# Inhibition of lysosomal protein degradation inhibits the basal degradation of 3-hydroxy-3-methylglutaryl coenzyme A reductase

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**Abstract** The effect of inhibiting lysosomal protein degradation on the activity of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase was determined using a mouse mammary cell line (TS-85) which expresses a temperature-sensitive mutation in the ubiquitin degradative pathway. Incubating cells for 18 hr in medium containing 20 mM $NH_4Cl$ did not alter total protein synthesis or cell growth, but it did inhibit the rate of total protein degradation by 19%, which is consistent with the known inhibitory effect of $NH_4Cl$ on lysosomal protein degradation. $NH_4Cl$ treatment also resulted in an increase (81% ± 20) in HMG-CoA reductase activity. The increase in reductase activity was not correlated with changes in the phosphorylation state of the enzyme or with alteration in the relative rate of reductase synthesis. However, the basal degradation rate of the reductase was significantly inhibited, and after $NH_4Cl$ treatment, the half-life of the enyzme increased from 4.0 ± 0.4 hr to 8.3 ± 0.8 hr. The change in the rate of reductase degradation can account completely for the increase in reductase activity observed in $NH_4Cl$-treated cells. The accelerated degradation of HMG-CoA reductase induced by 25-hydroxycholesterol treatment was not affected by either $NH_4Cl$ or by inactivation of the ubiquitin degradative pathway. Therefore, two different mechanisms may be responsible for the accelerated degradation and basal degradation of HMG-CoA reductase. The latter can be inhibited by $NH_4Cl$ and may imply that under basal conditions the enzyme may be degraded in lysosomes. — **Tanaka, R. D., A. C. Li, A. M. Fogelman, and P. A. Edwards.** Inhibition of lysosomal protein degradation inhibits the basal degradation of 3-hydroxy-3-methylglutaryl coenzyme A reductase. *J. Lipid Res.* 1986. **27:** 261–273.

**Supplementary key words** protein degradation • 25-hydroxycholesterol • lysosomes • ubiquitin pathway

HMG-CoA reductase is a transmembrane protein (1) present in the endoplasmic reticulum of animal cells which catalyzes the rate-limiting step in the cholesterol biosynthetic pathway (2). Several different mechanisms have been reported for regulating reductase activity in animal cells. These regulatory mechanisms include alterations in the rate of synthesis and degradation of the enzyme (3–8), in the degree of enzyme phosphorylation (9), and in changes in the membrane fluidity of the endoplasmic reticulum (10). Immunoprecipitation experiments with radiolabeled cells using anti-reductase antibody clearly demonstrate that the inhibition of HMG-CoA reductase activity resulting from incubating cells in the presence of either mevalonic acid (5), low density lipoprotein (4), or 25-hydroxycholesterol (7) is due to both a rapid decrease in the rate of reductase synthesis and a concomitant increase in the degradation rate of the enzyme. Conversely, the increase in reductase activity observed in rat hepatocytes due to treatment with either high density lipoprotein (8), liposomes (8), or mevinolin (6) is due to an increased rate of synthesis and a decreased rate of degradation of the enzyme. The importance of enzyme phosphorylation and/or changes in membrane fluidity in the physiological regulation of the enzyme have not been fully elucidated.

The mechanism(s) by which HMG-CoA reductase or other proteins of the endoplasmic reticulum are degraded in animal cells are not well understood. Parker, Miller, and Gibson (11) recently reported that phosphorylyation enhanced the susceptibility of the rat liver HMG-CoA reductase to in vitro proteolysis, but these experiments may not reflect the actual in vivo degradative pathway. Proteases that can cleave the reductase polypeptide after disruption of the cell have been reported (1, 12, 13), but it is not known whether these proteases have any similar function in vivo. Orci et al. (14) examined the LDL-induced degradation of the HMG-CoA reductase in the crystalloid endoplasmic reticulum present in the UT-1

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; HMG, 3-hydroxy-3-methylglutaryl; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid.

cell line, a compactin-resistant clone of Chinese hamster ovary cells (15), and noted that increased cholesterol content in the membranes of the endoplasmic reticulum may be involved in signaling reductase degradation. Lysosomes did not appear to be associated with the LDL-induced degradation of the crystalloid endoplasmic reticulum (16).

Amplification of the endoplasmic reticulum can also be induced in rat hepatocytes by phenobarbital treatment (17, 18); however, ultrastructural studies have yielded conflicting reports regarding the function of lysosomes in the degradation of the endoplasmic reticulum (19). Hence further research is needed to clarify the role of lysosomes in the degradation of proteins in the endoplasmic reticulum. In the current report, we have attempted to examine the mechanism(s) involved in the degradation of HMG-CoA reductase. To determine whether lysosomes are involved in the degradation of the enzyme, we have examined the effect of lysosomal inhibitors ($NH_4Cl$, chloroquine, leupeptin) on reductase activity, synthesis, and degradation.

The TS-85 cells used in our study express a temperature-sensitive mutation in the ubiquitin degradative pathway (20, 21). The ubiquitin pathway is a soluble, ATP-dependent proteolytic system that is nonlysosomal and degrades both abnormal and normal proteins in erythrocytes and other mammalian cells (22, 23). Using the temperature-sensitive mutant, we also examined whether the accelerated degradation of the reductase induced by 25-hydroxycholesterol treatment was mediated by either the ubiquitin pathway or by lysosomes. Therefore, in our study we have attempted to examine the mechanism(s) involved in both the basal degradation and accelerated degradation of HMG-CoA reductase.

## MATERIALS AND METHODS

### Materials

Fetal calf serum was purchased from either Gibco (Santa Clara, CA) or Hyclone (Logan, UT) and Dulbecco's modified Eagle's medium was from Gibco. The following were purchased from the indicated sources: Cabosil (United Technologies Packard, Downers Grove, IL); HEPES, Tricine, Pansorbin (Calbiochem-Behring, La Jolla, CA); uranyl acetate, lead citrate, glutaraldehyde, Epon (Polysciences, Warrington, PA) 25-hydroxycholesterol (Steraloids, Wilton, NH); bovine serum albumin (fraction V, Miles Scientific, Naperville, IL); methionine-free MEM (Flow Laboratories, Los Angeles, CA); Hydrofluor (National Diagnostics, Sommerville, NJ); and [$^{35}$S]methionine (Amersham, Arlington Heights, IL). The sources of all other material have been previously described (5–8).

### Cells

The wild-type FM3A cell line originated from a mouse mammary cell line (24). Cells were grown at 37°C in a mixture of 5% $CO_2$ and 95% air, as described by Finley, Ciechanover, and Varshavsky (20), in DMEM media containing penicillin (50 units/ml), streptomycin (50 μg/ml), and 10% FCS (DMEM-FCS). The TS-85 cell line, a temperature-sensitive mutant derived from the FM3A cell line (25), was grown at 30°C using the same DMEM media. To confirm the presence of the temperature-sensitive mutation in the ubiquitin pathway, the TS-85 cells were tested for their inability to grow at the non-permissive temperature (40°C) and to synthesize histone UH2A (26). The FM3A and TS-85 cell lines were kindly provided by Dr. Hideyo Yasuda (University of California, Davis).

