Discussion
The results described in the thesis clearly demonstrate that a rapid, sensitive, reproducible and inexpensive colorimetric method for the assay of cytosolic neutral cholesteryl ester hydrolase (CEH) has been developed. The assay is based on the CEH-mediated hydrolysis of substrate p-nitrophenyl (PNP) esters of long chain fatty acids such as palmitate and stearate to generate the product p-nitrophenol which has an absorption at 405 nm. The results also demonstrate that CEH-mediated hydrolysis of PNP-palmitate is significantly higher than cholesteryl oleate (CO) in cytosol, 0-40% ASF (69 kDa CEH) and 40-80% ASF (45 kDa CEH) of cytosol, when both the substrates were added as lipid droplet dispersion. The combined results represent an initial demonstration of additional CEH activity associated with low molecular weight (45 kDa) protein fractionated in 40-80% ASF of cytosol. Identification of low (45 kDa) molecular form of rat liver cytosolic CEH, associated with 40-80% ASF is consistent with an earlier report where a significant amount of CEH activity was detected in 40-60% ASF of rat liver cytosol by employing CO as substrate. However, no characterization of this form of CEH was reported (Ghosh et al., 1990). The comparison of substrate specificities of CE hydrolases in cytosol, 0-40% ASF (69 kDa CEH) and 40-80% ASF (45 kDa CEH) revealed that highest specific activity was obtained with PNP-acetate, followed by PNP-butyrate, PNP-caprylate, PNP-palmitate and PNP-stearate. The highest hydrolytic activity toward PNP-acetate and PNP-butyrate of the three fractions appears to be due to the presence of additional esterase(s) with greater specificity for these two short chain length PNP-fatty acyl esters.

Water solubility of PNP-acetate and PNP-butyrate appears to be an additional contributory factor for substantially higher CEH-mediated hydrolytic rate of these substrate. Similar results were obtained with purified 69 kDa and 45 kDa CEH toward PNP-esters with fatty acids of different chain lengths. It is interesting to note that increase in CEH specific activity with short chain substrates relative to specific activity with PNP-palmitate was highest in porcine liver CEH followed by rabbit liver CEH. The results confirm the earlier observations with cytosol and its
ASF that disproportionately higher activity of various purified esterases toward PNP-acetate and PNP-butyrate is due to water solubility of these substrates. These results are consistent with an earlier report where a very high liver cytosolic esterase activity was reported toward PNP-acetate followed by PNP-caprylate and CO, while PNP-butyrate was not used (Natarajan et al., 1996a). The results also indicate that PNP-palmitate and PNP-stearate are specific substrates for CEH in cytosol and ammonium sulfate fractions containing high (69 kDa) or low (45 kDa) molecular forms.

In order to establish that high level of CEH-mediated hydrolysis of PNP-palmitate, in comparison to CO, is specific, enzyme activities of CE hydrolases in cytosol, 0-40% ASF (69 kDa CEH) and 40-80% ASF (45 kDa CEH) were assayed in presence of taurocholate, which is known to activate CEH enzyme (Harrison et al., 1989). The results demonstrate that percent increase in CEH activities of these three fractions, in comparison to respective controls, mediated by taurocholate is similar, when assayed either with PNP-palmitate or CO. The results also indicate that like CO, the hydrolysis of PNP-palmitate is specific to cytosolic high (69 kDa) and low (45 kDa) molecular forms of CEH. Similarly, comparison of hydrolysis of PNP-palmitate and CO was done with cytosolic 69 kDa (0-40% ASF) and 45 kDa (40-80% ASF) CEH isolated from rats treated with tocotrienols which have been shown to inhibit CEH activity in vivo. The results confirm the earlier findings that cytosolic, high and low molecular forms of CEH from normal and TRF-treated rats preferentially hydrolyzed PNP-palmitate than CO which is due to lower hydrophobicity of PNP-palmitate. Nevertheless, the TRF-mediated inhibition level of CEH activity was similar, when assayed with both the substrates. The results provide evidence that all the measurable cytosolic CEH activity was associated with the two forms of CEH after non-SDS PAGE and gel permeation HPLC, when assayed either with PNP-palmitate or CO. Other than the two CEH peaks, no esterase activity was assayed with PNP-palmitate or CO. Consistent with earlier results, PNP-palmitate is hydrolyzed more efficiently than CO, indicating that accessibility
of PNP-palmitate towards enzyme is greater than CO apparently due to lower hydrophobicity of PNP-palmitate. In agreement with above results, PNP-palmitate is preferentially hydrolyzed at much faster rate in comparison to CO, by purified 69 kDa CEH, 45 kDa CEH, purified CE hydrolases from rabbit liver, porcine liver, bovine pancreas and pseudomonas. The combined results provide definitive evidence that PNP-palmitate is preferentially hydrolyzed at a faster rate in comparison to CO, by rat liver cytosolic, partially purified and purified 69 kDa and 45 kDa CE hydrolases and purified CE hydrolases from rabbit and porcine liver. Surprisingly, with purified CE hydrolases from rabbit and porcine liver, the efficiency of PNP-palmitate hydrolysis was several fold higher, in comparison to CO. Our results with purified rat liver cytosolic high molecular (69 kDa) form of CEH are in disagreement with an earlier report, where lowest purified cytosolic CEH activity was obtained with PNP-acetate followed by PNP-butyrate. Activity peaked with more lipophilic PNP-caprylate and then declined gradually with increasing chain length greater than caprylate (Natarajan et al., 1996a). However, the reported finding that purified CEH, in the presence of 0.25 mM taurocholate, catalyzed the hydrolysis of PNP-palmitate more efficiently than CO when added as lipid droplet dispersion is in complete agreement with our results (Natarajan et al., 1996a).

