Cycloheximide Sensitivity in Regulation of Acyl Coenzyme A:Cholesterol Acyltransferase Activity in Chinese Hamster Ovary Cells. 2. Effect of Sterol Endogenously Synthesized†

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ABSTRACT: We reported in another paper [Chang, C. C. Y., Doolittle, G. M., & Chang, T. Y. (1986) *Biochemistry* (preceding paper in this issue)] that in Chinese hamster ovary (CHO) cells activation of acyl coenzyme A:cholesterol acyltransferase (ACAT) activity by treating cells with cycloheximide was abolished by providing exogenous sterol in the medium. We now report that providing 20 mM DL-mevalonate to cells grown in sterol-free medium increases the ACAT activity by approximately 6-fold and diminishes the cycloheximide activation effect. The mevalonate supplement has no significant effect on the rate of triglyceride or polar lipid synthesis, [^{3}H]cholesterol efflux, or bulk protein degradation in cells. The activation of ACAT by mevalonate is prevented by adding a specific squalene oxide cyclase inhibitor to cells, indicating the requirement for endogenous sterol synthesis to mediate the mevalonate effect. In sterol-free medium, if sterol synthesis is blocked by specific enzyme inhibitors or through mutation, the ACAT activation by cycloheximide is again abolished. These results support the hypothesis that there may exist a short-lived factor(s) serving directly or indirectly as an endogenous ACAT inhibitor(s), the inhibitory action of this (these) factor(s) is (are) abolished, and its (their) turnover rate(s) is (are) increased by either exogenous sterol or by sterol endogenously synthesized. Conversely, removing exogenous sterol coupled with blocking endogenous sterol synthesis decreases the turnover rate(s) of the inhibitor(s), rendering its (their) action insensitive to intracellular degradation over the time period studied.

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)]. In cultured mammalian cells, its activity is highly regulated by exogenous sterol in the medium (Goldstein et al., 1974; Brown et al., 1975a). The mode of action of sterol on this enzyme remains largely unknown and needs to be investigated at the molecular level. In the preceding paper (Chang et al., 1986), we showed that in CHO cells the inactivation of ACAT activity in response to the removal of low-density lipoprotein (LDL) from the medium is an early, rapid, and specific event. We then showed that the ACAT activity was activated by protein synthesis inhibitors such as cycloheximide and emetine. The activation by cycloheximide could be diminished or abolished by LDL, cationized LDL, or 25-hydroxycholesterol in the medium. These and other supporting results lead us to propose that there may exist at least one specific short-lived factor that directly or indirectly inhibits ACAT activity. The inhibitory effect is maximally manifested in cells grown without sterol, and its activity is diminished or abolished by exogenous sterol; the turnover rate(s) of this (these) factor(s) seem(s) to be increased by exogenous sterol. In addition, we deduced that the principal mode of action of this (these) factor(s) on ACAT activity has to be a reversible inhibition at the posttranslational level, since reconstituting the ACAT activity in cholesterol-containing liposomes abolishes the inhibitory effect. The cycloheximide-sensitive factor(s) combined with a separate, cycloheximide-insensitive process may constitute the major mechanism for sterol-dependent regulation of ACAT activity in simple cell systems such as the CHO cells.

In this paper, we examine the possible relationship between the cycloheximide activation of ACAT activity and the rate of endogenous sterol synthesis in CHO cells. We take advantage of the fact that mevalonate [the immediate product of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase; for a review, see Change (1983)] present in the medium is able to serve as a biosynthetic precursor of sterol in amounts sufficient for cell growth (Goldstein et al., 1979; Schnitzer-Polokoff et al., 1982). Mevalonate supplements in rat hepatocytes is known to stimulate cholesterol synthesis and cholesterol ester synthesis (Nilsson, 1975; Havel et al., 1979). We also employ compactin as a specific inhibitor of HMG-CoA reductase (Endo et al., 1976) and *trans*-4,4,10β-trimethyldecal-3β-ol (TMD) as a specific inhibitor of squalene-oxide cyclase (Nelson et al., 1978; Chang et al., 1979) to study the effect of blocking endogenous sterol synthesis on cycloheximide activation of ACAT. Finally, the cycloheximide effect is examined in a CHO cell mutant (mutant 1) requiring exogenous cholesterol for growth (Limanek et al., 1978; Chin & Chang, 1981, 1982). The increase in the rate of sterol synthesis in response to LDL removal from the medium has been shown to be defective in this mutant.

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[1] Abbreviations: ACAT, acyl coenzyme A:cholesterol acyltransferase; CHO, Chinese hamster ovary; fcs, fetal calf serum; LDL, low-density lipoprotein; PBS, phosphate-buffered saline; TG, triglyceride; LDL$^-$ medium, F-12 + 10% delipidated fcs + 35 μM oleate; LDL$^+$ medium, LDL$^-$ medium plus 75 μg of protein/mL of LDL; Mev, mevalonate; TMD, *trans*-4,4,10β-trimethyldecal-3β-ol.