To induce high levels of HMG-CoA reductase activity, cells ($5 \times 10^5$/ml) were grown in DMEM media containing antibiotics and 10% Cabosil-treated fetal calf serum (DMEM-C). Cabosil treatment removed serum lipoproteins (27). Serum cholesterol concentration was measured (28) and Cabosil treatment removed 93–95% of the total serum cholesterol (data not shown).

### Enzyme assay

HMG-CoA reductase activity was assayed as previously described (7) with the following minor modifications. The reductase was solubilized in a buffer containing 0.16 M HEPES buffer (pH 7.4), 0.2 M KCl, 1 mM EDTA, 0.25% Kyro EOB (Proctor and Gamble), and 5 mM glutathione. Aliquots of the solubilized enzyme were assayed directly, without centrifugation (7). The concentration of [$^{14}$C]HMG-CoA (sp act 5 μCi/μmol) in the assay was 40 μM. Protein concentration was determined by the method of Bradford (29).

In experiments to determine the effect of 25-hydroxycholesterol on reductase activity, cells received either 25-hydroxycholesterol (added in ethanol, final concentration 5 μg/ml) or an equivalent volume of ethanol, and reductase activity was determined after varying time periods.

To measure the "expressed" enzyme activity, cells were solubilized and assayed in the presence of 50 mM NaF. Total enzyme activity was determined by isolating and assaying reductase activity in the absence of NaF.

### Immunoprecipitation

The anti-reductase serum was prepared in rabbits using the HMG-CoA reductase purified from rat liver (30). This antiserum also cross-reacts and inactivates the reductase present in mouse cells (data not shown). The anti-reductase serum specifically immunoprecipitated a radiolabeled polypeptide (mol wt 97,000) from TS-85 cells or FM3A cells (data not shown). This is similar to

the reported molecular weight of reductase present in rat (31), hamster (32, 33), and chicken (7) cells.

Procedures for pulse and pulse-chase experiments and immunoprecipitation of the reductase have been reported (5–8). The TS-85 cells used in these experiments were grown in DMEM-C at a concentration of $5 \times 10^5$ cells/ml for 18 hr at 30°C in the presence or absence of 20 mM $NH_4Cl$. Addition of the $NH_4Cl$ (in phosphate-buffered saline, pH 7.2) did not change the pH of the culture medium. Cells were collected by brief centrifugation and were resuspended at a concentration of $3 \times 10^6$/ml in MEM-M (methionine-free MEM containing 1.5% bovine serum albumin, 20 mM glutamine, and 17 mM Tricine buffer, pH 7.2). For the $NH_4Cl$-treated cells, 20 mM $NH_4Cl$ was also present in the MEM-M medium. All samples were gassed with 95% $O_2$:5% $CO_2$ and incubated at 30°C. [$^{35}$S]Methionine (sp act 1460 Ci/mmol, 30–100 $\mu$Ci/ml) was added and the cells were pulsed for either 30 or 60 min. To determine the relative rate of synthesis, cells were collected by centrifugation, the supernatant was carefully aspirated, and the cell pellets were solubilized in buffer A (1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 0.01% $NaN_3$, 0.1 M NaCl, 5 mM EDTA, 0.01 M phosphate buffer, pH 7.5, 0.1 mM leupeptin, and 1 mM phenylmethylsulfonyl fluoride) at a concentration of $3–6 \times 10^6$/ml. The cell lysates were centrifuged at 100,000 *g* for 45 min at 5°C, the supernatant was collected, and bovine serum albumin was added to a final concentration of 5 mg/ml. Samples were precleared by addition of 80 $\mu$l of Pansorbin (10% w/v, in buffer A) per ml of lysate followed by a 120-min incubation at room temperature. The samples were centrifuged for 3 min in a Brinkmann microfuge and the supernatants were collected and precleared a second time. Preimmune rabbit serum was added to a concentration of 4–8 $\mu$l/ml and the samples were incubated overnight at 5°C. Pansorbin was added (100 $\mu$l/ml of lysate) and the samples were incubated for 120 min at room temperature. After a 3-min centrifugation in the microfuge, the supernatants were collected and anti-reductase rabbit serum was added to a concentration of 4–8 $\mu$l/ml of lysate. After 16 hr at 4°C, the reductase-antibody complexes were precipitated with Pansorbin exactly as described for the preimmune serum. The Pansorbin pellets containing the preimmune or anti-reductase serum were washed three times with buffer A not containing leupeptin or phenylmethylsulfonyl fluoride and processed for polyacrylamide gel electrophoresis as described below. All procedures were optimized to quantitatively immunoprecipitate all of the radiolabeled HMG-CoA reductase. The total incorporation of [$^{35}$S]methionine into protein was determined by precipitation with trichloroacetic acid as previously described (7).

To determine the relative rate of degradation of the reductase, TS-85 cells were pulsed with [$^{35}$S]methionine for 30 or 60 min as stated above. The cells were collected by centrifugation and the cell pellets were resuspended at a concentration of $3 \times 10^6$/ml in DMEM-C containing 5 mM unlabeled methionine. For the $NH_4Cl$-treated cells, 20 mM $NH_4Cl$ was also present in all the media. All samples were gassed with 95% $O_2$:5% $CO_2$ and incubated at 30°C. Samples were pre-incubated 60–120 min and then samples were withdrawn at varying time intervals and processed for immunoprecipitation as stated above. Relative rates of synthesis and degradation of HMG-CoA reductase were calculated as described (5–8).

### Measurement of the total rate of protein degradation

The methods used to measure the total rate of protein degradation were based on the method described by Seglen, Grinde, and Solheim (34). Cells were labeled with [$^{35}$S]methionine and treated exactly as described for experiments to measure the rate of degradation of HMG-CoA reductase. However, at varying times during the chase period, aliquots were removed and the proteins were precipitated by addition of perchloric acid to a final concentration of 2%. Samples were incubated at 5°C for 15 min and centrifuged for 4 min in a microfuge. An aliquot (100 $\mu$l) of the supernatant was mixed with Hydrofluor and the radioactivity was measured in a Beckman LS 2800 scintillation counter. These data represented the amount of perchloric acid-soluble radioactivity present in the medium and in the cells. The pellets (perchloric acid-insoluble material) were washed three times with ice-cold 2% perchloric acid, dissolved in 0.3 M NaOH, and an aliquot was removed and counted in Hydrofluor as stated above. These data represented the amount of perchloric acid-insoluble radioactivity present in both the medium and cells. The rate of protein degradation was measured as the change in the amount of perchloric acid-soluble radioactivity, expressed as a percent of the total radioactivity (soluble and insoluble), occurring after a 4-hr incubation.