High and low molecular forms of cytosolic CEH, associated with 0-40% ASF and 40-80% ASF of cytosol, respectively, were purified to electrophoretic homogeneity. The method of purification employed a preparative non-SDS PAGE of cytosolic protein following which CEH activity associated with each of the two forms of CEH was eluted separately and pooled protein fractions were purified on 30,000 MW cut Centricon concentrators. On SDS-PAGE, a single protein band corresponding to molecular weight of 69 kDa for high molecular form of CEH and 45 kDa for low molecular form of CEH was obtained. An apparent molecular weight of 69 kDa for cytosolic high molecular form of CEH is consistent with a reported molecular weight of 66 kDa for the same enzyme (Ghosh and Grogan,
1991a). For the newly discovered low molecular form of cytosolic CEH, an apparent molecular weight of 45 kDa represents an initial demonstration. Neuraminidase treatment of purified 69 kDa CEH, reduced the molecular weight to 66 kDa, indicating removal of carbohydrate. In contrast, purified 45 kDa CEH does not seem to be a glycoprotein. Previously published reports also indicated that 66 kDa CEH is a glycoprotein (Ghosh and Grogan, 1991a).

CEH activities of cytosol, partially purified 69 kDa CEH in 0-40% ASF, partially purified 45 kDa CEH in 40-80% ASF and purified 69 kDa and 45 kDa CEH were significantly increased (40-70%) at 5 mM taurocholate. However, at higher concentration of taurocholate (6-20 mM), the activation was significantly reduced in all the CEH fractions. These results are in agreement with the findings of Ghosh and Grogan (1991a) that rat liver cytosolic and purified CEH, unlike pancreatic CEH (Harrison, 1988) is not bile salt dependent, whereas, our results are inconsistent with other studies where CEH activities in rat liver homogenates or cytosol required millimolar concentrations of bile salt for optimal activity (Harrison et al., 1979). In addition, the bile salt dependent CEH enzyme activity varied widely among rat liver homogenate from no stimulation to 70-80% inhibition or a ten fold stimulation of hepatic CEH (Harrison et al., 1979; Harrison, 1988; Camulli et al., 1989; Kissel et al., 1989). In addition, our results and that of Ghosh and Grogan (1991a) are in disagreement with the suggestion by Rojas and Harrison (1994) that both bile salt-dependent and independent CEH activities of rat liver cytosol may originate from the same enzyme. However, Ghosh and Grogan (1991a) demonstrated that purified rat liver cytosolic CEH aggregates to form a multimer of molecular weight 400 kDa and expresses no activity in absence of bile salt. Tuhackova et al. (1980) also observed aggregation of liver CEH in absence of bile salt. Activation by the lower concentrations (upto 5 mM) of taurocholate was much more pronounced with purified liver CEH than in cytosol (Natarajan et al., 1996a), suggesting that the activation may be a detergent effect rather than a specific effect on the enzyme. In contrast, in our hands, purified 69 kDa CEH expressed good
amount of basal activity in the absence of bile salt. However, addition of 5 mM taurocholate did activate the enzyme by 72%. Our results provide evidence that like 69 kDa CEH, partially purified and purified 45 kDa CEH was activated by 5 mM taurocholate and this activation was inhibited at taurocholate concentrations higher than 5 mM.