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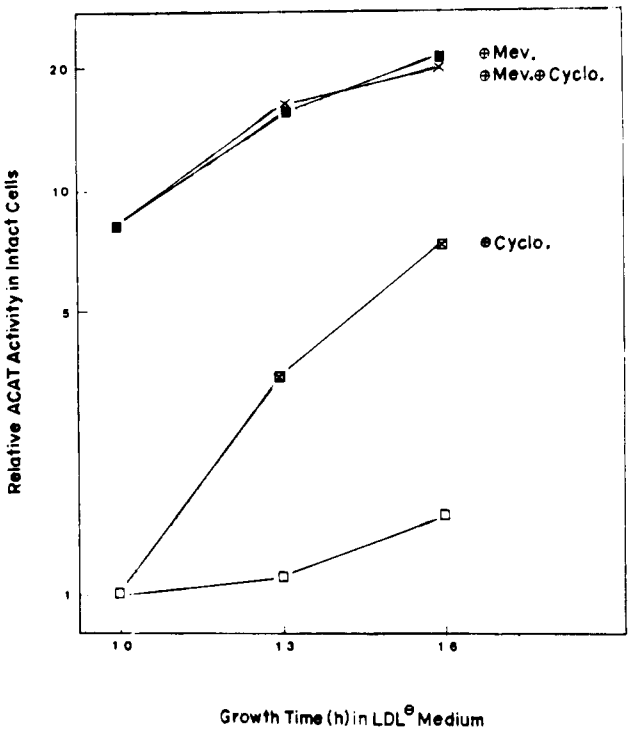

FIGURE 1: DL-Mevalonate (20 mM) abolishes the ACAT activation by cycloheximide. Monolayers of starting culture were incubated with 4 mL/flask LDL⁻ media with or without 20 mM DL-mevalonate for 8 h. The media were replaced, and the cells were incubated in a 37 °C H_2O bath for 2 h, after which either cycloheximide or PBS as the control was added. At the time indicated, each flask was pulse-labeled with [^{3}H]oleate as described under Materials and Methods in the preceding paper. The 10th hour culture grown without mevalonate contained 4 pmol min^{-1} mg^{-1} for ACAT activity. Symbols: LDL⁻ medium with (box with ×) or without (□) cycloheximide; LDL⁻ medium plus 20 mM DL-mevalonate with (×) or without (■) cycloheximide.

Materials and Methods

Sodium DL-mevalonate was prepared from DL-mevalonolactone (Sigma Chemical Co.) according to the procedure described in Brown et al. (1978). The concentration of NaCl in F-12 medium containing varying amounts of mevalonate was adjusted so that the final concentration of NaCl and mevalonate remained constant (130 mM). Compactin and DL-TMD were added to medium as previously described (Chin & Chang, 1982; Chang et al., 1979). DL-TMD, 99% pure by gas–liquid chromatographic analysis, was a gift from Dr. Thomas Spencer, Professor at the Chemistry Department of Dartmouth College. All other materials and methods were described in the preceding paper (Chang et al., 1986).

Results

In four different experiments, the ACAT activity in CHO cells grown in LDL⁻ medium was found to be stimulated [6 ± 1 (SD)]-fold by providing 20 mM DL-mevalonate in the medium. Meanwhile, the mevalonate supplement diminished the extent of ACAT activation by cycloheximide from [4.5 ± 0.7 (SD)]-fold to [1.3 ± 0.3 (SD)]-fold. One of these four experiments is shown in Figure 1. Results in Figure 2 showed that the effect of mevalonate was concentration-dependent. Control experiments showed that the mevalonate supplement had no significant effect on cellular triglyceride synthesis or polar lipid synthesis from [^{3}H]oleate (results not shown). The mevalonate stimulation of ACAT activity was blocked by TMD in a concentration-dependent manner (Figure 3). Control experiments using a [^{14}C]acetate pulse to monitor the rate of ^{14}C-labeled sterol synthesis showed that endogenous

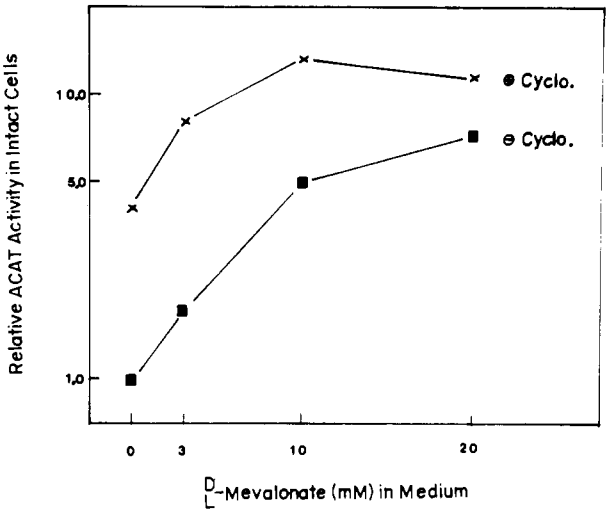

FIGURE 2: Effect of DL-mevalonate concentration on ACAT activation by cycloheximide in intact cells. Monolayers of starting culture were incubated with 4 mL/flask LDL⁻ media containing the indicated amounts of DL-mevalonate for 8 h. The media were replaced, and the cells were incubated in a 37 °C H_2O bath for 2 h. Cycloheximide or PBS as the control was then added, and the cells were incubated for 4 h. Each flask was pulse-labeled with [^{3}H]oleate to measure ACAT activity, the rate of TG synthesis, and the rate of polar lipid synthesis as described under Materials and Methods in the preceding paper. Cell cultures grown in medium without mevalonate or cycloheximide expressed an ACAT activity of 3 pmol min^{-1} mg^{-1}, 18 × 10^3 dpm min^{-1} mg^{-1} for [^{3}H]TG synthesis, and 11 × 10^3 dpm min^{-1} mg^{-1} for polar lipid synthesis.