### Polyacrylamide gel electrophoresis

The immunoprecipitated HMG-CoA reductase was analyzed by SDS–8 M urea polyacrylamide gel electrophoresis. Methods for preparing the samples and making the polyacrylamide gels were previously reported (5–8). After electrophoresis the radiolabeled reductase polypeptide was visualized by fluorography (35) and the appropriate bands were cut out of the gel and dissolved in 1 ml of 4% periodate. Samples were mixed with 10 ml of Hydrofluor and the radioactivity was measured on a Beckman scintillation counter. The total radioactivity in the reductase polypeptide was calculated as the difference in cpm between identical portions of the gel obtained after immunoprecipitation with anti-reductase and preimmune serum.

### Electron microscopy

TS-85 cells were washed three times in ice-cold phosphate-buffered saline (pH 7.4) and fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 1 hr at 4°C. Samples were then postfixed in 1% $OsO_4$ in 0.1 M cacodylate buffer (pH 7.4) for 1 hr at 4°C, dehydrated in ethanol, and embedded in an Epon/Araldite mixture. Silver sections were cut on an LKB ultrotone and subsequently stained with uranyl acetate and lead citrate. The sections were examined using a Philips 300 electron microscope at 60 KV.

## RESULTS

### Effect of $NH_4Cl$ concentration on total protein synthesis

Ammonia, or $NH_4Cl$, is a very effective inhibitor of lysosomal function and inhibits the function of lysosomal proteases by increasing the intralysosomal pH (36, 37). Due to its relatively low cytotoxicity (37), $NH_4Cl$ was used primarily in our study. Incubating cells in the presence of high concentrations of lysosomal inhibitors can inhibit total protein synthesis (38, 39), and inhibition of protein synthesis has been reported to alter the regulation of HMG-CoA reductase (40, 41). Therefore, experiments were conducted to measure protein synthesis in TS-85 cells after an 18-hr incubation at 30°C in DMEM-C containing different concentrations (0–60 mM) of $NH_4Cl$. As shown in **Fig. 1,** concentrations of $NH_4Cl$ as high as 20 mM showed no inhibition of total protein synthesis when compared to control cells grown in the absence of $NH_4Cl$. Substantial inhibition of protein synthesis occurred at concentrations of 30, 40, and 60 mM $NH_4Cl$ (Fig. 1). Similar results were obtained in three separate experiments. Therefore, a concentration of 20 mMCl was used in all subsequent experiments.

### Cell morphology

Based on the trypan blue exclusion test, no apparent cytotoxicity was detected in TS-85 cells after incubation for 18 hr at 30°C in the presence of 20 mM $NH_4Cl$. Cell viability was approximately 95% in both untreated and $NH_4Cl$-treated cells. Other investigators studying protein degradation have incubated cells in the presence of 20 mM $NH_4Cl$ and also noted no apparent cytotoxicity (42–44). Ultrastructural studied on TS-85 cells treated with 20 mM $NH_4Cl$ confirmed the absence of cytotoxicity (**Fig. 2**). No cellular degeneration was apparent in the $NH_4Cl$-treated cells, although numerous large vacuoles containing electron-dense material were consistently observed (Fig. 2). These large vacuoles are commonly observed in cells treated with lysosomotrophic agents and result from accumulation of the lysosomotrophic agent in lysosomes and subsequent inhibition of degradation (45, 46).

### Effect of 20 mM $NH_4Cl$ on HMG-CoA reductase activity

Incubating TS-85 cells for 18 hr at 30°C in DMEM-C containing 20 mM $NH_4Cl$ consistently induced an increase in HMG-CoA reductase activity when compared to controls. The observed increase in reductase activity was not due to the presence of small amounts of $NH_4Cl$ in the enzyme assay since addition of $NH_4Cl$ (0–40 mM) to the reductase assay had no effect on the enzyme activity (data not shown).

In nine separate experiments, treatment of cells with 20 mM $NH_4Cl$ resulted in an 81% ± 20 (range 50–115%) increase in reductase activity compared to controls. When cells were grown in DMEM-C, the enzyme activity increased approximately 7-fold, compared to cells grown in DMEM-FCS (**Table 1**). The increase in reductase activity presumably results from the increased requirements for de novo synthesis of cholesterol, which results when cells are grown in medium devoid of cholesterol-rich lipoproteins (47). Addition of 20 mM $NH_4Cl$ to cells growing in either DMEM-FCS or DMEM-C also induced higher

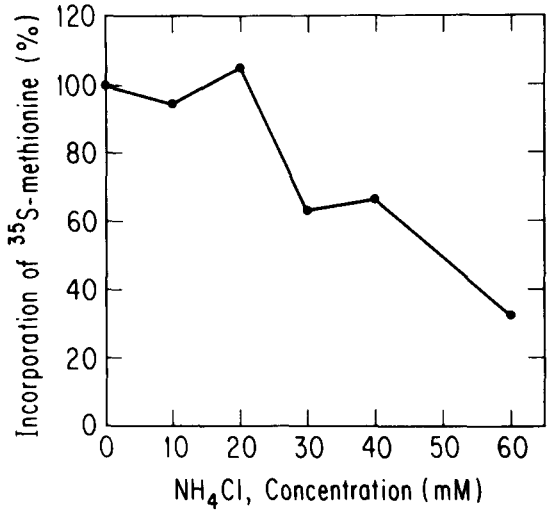


**Fig. 1.** The effect of different concentrations of $NH_4Cl$ on the relative rate of total protein synthesis in TS-85 cells. Cells were suspended ($5 \times 10^5$ cells/ml) in DMEM-C containing different concentrations (0–60 mM) of $NH_4Cl$ and incubated for 18 hr at 30°C. The cells were then collected by centrifugation and suspended ($2 \times 10^6$ cells/ml) in MEM-M containing [$^{35}$S]methionine (6 μCi/ml) and an identical concentration of $NH_4Cl$ used in the prior 18-hr incubation. The cells were collected after a 30-min incubation at 30°C and solubilized in buffer A, and protein synthesis was measured by the incorporation of [$^{35}$S]methionine into TCA-insoluble protein as described in Methods. Results are presented as the amount of TCA-insoluble radioactivity calculated as a percent of the TCA-insoluble radioactivity incorporated by control cells grown in the absence of $NH_4Cl$. The results are the mean values from two samples and, at each concentration of $NH_4Cl$, the results varied by less than 15%. After a 30-min incubation, control cells incorporated $3.3 \times 10^4$ cpm/$10^6$ cells into TCA-insoluble protein.

