [^{3}H]leucine-pulse-labeled proteins in intact cells (Figure 3B). It is unlikely that this factor bears any direct relationship with the presumptive mediator protein, which mediates the sterol effect on posttranslational inhibition of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity in CHO cells (Chang et al., 1981a), since the time required for cellular depletion of these two factors in cells grown in delipidated serum differ from each other [6–8 vs. 2 h or less; Figures 3 and 6 in this paper and Figure 5 in Chang et al. (1981a)]. The short-lived factor reported here may act as an inhibitor of ACAT activity by competing against LDL-derived cholesterol. The cycloheximide-insensitive factor is probably cholesterol serving as the ACAT substrate.

This hypothesis does not provide a molecular mechanism to explain how the short-lived factor may act on ACAT. Nevertheless, it is conceivable that two types of mechanisms may be operating. The first is that the short-lived factor may directly inhibit ACAT by binding to an allosteric site or modifying an enzymic site on ACAT. Its action is abolished by cholesterol or analogues of cholesterol. Removal of LDL decreases the cholesterol pool near ACAT causing stronger

interaction between the short-lived factor and ACAT, resulting in rapid inactivation of ACAT. Removal of LDL after depletion of this factor by cycloheximide still causes a decrease in the cholesterol pool; however, the ACAT inactivation rate is slower due to the absence of the short-lived inhibitor. The alternate possibility is that the short-lived factor may play a role in an intracellular cholesterol translocation process that depletes the substrate pool for ACAT, resulting in inactivation of ACAT. There may be a continuous flow of cholesterol entering and exiting a specific pool, which serves as the substrate for ACAT. A decrease in exiting rate, or an increase in entering rate, would "activate" the ACAT activity. In this model, the short-lived factor would be involved in removing cholesterol from the ACAT substrate pool. When this factor is depleted by cycloheximide, a second, cycloheximide-insensitive process still operates in removing cholesterol from the ACAT substrate pool, but at a slower rate.

Three other possibilities, none of which is consistent with the current data, are nonetheless worthwhile discussing. The first is a direct involvement of the short-lived factor in sterol entrance to the ACAT substrate pool. This would have caused a *decrease* in ACAT activity after the cycloheximide treatment, a result inconsistent with the current observation. The second possibility is that there may exist an unknown cycloheximide-sensitive, cholesterol utilization process that shares the LDL-derived cholesterol with the ACAT substrate pool. Halting this process by adding cycloheximide increases the ACAT activity by shunting more cholesterol available to the ACAT substrate pool. This is not a viable possibility since CHO cells, a fibroblast-like cell line, unlike the intestinal organ culture (Field et al., 1982) or the intact rat ovary (Tavani et al., 1982), are not capable of lipoprotein synthesis or steroid hormone production. Cholesterol ester is the only detectable metabolite after prolonged [^{3}H]cholesterol incorporation into CHO cells (results described under Measurement of Sterol Efflux). The third possibility would be to attribute the cycloheximide activation of ACAT activity as a nonspecific consequence of halting cellular protein synthesis without having to postulate any specific, short-lived cellular factor serving as the ACAT inhibitor. The fact that we are able to show the cycloheximide activation on ACAT is a specific, sterol-dependent event lends no support to this type of interpretation.

In conclusion, the simplest and the only logical interpretation of our result is to postulate an exogenous sterol-regulated short-lived factor either serving as a direct inhibitor on the enzyme ACAT or participating in removing cholesterol from the ACAT substrate pool, thereby inactivating ACAT activity. The relationship between the effect of endogenous cholesterol biosynthesis and the cycloheximide activation effect on ACAT is reported in the accompanying paper (Chang & Chang, 1986).

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