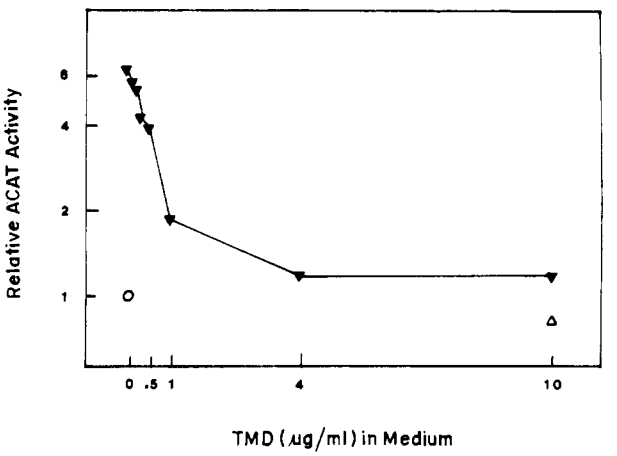

FIGURE 3: TMD prevents the ACAT activation by DL-mevalonate. Monolayers of starting culture were incubated with 4 mL/flask LDL⁻ media containing 20 mM DL-mevalonate with various amounts of DL-TMD as indicated for 8 h. The media were then replaced, and the cells were incubated in a 37 °C H_2O bath for 2 h. Each flask was then pulse-labeled with [^{3}H]oleate to measure ACAT activity as described under Materials and Methods in the preceding paper. Cell cultures grown in medium without mevalonate or TMD (○) expressed an ACAT activity of 2.8 pmol min^{-1} mg^{-1}. (△) Cells grown in medium containing 10 μg/mL TMD only.

sterol synthesis in intact CHO cells was inhibited approximately 85% at 4 μg/mL TMD and approximately 95% at 10 μg/mL TMD (results not shown).

Cells grown in LDL⁻ medium are known to produce an increased amount of endogenous sterol [for a review, see Goldstein & Brown (1977)]. We examined the cycloheximide sensitivity of ACAT activity in cells grown in LDL⁻ medium but not able to produce endogenous sterol and found that the cycloheximide activation of ACAT in LDL⁻ medium grown cells (Figure 4A) could be abolished by including the sterol synthesis inhibitor compactin (Figure 4B) or TMD (Figure 4C) in the medium. Control experiments showed that com-

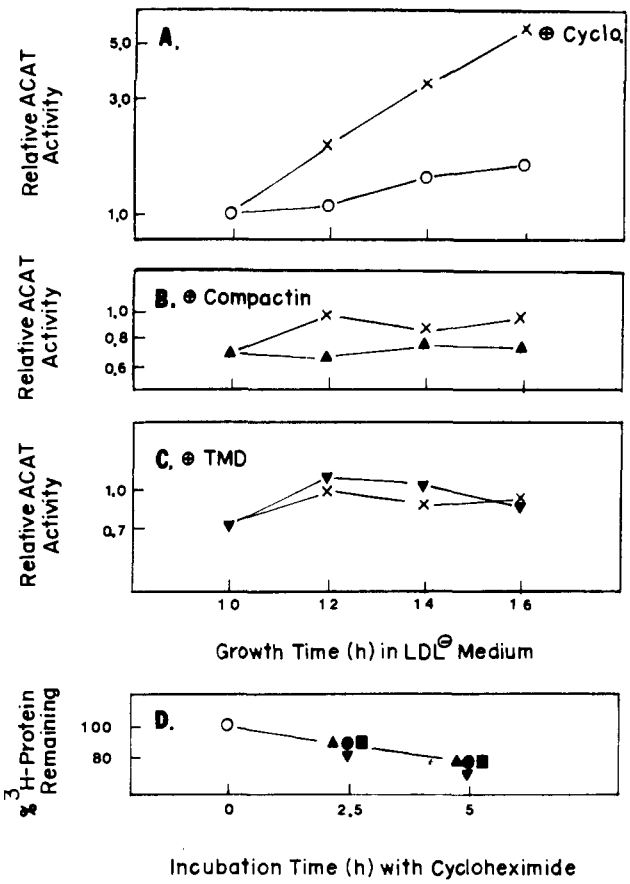

FIGURE 4: ACAT activation by cycloheximide (A) can be abolished by incubating cells with (B) compactin or (C) TMD without affecting (D) the rate of disappearance of [^{3}H]leucine-pulse-labeled proteins in intact cells. Monolayers of starting culture were incubated with 4 mL/flask LDL$^-$ media containing (A) no sterol synthesis inhibitor (○), (B) 1 μM compactin (▲), or (C) 4 μg/mL DL-TMD (▼) for 8 h. The media were then replaced, and the cells were incubated in a 37 °C H_2O bath for 2 h. For (A), (B), and (C), cycloheximide (×) or PBS as the control was added, and the cells were incubated for 4 h. Each flask was pulse-labeled with [^{3}H]oleate to measure ACAT activity. Zero-time cultures without compactin or TMD contained 3.9 pmol min^{-1} mg^{-1} for ACAT activity. For (D), after cells were incubated without sterol inhibitor (●), with compactin (▲), with TMD (▼), or with 20 mM DL-mevalonate (■) for 8 h as described above, the cells were incubated in a 37 °C H_2O bath for 1 h, 6 μL/flask L-[4,5-^{3}H]leucine (Amersham, 25 Ci/mmol, 0.14 μCi/μL) was added, and the cultures were incubated for 1 h. At time zero, cycloheximide was added. At the time indicated, cultures were harvested as described under Materials and Methods in the preceding paper. Data are expressed as percentages of the value obtained immediately after the 1-h pulse, which was 26.8 × 10^3 dpm/flask.