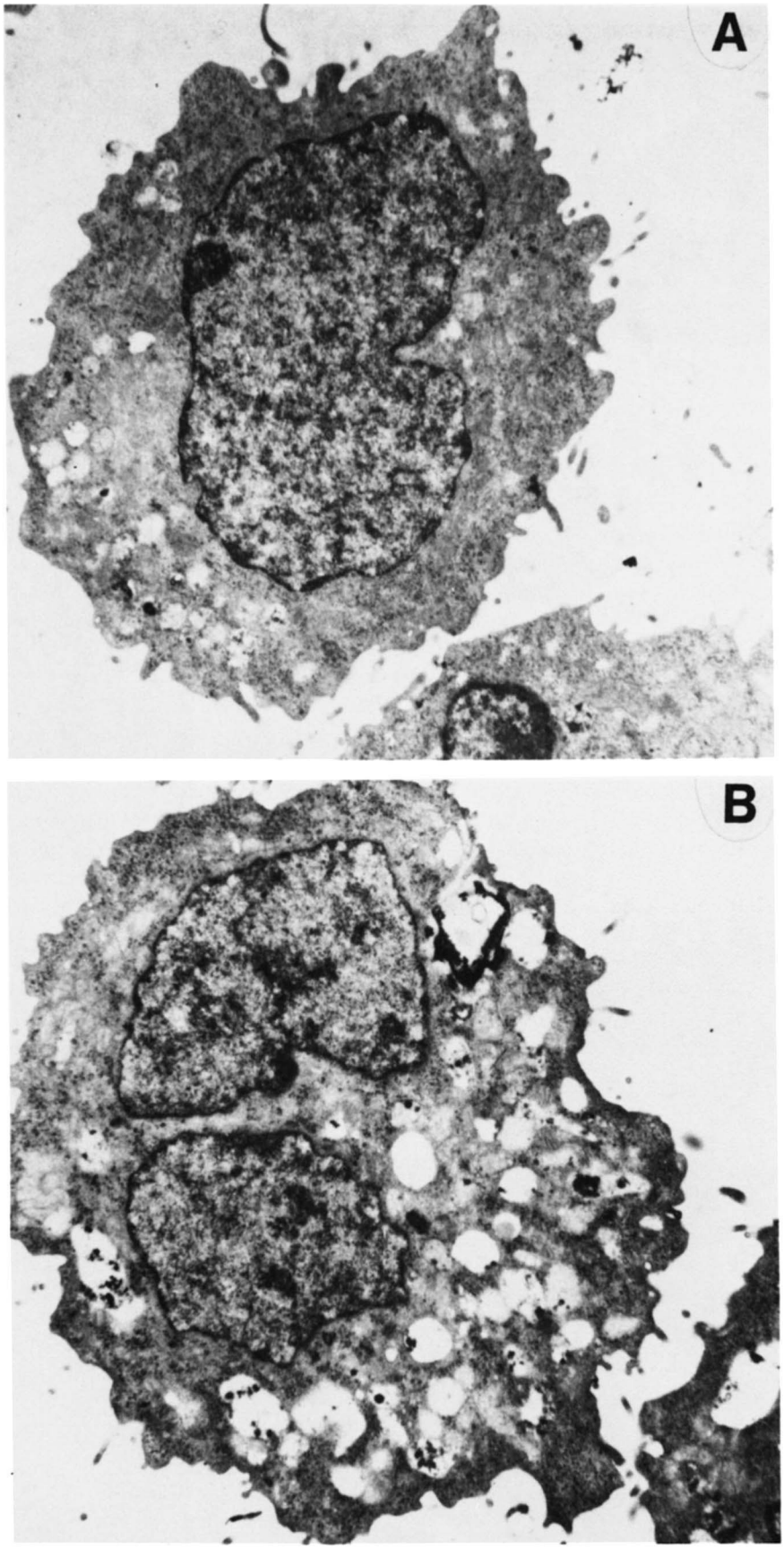


**Fig. 2.** The effect 20 mM $NH_4Cl$ on cell ultrastructure. TS-85 cells were suspended ($5 \times 10^5$ cells/ml) in DMEM-C with or without 20 mM $NH_4Cl$ and the cells were incubated for 18 hr at 30°C. After the incubation, the cells were washed three times in phosphate-buffered saline (pH 7.2) and fixed in 0.1 M sodium cacodylate buffer (pH 7.4) containing 0.1 M sucrose and 2% glutaraldehyde. Cells were postfixed in a 1% solution of $OsO_4$ for 1 hr at 4°C, dehydrated in ethanol, and embedded in an Epon/Araldite mixture. Blocks were cut on an LKB ultrotone and sections were stained with uranyl acetate and lead citrate. Samples were examined with a Philips 300 electron microscope. Pictures are magnified by a factor of 13,400. The activities of HMG-CoA reductase in the control (A) and $NH_4Cl$-treated (B) cells were 0.025 and 0.043 nmol/min per mg protein, respectively.

TABLE 1. Effect of 20 mM $NH_4Cl$ on the activity of HMG-CoA reductase and on the growth of TS-85 cells

| Condition | Reductase Activity | Cell Concentration |
|---|---|---|
| | *nmol/min per mg protein* | *cells × $10^5$/ml* |
| 1. 10% Cabosil - DMEM (DMEM-C) | 0.067 ± 0.005[a] | 6.77 ± 0.99[a] |
| 2. DMEM-C + 20 mM $NH_4Cl$ | 0.112 ± 0.008 | 6.61 ± 0.37 |
| 3. 10% FCS - DMEM (DMEM-F) | 0.009 ± 0.002 | 6.99 ± 1.18 |
| 4. DMEM-F + 20 mM $NH_4Cl$ | 0.104 ± 0.006 | 7.21 ± 0.72 |

Cells were suspended (5 × $10^5$ cells/ml) in either DMEM containing 10% FCS (DMEM-FCS), DMEM-FCS + 20 mM $NH_4Cl$, DMEM containing 10% Cabosil-treated serum (DMEM-C), or DMEM-C + 20 mM $NH_4Cl$. The cell cultures were incubated 18 hr at 30°C with 95% air/5% $CO_2$, and then the cells were collected, assayed for HMG-CoA reductase activity, and the cell concentration was determined using a hemocytometer. Reductase activity was assayed as described in Methods. Cell viability after the 18-hr incubation was greater than 93% in all groups. Results are the mean values from five different cell samples.

[a]Standard deviation, n = 5.

levels of reductase activity compared to cells grown in the absence of $NH_4Cl$ (Table 1). Enzyme activity increased nearly 12-fold in cells treated with DMEM-FCS + 20 mM $NH_4Cl$ as compared to DMEM-FCS alone. The levels of reductase activity were essentially identical when cells were grown in either medium (DMEM-FCS or DMEM-C) supplemented with 20 mM $NH_4Cl$ (Table 1). The 12-fold increase in reductase activity observed in cells grown in DMEM-FCS + 20 mM $NH_4Cl$ may result in part from the reported inhibition of low density lipoprotein degradation by a lysosomotrophic agent (48). Such inhibition of low density lipoprotein degradation would be expected to block the influx of exogenous cholesterol into the cytoplasm and would be expected to lead to increased activity of HMG-CoA reductase.

The presence of 20 mM $NH_4Cl$ during the 18-hr incubation at 30°C did not inhibit the growth of the cells in either DMEM-FCS or DMEM-C (Table 1). In all the conditions tested, the cell concentration increased approximately 30–40% after 18 hr and no significant difference was apparent (Table 1). These results were corroborated in two other separate experiments (data not shown).

Phosphorylation of HMG-CoA reductase has been postulated as a regulatory mechanism for controlling reductase activity (9). Treatment with 20 mM $NH_4Cl$ may increase reductase activity by altering the phosphorylation state of the enzyme. To examine this point, TS-85 cells were grown for 18 hr at 30°C in either DMEM-C or DMEM-C + 20 mM $NH_4Cl$; in half of the cells from each group the reductase was solubilized in detergent buffer alone and in the remaining cells the reductase was solubilized in detergent buffer containing 50 mM NaF. The NaF inhibits endogenous phosphatase activity and the reductase activity measured in the presence of NaF reflects the "expressed" enzyme activity (9). Reductase activity in cell extracts isolated and assayed in the absence of NaF reflects the "total" enzyme activity (**Table 2**). The ratio of the expressed/total activity in the cells grown in DMEM-C alone or DMEM-C + 20 mM $NH_4Cl$ was 0.46 ± 0.01 and 0.49 ± 0.05, respectively. These values are not significantly different. Therefore, the

TABLE 2. Effect of 20 mM $NH_4Cl$ on the phosphorylation of HMG-CoA reductase

| Condition | 50 mM NaF | HMG-CoA Reductase Activity | Ratio Expressed/Total |
|---|---|---|---|
| | | *nmol/min per mg protein* | |
| 1. 10% Cabosil - DMEM (DMEM-C) | – | 0.063 ± 0.004[a] | 0.46 ± 0.01[a] |
| 2. DMEM-C | + | 0.029 ± 0.001 | |
| 3. DMEM-C + 20 mM $NH_4Cl$ | – | 0.113 ± 0.007 | 0.49 ± 0.05 |
| 4. DMEM-C + 20 mM $NH_4Cl$ | + | 0.055 ± 0.003 | |

TS-85 cells were grown for 18 hr in the presence or absence of 20 mM $NH_4Cl$ as described in Table 1. Cells were collected and solubilized in either detergent buffer alone (0.25% Kyro EOB, 0.2 M KCl, 1 mM EDTA, 5 mM glutathione, and 0.16 mM HEPES, pH 7.4) or in detergent buffer containing 50 mM NaF. HMG-CoA reductase activity was assayed in the presence or absence of 50 mM NaF as described in the text. The expressed activity was the reductase activity determined in the presence of NaF, and the total activity was the enzyme activity determined in the absence of NaF. Results are the average value from three different groups of cells.

[a]Standard deviation, n = 3.

effect of $NH_4Cl$ on reductase activity does not appear to be correlated with any $NH_4Cl$-induced changes in the relative phosphorylation state of the enzyme.

### Effect of other lysosomal inhibitors on reductase activity

Chloroquine is another lysosomotrophic agent and is commonly used to inhibit lysosome function (37, 49). Incubating TS-85 cells for 18 hr in DMEM-C containing either 25, 50, or 100 mM chloroquine induced a 47%, 188%, and 253% increase, respectively, in reductase activity compared to cells grown in DMEM-C alone. These data suggest that chloroquine may be more effective than 20 mM $NH_4Cl$ in inducing reductase activity. However, chloroquine is a very potent inhibitor of protein synthesis. Chen and Leonard (39) reported that incubation of Chinese hamster overy cells for 120 min in the presence of 20 and 50 mM chloroquine resulted in the inhibition of total protein synthesis by 56% and 70%, respectively. Chloroquine is also known to inhibit both cholesterol biosynthesis (50) and the conversion of squalene oxide to lanosterol (39). Therefore, chloroquine was not suitable for our experiments and was not used in further studies because of possible problems in valid interpretation of the results.

Leupeptin has been used extensively as an inhibitor of lysosomal function (22, 51) and its primary mode of action is believed to be inhibition of thiol proteases, such as cathepsin D, present in lysosomes (22, 52). Inhibition of other cellular proteases has also been reported (53, 54). Incubating cells for 18 hr in the DMEM-C containing 0.45 mM leupeptin had no effect on the activity of HMG-CoA reductase. The absence of any increase in reductase activity after leupeptin treatment may be due to the fact that leupeptin may be incapable of inhibiting the activity of all the proteases present in lysosomes or that entry of leupeptin into the cells is not complete.

### Effect of $NH_4Cl$ on the rate of total protein degradation

The increase in reductase activity observed when TS-85 cells were grown in the presence of 20 mM $NH_4Cl$ could result from the known inhibitory effect of $NH_4Cl$ on lysosomal protein degradation and suggested that lysosomes might be involved in the degradation of the reductase. To ensure that under our experimental conditions the concentration of $NH_4Cl$ was sufficient to inhibit lysosomal protein degradation, the effect of 20 mM $NH_4Cl$ on the rate of total cellular protein degradation was determined. Cells were incubated for 18 hr at 30°C in the presence or absence of 20 mM $NH_4Cl$, pulsed with [$^{35}$S]methionine, and then the rate of protein degradation was measured by the relative increase in perchloric acid-soluble radioactivity. The rate of total protein degradation in cells grown in DMEM-C alone was 1.85%/hr which is similar to values reported for other murine cells grown in culture (43, 44, 51, 55, 56). When cells were grown in DMEM-C containing 20 mM $NH_4Cl$, the rate of protein degradation was inhibited 19%. This value is similar to that reported by other investigators using similar incubation conditions (43, 44, 54, 56). These data suggested that in TS-85 cells the increase in reductase activity associated with $NH_4Cl$ treatment was correlated with inhibition of lysosomal protein degradation.

### Effect of $NH_4Cl$ on the relative rate of synthesis of HMG-CoA reductase

In three experiments, cells treated for 18 hr with 20 mM $NH_4Cl$ had higher reductase activities (85%, 51%, and 77% increase) compared to controls. However, the relative rate of synthesis of the reductase was identical in experimentals and controls (0.022% ± 0.003) (**Table 3**). We conclude that the increase in reductase activity induced by treatment with 20 mM $NH_4Cl$ is not due to an increase in the rate of synthesis of the enzyme.