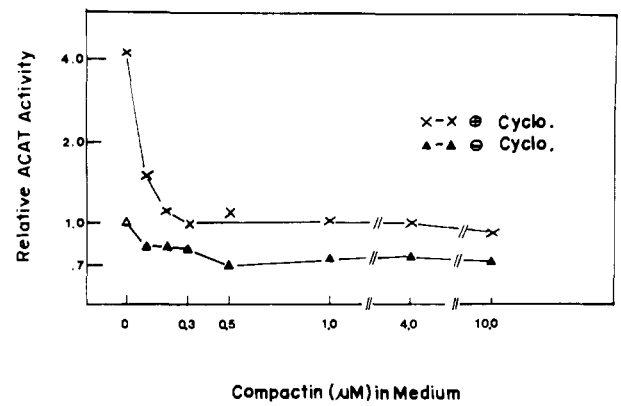

FIGURE 5: Effect of compactin concentration on ACAT activation by cycloheximide. Monolayers of starting culture were incubated with 4 mL/flask LDL$^-$ media containing the indicated amounts of compactin for 8 h. The media were replaced, and the cells were incubated in a 37 °C H_2O bath for 2 h. Cycloheximide or PBS as the control was then added, and the cells were incubated for 4 h. Each flask was pulse-labeled with [^{3}H]oleate to measure ACAT activity, the rate of TG synthesis, and the rate of polar lipid synthesis as described under Materials and Methods in the preceding paper. Cell cultures grown in medium without compactin or cycloheximide expressed an ACAT activity of 4.9 pmol min^{-1} mg^{-1}, 16.7 × 10^3 dpm min^{-1} mg^{-1} for [^{3}H]TG synthesis, and 7.3 × 10^3 dpm min^{-1} mg^{-1} for polar lipid synthesis.

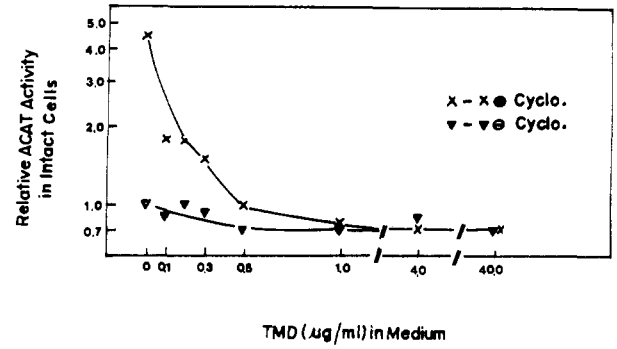

FIGURE 6: Effect of TMD concentration on ACAT activation by cycloheximide. Monolayers of starting culture were incubated with 4 mL/flask LDL$^-$ media containing the indicated amounts of DL-TMD for 8 h. The media were replaced, and the cells were incubated in a 37 °C H_2O bath for 2 h. Cycloheximide or PBS as the control was then added, and the 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 preceding paper. Cell cultures grown in medium without TMD or cycloheximide expressed an ACAT activity of 4.9 pmol min^{-1} mg^{-1}.

pactin, TMD, and mevalonate did not alter the rate of [^{3}H]leucine-pulse-labeled protein degradation after cycloheximide administration (Figure 4D). Another control experiment showed that neither compactin, TMD, nor mevalonate had any detectable effect on the rate of [^{3}H]cholesterol released into the medium (data not shown). Figures 5 and 6 showed the concentration dependency of the compactin and TMD effect. Control experiments showed that neither compactin nor TMD had any significant effect on TG synthesis or polar lipid synthesis (data not shown).

When ACAT activity became cycloheximide insensitive by addition of the sterol synthesis inhibitor compactin or TMD, adding low concentrations of cationized LDL (≤4 μg of protein/mL) to the medium caused the cycloheximide activation of ACAT to reappear. However, this activation diminished as the cationized LDL concentration increased to ≥10 μg of protein/mL, as shown in Figure 7B,C. Namely, in the absence of endogenous sterol synthesis, the relationship between the fold activation of ACAT due to cycloheximide and cationized LDL concentration was biphasic (Figure 7B-b,C-b), instead of the monophasic relationship found in control cell cultures (Figure 7A-b).

Our previous work showed that CHO cell mutant 1 was defective in the increase in rate of sterol synthesis and LDL binding activity normally observed upon removal of LDL from the medium (Chin & Chang, 1981, 1982). As expected, we found that in this mutant the LDL-mediated activation of ACAT activity was much less than what was found in normal (parental) CHO cells (Figure 8, bottom two curves). However, its cationized LDL-mediated activation was almost identical with that in normal cells (Figure 8, top two curves). This mutant was therefore employed to further examine the relationship between fold activation of ACAT due to cycloheximide and cationized LDL concentration in the absence of sterol synthesis. As shown in the right panel of Figure 9, a biphasic curve was obtained, confirming the results presented in Figure 7.