### Effect of $NH_4Cl$ on the relative rate of degradation of HMG-CoA reductase

Pretreatment of cells with $NH_4Cl$ resulted in increased reductase activities of approximately 69% and a decrease in the relative rate of degradation of HMG-CoA reductase of approximately 50% (**Table 4, Fig. 3**). The half-life of the reductase in cells grown in DMEM-C alone was 4.0 hr ± 0.4 which is similar to the half-life of the enzyme reported in Chinese hamster ovary cells (57), $t_{1/2}$ = 5 hr, and in chicken myeloblasts (7), $t_{1/2}$ = 3.3 hr. When cells were grown in DMEM-C containing 20 mM $NH_4Cl$, the half-life of the reductase increased to 8.3 hr ± 0.8 (Table 4, Fig. 3). Cells used in experiments 2 and 3 (Table 4) were the same group of cells used in experiments 2 and 3 (Table 3) to measure the relative rate of synthesis of the reductase. If the relative rate of synthesis of the enzyme is unchanged while the rate of degradation is decreased approximately 50%, the activity of HMG-CoA reductase would be predicted to increase approximately 100% after an 18-hr incubation (58). The reductase activity in cells grown for 18 hr in DMEM-C + 20 mM $NH_4Cl$ actually increased 81% ± 20, which is qualitatively similar to the theoretical predicted value. Therefore, the increase in reductase activity observed after incubation with 20 mM $NH_4Cl$ can be completely accounted for by the relative change in degradation rate of the enzyme. We conclude from these data that $NH_4Cl$ treatment inhibits the basal rate of degradation of HMG-CoA reductase by inhibiting some $NH_4Cl$-sensitive step in either the lysosomal protein degradation pathway or in some non-lysosomal degradative pathway that is yet to be elucidated.

TABLE 3. Effect of 20 mM $NH_4Cl$ on the rate of synthesis of HMG-CoA reductase in TS-85 cells

| Experiment | HMG-CoA Reductase Activity | % Change in Activity | Rate of Reductase Synthesis |
|---|---|---|---|
| | *nmol/min per mg protein* | | |
| 1. Control | 0.086 (n = 1) | | 0.021 ± 0.001[a] (n = 3) |
| + 20 mM $NH_4Cl$ | 0.159 (n = 1) | 85% | 0.024 ± 0.002 (n = 3) |
| 2. Control | 0.096 ± 0.004[a] (n = 2) | | 0.019 (n = 1) |
| + 20 mM $NH_4Cl$ | 0.145 ± 0.004 (n = 2) | 51% | 0.017 (n = 1) |
| 3. Control | 0.034 ± 0.012 (n = 3) | | 0.025 ± 0.001 (n = 2) |
| + 20 mM $NH_4Cl$ | 0.060 ± 0.004 (n = 3) | 77% | 0.021 ± 0.001 (n = 2) |
| Average: | | | |
| Control | | | 0.022 ± 0.003 (n = 6) |
| + 20 mM $NH_4$ | | 71% | 0.022 ± 0.003 (n = 6) |

TS-85 cells ($5 \times 10^5$ cells/ml) were grown for 18 hr at 30°C in DMEM + 10% Cabosil-treated serum (DMEM-C) alone or DMEM-C containing 20 mM $NH_4Cl$. Cells were transferred to methionine-free MEM media and were incubated with [$^{35}$S]methionine for 30 min at 30°C. The radiolabeled HMG-CoA reductase was immunoprecipitated and analyzed on SDS–8 M urea polyacrylamide gels as described in Methods. The rate of reductase synthesis was expressed as the percent of the total TCA-precipitable radioactivity present in the radiolabeled reductase polypeptide. HMG-CoA reductase activity was also determined by radioassay. Data from three separate experiments are presented.

[a]Standard deviation.

## Accelerated degradation of HMG-CoA reductase induced by 25-hydroxycholesterol

Since $NH_4Cl$ inhibits the basal degradation rate of HMG-CoA reductase, we were interested in determining whether $NH_4Cl$-treatment also inhibited the effect of 25-hydroxycholesterol on the enzyme. 25-Hydroxycholesterol is a potent inhibitor of reductase activity and addition of 25-hydroxycholesterol (5 μg/ml) to TS-85 cells rapidly inhibited reductase activity in cells grown at the permissive temperature (30°C) and in cells grown for 18 hr at 30°C in the presence of 20 mM $NH_4Cl$ (**Fig. 4**, panels A and C). Reductase activity in FM3A cells was also inhibited by 25-hydroxycholesterol (data not shown).

Addition of 25-hydroxycholesterol to either chicken (7) or hamster (4) cells rapidly lowered reductase activity by inhibiting the synthesis of the enzyme and by inducing a 3- to 4-fold increase in the rate of degradation of the enzyme. When 25-hydroxycholesterol was added to TS-85 cells, it also increased the relative degradation rate of the reductase approximately 3- to 4-fold in cells grown for 18 hr in either DMEM-C alone or DMEMC + 20 mM $NH_4Cl$ (data not shown). These data demonstrated that 25-hydroxycholesterol can still inhibit reductase activity in TS-85 cells treated with 20 mM $NH_4Cl$ and that the inhibition of activity is due in part to an increase in the rate of degradation of the reductase. Therefore, the accelerated degradation of the enzyme induced by 25-hydroxy-

TABLE 4. Effect of 20 mM $NH_4Cl$ on the rate of degradation of HMG-CoA reductase in TS-85 cells

| Experiment | HMG-CoA Reductase Activity | % Change Activity | Half-life of the Reductase | % Change in Half-life |
|---|---|---|---|---|
| | *nmol/min per mg protein* | | *hr* | |
| 1. Control | 0.009 ± 0.002[a] (n = 5) | | 3.6 | |
| + 20 mM $NH_4Cl$ | 0.016 ± 0.001 (n = 5) | 78% | 7.4 | 106% |
| 2. Control | 0.096 ± 0.004 (n = 2) | | 4.3 | |
| + 20 mM $NH_4Cl$ | 0.145 ± 0.004 (n = 2) | 51% | 8.8 | 105% |
| 3. Control | 0.034 ± 0.012 (n = 3) | | 4.2 | |
| + 20 mM $NH_4Cl$ | 0.060 ± 0.004 (n = 3) | 77% | 8.6 | 105% |
| Average: | | | | |
| Control | | | 4.0 ± 0.4 | |
| + 20 mM $NH_4Cl$ | | 69% | 8.3 ± 0.8 | 105% |

TS-85 cells ($5 \times 10^5$ cells/ml) were grown for 18 hr at 30°C in DMEM + 10% Cabosil-treated serum (DMEM-C) or DMEM-C containing 20 mM $NH_4Cl$. Cells were transferred to methionine-free MEM, labeled with [$^{35}$S]methionine, and then transferred to DMEM-C media containing 5 mM unlabeled methionine. The radiolabeled HMG-CoA reductase was immunoprecipitated and analyzed on SDS–8 M urea gels as described in Methods. The activity of HMG-CoA reductase was also determined and the rate of reductase degradation was calculated as half-life ($t_{1/2}$) of the radiolabeled enzyme. Data from three separation experiments are presented.

[a]Standard deviation.

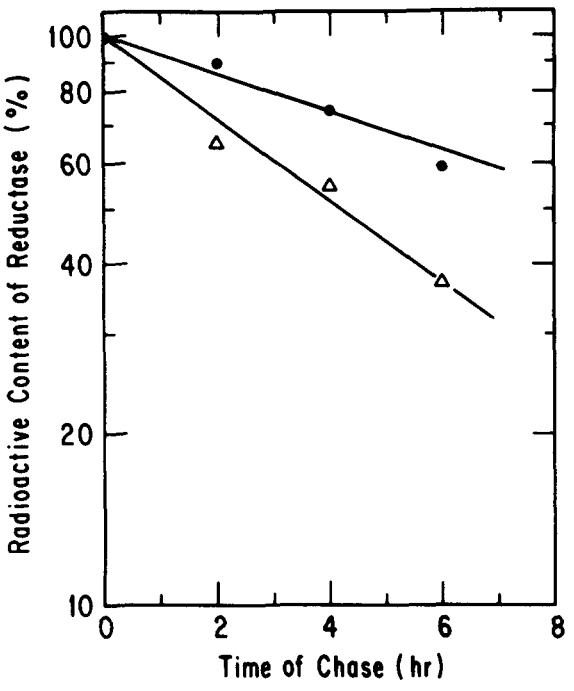


**Fig. 3.** Relative rate of degradation of HMG-CoA reductase in cells grown in the presence or absence of 20 mM $NH_4Cl$. TS-85 cells ($5 \times 10^5$ cells/ml) were grown for 18 hr in either DMEM-C alone or DMEM-C + 20 mM $NH_4Cl$. Cells were labeled with [$^{35}$S]methionine and the radiolabeled reductase was immunoprecipitated and analyzed by electrophoresis on SDS–8 M urea gels after varying times during the chase period as described in Methods. The radioactivity in the reductase polypeptide is expressed as a percent of the radioactivity present in the reductase at the first time point (time 0) which was 4968 cpm and 4002 cpm for cells grown in DMEM-C alone (△) or DMEM-C containing 20 mM $NH_4Cl$ (●), respectively. Reductase activity in cells incubated in DMEM-C alone and DMEM-C + 20 mM $NH_4Cl$ was 0.034 and 0.060 nmol/min per mg protein, respectively.

cholesterol treatment may not involve lysosomes and may be mediated by a pathway different from the one used for the basal degradation rate since the latter appears to be blocked with 20 mM $NH_4Cl$.

### Role of the ubiquitin pathway

Finley et al. (20) and Ciechanover, Finley, and Varshavsky (21) reported that the TS-85 cell line expresses a temperature-sensitive mutation in the ubiquitin pathway. Incubating cells for 2 hr at the nonpermissive temperature (40°C) inactivated the ubiquitin pathway for protein degradation in the TS-85 cells but did not inhibit the pathway in wild-type FM3A cells (21). This temperature-sensitive mutation gave us an opportunity to determine whether the accelerated degradation of HMG-CoA reductase induced by 25-hydroxycholesterol was mediated by the ubiquitin pathway. Before conducting experiments, the phenotypic characteristics of the TS-85 cells were measured to ensure that the cells still retained the temperature-sensitive mutation and that the reductase was similar to the enzyme in wild-type cells. The relative molecular weight of the reductase was identical in TS-85 and FM3A cells ($M_r$ = 97,000) and the $K_m$ for DL-HMG-CoA was 5.1 ± 0.95 μM and 6.0 ± 2.3 μM (n = 3), respectively. These latter values are similar to the $K_m$ reported for the enzyme in mouse uterine epithelial cells (59). Hence the HMG-CoA reductase was similar in both cell types. Incubating TS-85 cells at 40°C completely blocked cell growth and, also in agreement with earlier studies (26), inhibited the formation of the ubiquitin-conjugated histone, UH2A (data not shown). These data demonstrate that the TS-85 cells used in our experiments still retain their temperature-sensitive mutation in the ubiquitin pathway. Incubating TS-85 cells at the non-

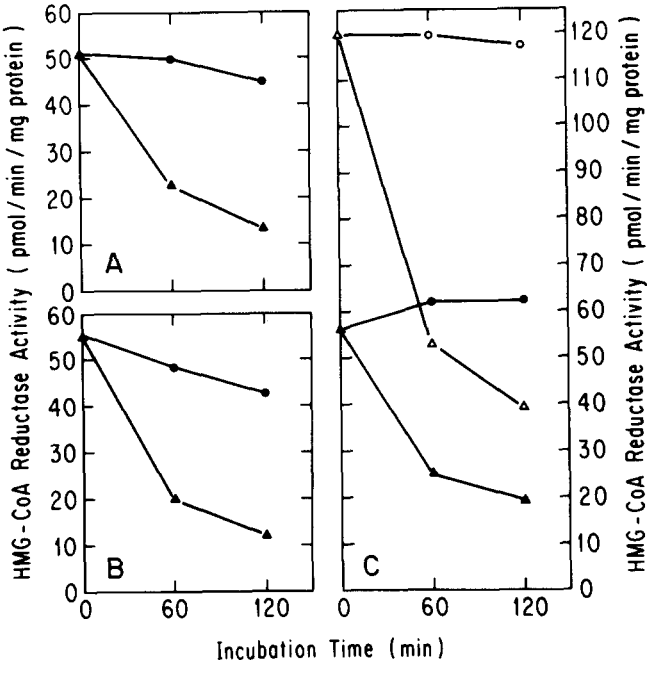


**Fig. 4.** Effect of 25-hydroxycholesterol on HMG-CoA reductase activity in TS-85 cells grown at permissive temperature, nonpermissive temperature, or incubated in the presence of 20 mM $NH_4Cl$. Panel A, TS-85 cells were grown in DMEM-C at 30°C for 18 hr and then treated with either 25-hydroxycholesterol (5 μg/ml, added ethanol) or, in control cells, an equivalent volume of ethanol. Reductase activity was determined at the specified times after addition of the 25-hydroxycholesterol (▲) or ethanol (●). Panel B, TS-85 cells were grown in DMEM-C at 30°C for 18 hr and then the cells were incubated at 40°C for 2 hr. 25-Hydroxycholesterol (5 μg/ml), or, in control cells, an equivalent amount of ethanol was added and the reductase activity was determined at the specified times. Cells were maintained at 40°C after the addition of either the 25-hydroxycholesterol (▲) or ethanol (●). Panel C, TS-85 cells ($5 \times 10^5$ cells/ml) were incubated for 18 hr at 30°C in DMEM-C alone or DEM-C + 20 mM $NH_4Cl$. Cells were treated with either 25-hydroxycholesterol (5 μg/ml) or, in the controls, an equivalent amount of ethanol and the reductase activity was determined at the specified times after addition. All cells were incubated at 30°C and, in the $NH_4Cl$-treated cells, 20 mM $NH_4Cl$ was also present during the incubation. Symbols represent cells grown in DMEM-C alone and treated with 25-hydroxycholesterol (▲) or ethanol (●); cells grown in DMEM-C containing 20 mM $NH_4Cl$ and treated with 25-hydroxycholesterol (△) or ethanol (○).

permissive temperature (40°C) did not inhibit the effect of 25-hydroxycholesterol on enzyme activity compared to cells grown at the permissive temperature (Fig. 4), panel B). Since 25-hydroxycholesterol has been shown to increase the rate of degradation of the reductase, we conclude that the accelerated loss of HMG-CoA reductase activity that occurs in the present study upon addition of 25-hydroxycholesterol is not dependent on the ubiquitin pathway.