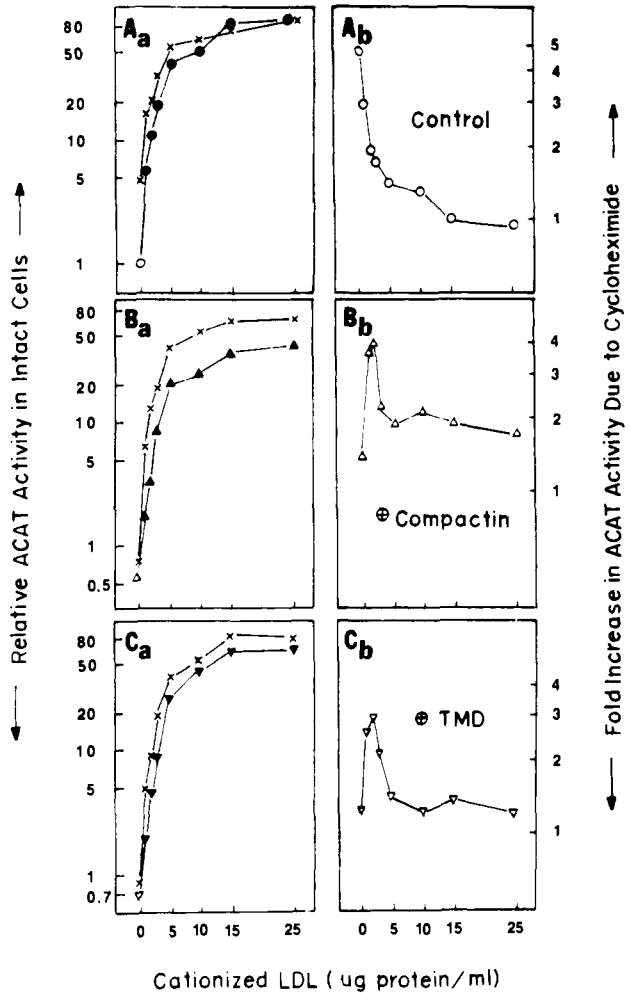

FIGURE 7: Magnitude of cycloheximide activation of ACAT activity as a function of cationized LDL concentration (A) without sterol synthesis inhibitor, (B) with the HMG-CoA reductase inhibitor compactin, or (C) with the 2,3-oxidosqualene cyclase inhibitor TMD. Monolayers of starting culture were incubated with 4 mL/flask LDL⁻ media containing the indicated amounts 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 the control was then added, and the 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 preceding paper. Cell cultures grown in medium without sterol synthesis inhibitors or cycloheximide expressed an ACAT activity of 4.6 pmol min^{-1} mg^{-1}. In the right panels, values for the fold increase in ACAT activity due to cycloheximide were obtained by using ACAT activity in cycloheximide-treated cells divided by that in nontreated cells at a given concentration of cationized LDL.

The increase in ACAT activity and the decrease in cycloheximide activation of ACAT by addition of mevalonate in the medium (Figures 1 and 2), as well as the lack of cycloheximide activation in mutant 1 cells incubated with or without 25 μg of protein/mL of catonized LDL (Figure 9), could all be qualitatively confirmed with an in vitro assay to measure ACAT activity, as shown in the open bars of Figure 10a,d,e,f. The lack of cycloheximide activation after compactin or TMD treatment, as shown in Figures 4, 5, and 6 with an [^{3}H]oleate pulse, could only be partially confirmed by the in vitro assay (open bars of Figure 10b,c). This minor discrepancy persisted in several seperate experiments, and the cause is unknown at present. Nevertheless, when the cell homogenates, obtained in conditions described in Figure 10a–f, were reconstituted into cholesterol-containing liposomes according to the method described in the preceding paper (Chang et al., 1986), the differences in ACAT activities in these cell extracts were all

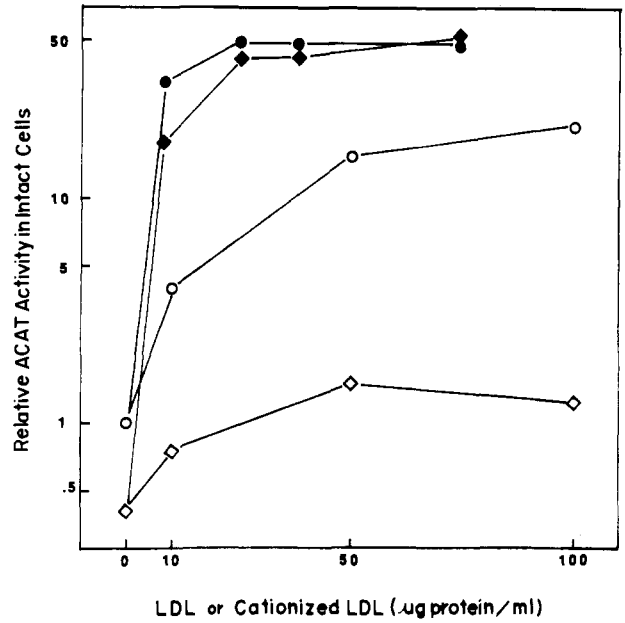

FIGURE 8: Effect of LDL [(○) normal cell; (◇), mutant 1 cells] or cationized LDL [(●) normal cells; (◆) mutant 1 cells] concentration on ACAT activity. Monolayers of two types of starting culture cells were incubated with 4 mL/flask LDL⁻ media containing the indicated amounts of LDL or cationized LDL for 8 h. The media were replaced, and the cells were incubated in a 37 °C H_2O bath for 2 h. Each flask was then pulse-labeled with [^{3}H]oleate as described under Materials and Methods in the preceding paper. ACAT activity for normal cells grown in medium without LDL or cationized LDL was 4.24 pmol min^{-1} mg^{-1}, while for mutant 1 cells, it was 1.7 pmol min^{-1} mg^{-1}.

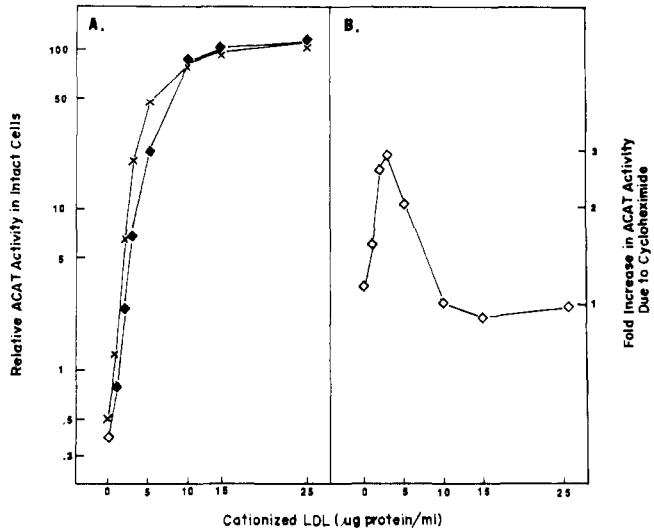

FIGURE 9: Effect of cationized LDL concentration on ACAT activation by cycloheximide in CHO cell mutant 1. Monolayers of mutant 1 cell culture were employed to perform the experiment in the same manner as described in Figure 7A. Cell cultures grown in medium without cationized LDL expressed an ACAT activity of 1.8 pmol min^{-1} mg^{-1}.

shown to be essentially abolished (Figure 10a–f solid bars).

Discussion

Previous work from this laboratory (Chang & Limanek, 1980; Chang et al., 1985) showed that when CHO cells were switched to grow from LDL⁺ medium to LDL⁻ medium, a time lag of 8 h or longer was observed before a large increase in the rate of sterol synthesis would occur, due to the time lag in activity increases of rate-controlling enzymes such as HMG-CoA reductase and HMG-CoA synthase (Change & Limanek, 1980). During this time period, providing meva-

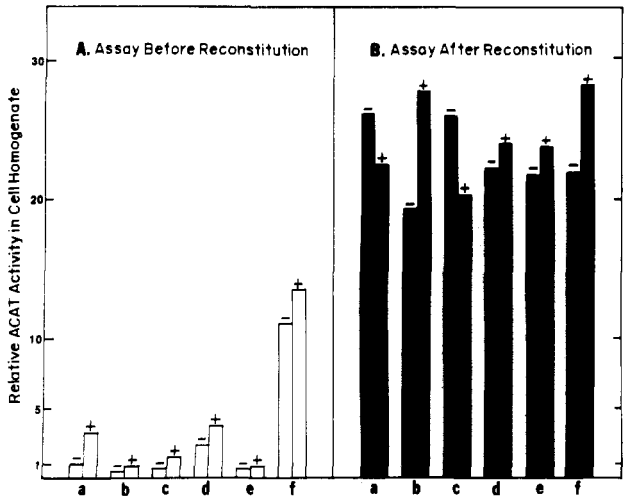

FIGURE 10: Comparison of ACAT activities from cells grown in the absence (−) or presence (+) of cycloheximide with reconstituted (solid bars) or nonreconstituted (open bars) enzyme assays. Monolayers of starting culture were incubated with 12 mL/flask LDL^- media containing (a and e) no additions, (b) 4 μg/mL TMD, (c) 1 μM compactin, (d) 20 mM mevalonate, and (f) 25 μg of protein/mL of cationized LDL for 8 h. For (a) through (d), normal cells were employed as the cell source, but for (e) and (f), mutant 1 cells were employed. The media were replaced, and the cells were incubated in a 37 °C H_2O bath for 2 h. Cycloheximide or PBS as the control was then added, and the cells were incubated for 4 h. Cell homogenates without reconstitution were assayed immediately. For the reconstitution assay, the method described in the preceding paper (Chang et al., 1986) was used. Results were plotted as the fold increase relative to the nonreconstituted ACAT activity from normal cells without LDL or cycloheximide treatment, which has the enzyme specific activity of 6.9 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%.

lonate in the medium apparently expanded the cellular pool size of mevalonate, which resulted in activating ACAT activity (Figures 1 and 2). The mevalonate activation of ACAT was prevented by an appropriate concentration of TMD (Figure 3), strongly suggesting that it was the sterol endogenously produced from mevalonate, rather than other mevalonate-derived, non-sterol metabolites [for a review, see Brown & Goldstein (1980)], that served as the signal for ACAT activation. The fact that a high concentration of mevalonate (≥10 mM) was needed for ACAT activation (Figures 1 and 2) is also consistent with this interpretation (Brown et al., 1978; Brown & Goldstein, 1980; Schnitzer-Polokoff et al., 1982). These results therefore extend the results of the preceding paper (Chang et al., 1985) suggesting that the activity of the short-lived, inhibitory factor(s) of ACAT can be abolished by sterol endogenously synthesized in the cells as well as by sterol present in the medium.

In the preceding paper, we showed that the time required to reach maximal activation by cycloheximide on ACAT was lengthened from 2.5 h in LDL^+ medium to 7 h in LDL^- medium [Figure 3 in Chang et al. (1986)], implying that the turnover rate(s) of the short-lived inhibitor(s) may be lengthened by deleting the sterol source from the medium. The data in Figures 4, 5, and 6 showed that the ACAT activity in cells grown in LDL^- media containing compactin or TMD became cycloheximide insensitive. This observation was confirmed by results with mutant 1 cells grown in LDL^- medium (Figure 9). These results are consistent with the following interpretation: blocking endogenous sterol synthesis coupled with deleting exogenous sterol lengthens the turnover rate(s) of the inhibitory factor(s) such that it is insensitive to intracellular degradation during the period of time studied. This interpretation is supported by the finding that low concentrations of cationized LDL in the medium causes the cycloheximide activation of ACAT to reappear (Figures 7B-b,C-b and 9B), strengthening the notion that the activity of the short-lived factor is controlled by both exogenous sterol and sterol endogenously synthesized. The effect of this factor on ACAT activity in crude cell homogenates is again found to be reversible, since its effect is abolished after the ACAT activity has been reconstituted into cholesterol-containing liposomes (Figure 10). The biochemical nature of this inhibitor is unknown at present, but the results in Figure 9 showed that this factor could not be HMG-CoA reductase, since the apparent turnover rates of HMG-CoA reductase in normal (parental) CHO cells and in mutant 1 cells grown in LDL^- medium were found to be identical [Table I in Chin & Chang (1982)]. This conclusion is in agreement with the results of Sexton et al. (1985), who reported that in rat intestinal epithelial IEC-6 cells, LDL suppression of HMG-CoA reductase activity was not prevented by compound 58-035, a specific ACAT inhibitor (Ross et al., 1984), suggesting that the detailed molecular mechanisms for mediating the LDL effect on reductase and on ACAT diverge at certain point beyond the lysosomal release of the sterol from hydrolytic cleavage of LDL-bound cholesterol ester.