## DISCUSSION

Very little is known regarding how proteins in the endoplasmic reticulum are proteolytically degraded in the cell. Numerous morphological studies have described the role of lysosomes in the degradation of the endoplasmic reticulum (17, 18, 45, 60). However, the differences in the turnover rates of microsomal proteins do not appear to support the concept of unit membrane degradation by lysosomes (61, 62). Therefore, multiple pathways may exist in cells for proteolytic degradation of proteins in the endoplasmic reticulum.

Our results on the degradation of HMG-CoA reductase also suggest the possibility of two different pathways for the basal and accelerated proteolytic degradation of the enzyme. The basal degradation rate of the reductase was inhibited by $NH_4Cl$ treatment and resulted, after 18 hr, in a 50% inhibition of the relative rate of reductase degradation. This altered rate of degradation can account for the 81% increase in enzyme activity that was observed after $NH_4Cl$ treatment. Inhibition of protein degradation by lysosomotrophic agents, such as $NH_4Cl$, is generally regarded as reflecting the inhibition of protein degradation in lysosomes. Hence the observed effect of $NH_4Cl$ on the degradation rate of HMG-CoA reductase may be the first indication that, under basal conditions, the enzyme is being degraded in lysosomes.

$NH_4Cl$-treatment might also affect reductase activity by inhibiting the lysosomal degradation of the small amount (2 μg/ml) of cholesterol present in the DMEM-C medium. Faust et al. (4) have shown that LDL cholesterol inhibits the synthesis and enhances the degradation of the reductase in Chinese hamster ovary cells. In our study $NH_4Cl$ treatment inhibited reductase degradation but had no effect on the rate of reductase synthesis (Table 3). If the residual LDL cholesterol in the DMEM-C medium was regulating reductase activity, we would predict that inhibition of lysosomal degradation, or cholesterol uptake, would also alter the rate of reductase synthesis. Since $NH_4Cl$ treatment had no effect on reductase synthesis, we conclude that lysosomal degradation of any residual LDL cholesterol present in the DMEM-C medium does not play a role in the regulation of the synthesis and degradation of the enzyme.

Our results on the effect of $NH_4Cl$ treatment on the relative rate of degradation of HMG-CoA reductase are very similar to the results reported by Chandler and Ballard (43) on the effect of lysosomotrophic agents on the degradation of the mitochondrial enzyme, pyruvate carboxylase. They noted that incubation of mouse 3T3-1 cells for 24 hr in medium containing 5 mM $NH_4Cl$ caused a 27% inhibition of total protein degradation and a 47% inhibition of the relative rate of degradation of pyruvate carboxylase. These investigators also concluded that pyruvate carboxylase may be degraded in the lysosomal system. However, other degradative pathways must also be functional in order to account for the degradation of other mitochondrial proteins which have a relatively short half-life. Degradation of mitochondria in lysosomes is well documented (41, 60), but $NH_4Cl$ inhibition of lysosomal protein degradation only induced a 47% inhibition in the relative rate of degradation of pyruvate carboxylase. Hence $NH_4Cl$ treatment may not completely block lysosomal function. Indeed, recent reports have shown that lysosomal inhibitors only partially inhibit (40–75%) the normal degradation of proteins by lysosomes (63, 64).

An important question regarding the degradation of HMG-CoA reductase is how the enzyme is marked or signaled for proteolytic degradation. Recently, Parker et al. (11) reported that phosphorylation of the reductase present in rat liver microsomes made the enzyme more susceptible to in vitro proteolysis. This may suggest that phosphorylation of the reductase might be a signal for degradation of the enzyme. Our results on the inhibition of reductase degradation by $NH_4Cl$ treatment would not support this conclusion. Inhibition of the relative rate of reductase degradation was not associated with any change in the ratio of expressed/total reductase activity (Table 2). If $NH_4Cl$ treatment selectively inhibited the degradation of the phosphorylated reductase, then we might expect to observe significant changes in the ratio of expressed/total activity.

By using the TS-85 cell line, which expresses a temperature-sensitive mutation in the ubiquitin degradation pathway, we were able to examine the role of a non-lysosomal proteolytic system in the accelerated degradation of the reductase induced by treatment with 25-hydroxycholesterol. TS-85 cells incubated at the non-permissive temperature (40°C), and not possessing a functional ubiquitin pathway, were still capable of responding to 25-hydroxycholesterol treatment (Fig. 4). These data clearly demonstrate that the rapid degradation of the reductase induced by 25-hydroxycholesterol is not mediated by the ubiquitin pathway.

An interesting observation was that 25-hydroxycholesterol treatment inhibited reductase activity in cells grown in the presence of 20 mM $NH_4Cl$ for 18 hr (Fig. 4). These

data imply that the accelerated degradation of HMG-CoA reductase induced by 25-hydroxycholesterol is not sensitive to $NH_4Cl$ inhibition and is mediated by a non-lysosomal, or $NH_4Cl$-insensitive, proteolytic pathway. In addition, the higher level of reductase activity observed in the $NH_4Cl$-treated cells is probably not due to sequestration of the enzyme in $NH_4Cl$-inhibited lysosomes since the reductase in these cells can still be rapidly degraded after 25-hydroxycholesterol treatment. Our results suggest that possibly two different degradative pathways may be functioning in the basal and accelerated degradation of HMG-CoA reductase. This may indicate that a complex regulatory system may control the degradation of the enzyme, which might be expected if degradation of the reductase was a significant regulatory mechanism for controlling enzyme activity. In future studies the differential sensitivity to $NH_4Cl$ inhibition may provide an important tool for dissecting the two degradative pathways.

Chen and Leonard (39) reported that chloroquine treatment inhibited the effect of 25-hydroxycholesterol on reductase activity in mouse L cells. These results may appear to contradict our study. However, the concentration of chloroquine used in their study also blocked total protein synthesis (39). Chang, Limanek, and Chang (40) noted that in Chinese hamster ovary cells inhibition of protein synthesis could significantly reduce or nearly abolish the effect of 25-hydroxycholesterol on reductase activity. These investigators postulated the presence of a rapidly turning over protein whose presence was required for the hydroxycholesterol to be inhibitory. Cavenee, Chen, and Kandutsch (41) also reported that inhibition of protein synthesis altered the regulation of HMG-CoA reductase by 25-hydroxycholesterol. Therefore, the effect of chloroquine treatment described by Chen and Leonard (39) may be due primarily to inhibition of total protein synthesis and, in future studies on the degradation of HMG-CoA reductase, care must be taken to ensure that total protein synthesis is not compromised by inhibitors of proteolytic degradation.

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