It may seem confusing to readers if one compares the results in this paper and the preceding one (Chang et al., 1985) with results published earlier from this laboratory [Table II in Doolittle & Chang (1982)], where no cycloheximide activation of ACAT was shown in cells grown in LDL^- medium (measured by in vitro assay). We should point out that all experiments reported in our current work were performed during the 10th to the 16th hour time period after cells have been switched to grow from LDL^+ to LDL^- medium, instead of during the 18th to the 24th hour time period used in our early work (Doolittle & Chang, 1982). The result in Figure 11 (panel B, open circles) showed that after cells were grown in LDL^- medium for more than 8 h the rate of endogenous sterol synthesis increased sharply, which caused the ACAT activity in intact cells to increase (Figure 11, panel A, open triangles). This interpretation was supported by the finding that the increase in ACAT activity was blocked by TMD at 4 μg/mL (solid inverted triangles), by 1 μM compactin (data not shown for clarity), or by a medium replacement at the eighth hour (open triangles). While the medium replacement caused a higher rate of sterol synthesis (Figure 11, panel B, open triangles), the amount of cholesterol released into the medium due to medium replacement[2] was apparently large enough to maintain the basal ACAT activity at a low level. This was the main reason why we chose the 10th to the 16th hour period for our current work and why there was always a medium replacement at 2 h before any experiment began.

By comparison of the cycloheximide effect of ACAT activity in cells grown in LDL^- medium during the 10th to the 16th hour (as shown in Figure 12, left curve) to those during the 18th to the 24th hour (as shown in Figure 12, right curve), it was clear that the activation of ACAT by cycloheximide was much smaller at the 24th hour (2.5-fold) as compared with the results at the 16th hour (5.3-fold). This difference was caused by the steeper rise in basal ACAT activity from the 18th to the 24th hour (Figure 12, open circles). Most likely, this was due to the large accumulation of endogenously syn-

[2] Under this condition, we found that with a medium replacement 8.0% of total ^3H-labeled cellular cholesterol was released from the cells into the medium in 8 h, while without medium replacement the value was only 2.5% (results not shown for clarity).

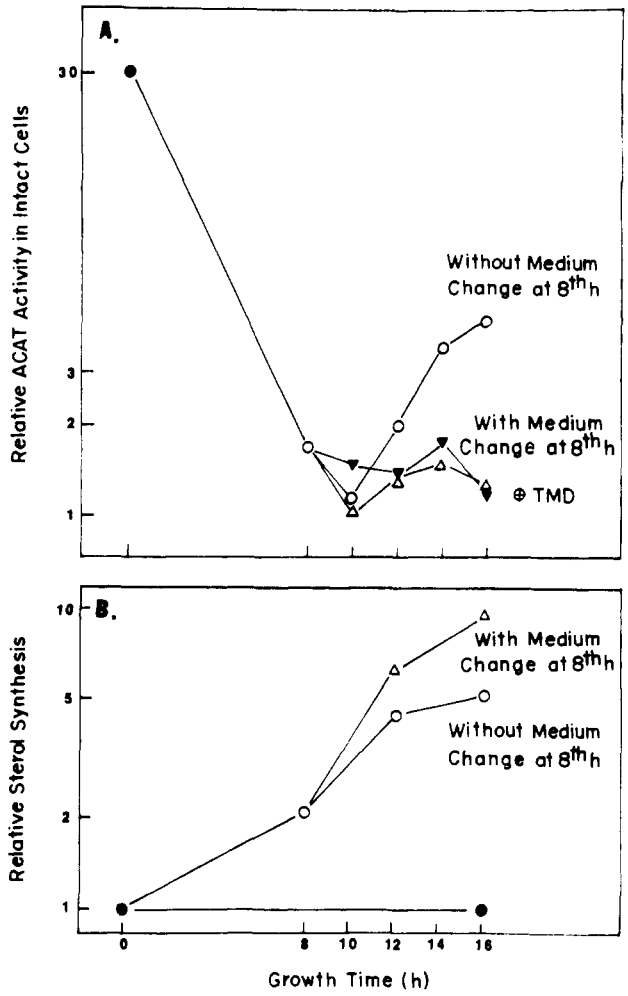

FIGURE 11: Effects of endogenous sterol synthesis and medium replacement on ACAT activity. At zero time, monolayers of starting culture were incubated with 4 mL/flask of LDL$^-$ medium, with or without 4 μg/mL TMD or 1 μM compactin. At the eighth hour, the media were replaced for a certain number of flasks (○). At the time indicated, ACAT activity and rate of sterol synthesis were measured by an [^{3}H]oleate pulse and an [^{14}C]acetate pulse as described (Chang et al., 1986). Results obtained with compactin (data not shown for clarity) were the same as those with TMD (▼). (●) Values obtained from cells grown in LDL$^+$ medium.

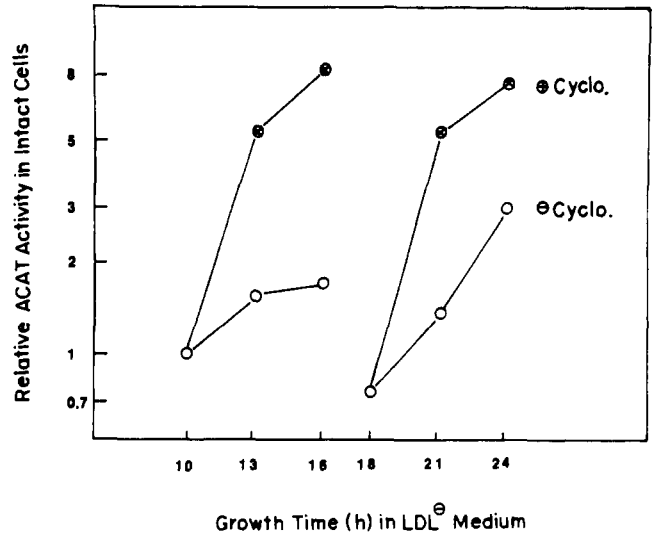

FIGURE 12: Difference in the extent of cycloheximide activation on ACAT activity between cell cultures grown in LDL$^-$ medium for 16 h and those for 24 h. Starting cultures were grown in LDL$^-$ medium for 10 h or longer as indicated. Media were replaced at the 8th and the 16th hours. Cycloheximide or PBS as the control was added at 10th or 18th hour. At the time indicated, ACAT activity as well as TG and polar lipid synthesis was measured by an [^{3}H]oleate pulse as described (Chang et al., 1986).

thesized sterol during this time period, the effect of which could no longer be compensated for by medium replacement at the 16th hour (Figure 12). Control experiments showed that the rates of triglyceride and polar lipid synthesis did not change significantly between these two time periods (results not shown). The much smaller ACAT activation effect by cycloheximide in cells grown at later time periods was still detectable by an [^{3}H]oleate pulse in intact cells but could have gone nondetectable by a less sensitive in vitro ACAT assay, as was used in our previous work (Doolittle & Chang, 1982). Thus, the evidence presented in Figures 11 and 12 together with our early work (Doolittle & Chang, 1982) supports the interpretation that the activity of the short-lived inhibitory factor on ACAT can be abolished by the increase in the rate of endogenous sterol synthesis.

In conclusion, we believe the work presented in this and the preceding paper has provided a framework for future biochemical research on ACAT regulation by sterol in cultured mammalian cells. To further delineate the precise nature of this regulatory mechanism, it is necessary to isolate and characterize the short-lived factor in vitro. It will also be necessary to isolate a subpopulation of microsomal vesicles enriched in ACAT activity free of contaminating vesicles and to examine the cholesterol content of these vesicles before and after the cycloheximide treatment. The feasibility of these studies depends on the availability of specific antibodies against the pure ACAT protein for performing immunoprecipitation or immunoadsorption experiments. These lines of research are currently being pursued in this laboratory.

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Assessment of Roles of β146-Histidyl and Other Histidyl Residues in the Bohr Effect of Human Normal Adult Hemoglobin†

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ABSTRACT: The contribution of the carboxyl-terminal histidines of the β chains, β146(HC3), to the alkaline Bohr effect of human normal adult hemoglobin has been shown by this laboratory to depend upon the solvent composition. Using high-resolution proton nuclear magnetic resonance spectroscopy, we have found that the pK_a value of the β146-histidine is 8.0 in the deoxy form, while in the carbonmonoxy form it ranges from 7.1 to 7.85 depending upon the concentration of inorganic phosphate and chloride ions present. These conclusions have been questioned by Perutz and co-workers on the basis of biochemical, structural, and proton nuclear magnetic resonance studies of mutant and enzymatically or chemically modified hemoglobins [Perutz, M. F., Kilmartin, J. V., Nishikura, K., Fogg, J. H., Butler, P. J., & Rollema, H. S. (1980) *J. Mol. Biol. 138*, 649-670; Kilmartin, J. V., Fogg, J. H., & Perutz, M. F. (1980) *Biochemistry 19*, 3189-3193; Perutz, M. F., Gronenborn, A. M., Clore, G. M., Fogg, J. H., & Shih, D. T.-b. (1985) *J. Mol. Biol. 183*, 491-498]. In this work, we use proton nuclear magnetic resonance spectroscopy to assess the effects of structural modifications on the histidyl residues and on the overall conformation of the hemoglobin molecule in solution. The structural perturbations investigated all occur within the tertiary domains around the carboxyl-terminal region of the β chain as follows: Hb Cowtown (β146His → Leu); Hb Wood (β97His → Leu); Hb Malmö (β97His → Gln); Hb Abruzzo (β143His → Arg). Our results demonstrate that the conformational effects of single-site structural modifications upon the conformation and dynamics of hemoglobin depend strongly on their location in the three-dimensional structure of the protein molecule and also on their chemical nature. Furthermore, in normal hemoglobin, the spectral properties of several surface histidyl residues are found to depend, in the ligated state, upon the nature of the ligand. Our present findings do not support the recent spectral assignments proposed by Perutz et al. (1985) for the proton resonances of the β146- and β97-histidines and their suggestion that the enzymatic removal of the carboxyl-terminal β146-histidyl residues induces a conformational equilibrium for the β97-histidines in the des-β146His hemoglobin molecule in the carbonmonoxy form.

A main functional property of the hemoglobin (Hb)[1] molecule is to release hydrogen ions upon oxygenation at pH above 6.0. This property, known as the alkaline Bohr effect, results from the conformational changes induced in the Hb molecule by ligand binding. These structural alterations affect the ionization properties of a number of amino acid residues such that they release or capture hydrogen ions upon ligation. The identification of those amino acid residues whose pK_a values are altered in going from deoxy- to oxy- (or carbon-

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[1] Abbreviations: Hb, hemoglobin; Hb A, human normal adult hemoglobin; Hb des-His, Hb A with the β146-histidyl residues deleted; met-Hb, methemoglobin; NMR, nuclear magnetic resonance; ppm, parts per million; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Bis-Tris, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane; NOE, nuclear Overhauser effect.

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