Maximum activities of CEH in cytosol, 69 kDa CEH in 0-40% ASF and 45 kDa CEH in 40-80% ASF of cytosol, were obtained at 37°C. The pH optima for the cytosolic CEH and partially purified 69 kDa CEH was between 7.0 and 7.3, whereas, the optimum pH for the low (45 kDa) molecular form associated with 40-80% ASF was between pH 7.0 and 7.2. The results are consistent with earlier reports indicating that purified high molecular form of CEH exhibited maximum activity between pH 7.0 and 7.4 (Ghosh and Grogan, 1991a). These results provide further evidence that newly identified rat liver cytosolic 45 kDa CEH is a minor CEH isoform and belongs to the category of neutral CE hydrolases.

Kinetic parameters of CEH activities in cytosol, partially purified 69 kDa CEH in 0-40% ASF and 45 kDa CEH in 40-80% ASF, revealed two fold higher $K_m$ values for PNP-palmitate than for CO. However, $V_{max}$ values of these three fractions for the hydrolysis of PNP-palmitate were higher than that of CO. These results further suggest that high CEH specific activity obtained with PNP-palmitate than CO is directly related to lower hydrophobicity and greater accessibility to the enzyme. On the other hand, as expected, CEH has higher affinity towards CO than PNP-palmitate.

The divalent metal ions, $\text{Fe}^{2+}$, $\text{Cu}^{2+}$, $\text{Zn}^{2+}$, $\text{Cd}^{2+}$, $\text{Ni}^{2+}$, $\text{Co}^{2+}$ and $\text{Hg}^{2+}$ (at 1-20 mM), on the CEH activities of cytosol, 0-40% ASF containing 69 kDa CEH and 40-80% ASF containing 45 kDa CEH, exhibited a concentration dependent inhibition. These results indicate that inhibition reflects a general effect of these divalent metal ions. Preincubation of cytosol, 0-40% ASF containing 69 kDa CEH and 40-80% ASF containing 45 kDa CEH with $\text{Mn}^{2+}$ caused an optimal activation at 5 mM. Similarly, 5 mM $\text{Mg}^{2+}$ also activated cytosolic and 69 kDa CEH, whereas,
45 kDa CEH remained unaffected. Based on these results it can be concluded that Mn\(^{2+}\) appears to directly affect the hydrolysis of substrate by CEH. The results also indicate that unlike Mn\(^{2+}\), the activating effect of Mg\(^{2+}\) on CEH is time-dependent, requires 20 min. preincubation to fully activate the CEH enzyme. The CEH activation is apparently mediated by endogenous phosphorylating agents including cAMP-dependent protein kinase, involving activation (phosphorylation) of CEH (Khan, 1999). The differential effect of Mn\(^{2+}\) and Mg\(^{2+}\) on CEH activity was consistent with the \(V_{\text{max}}\) and \(K_m\) values obtained in the presence of each cation. Mg\(^{2+}\)-mediated activation of CEH is inconsistent with the finding reported by Ghosh and Grogan (1989), where 4 mM Mg\(^{2+}\) resulted in dephosphorylation and inactivation of cytosolic CEH, involving Mg\(^{2+}\)-dependent phosphatase.

The data presented in the thesis demonstrates that the preincubation of cytosolic and partially purified 69 kDa CEH in 0-40% ASF, with 3 mM ATP or 2 mM Mg-ATP significantly activated the CEH which is mediated by endogenous cAMP-dependent kinase and other cofactors required for phosphorylation and activation (Khan, 1999). The results also indicate that inactivating effect of ATP (above 3 mM) and Mg-ATP (above 2 mM) on CEH activation and almost complete inhibition of basal CEH activity at 5-10 mM of ATP and Mg-ATP, might be associated with endogenous Mg-ATP-dependent phosphatase-mediated dephosphorylation and inactivation of CEH enzyme in cytosol and 0-40% ASF. Activation of such type of phosphatase at high levels of ATP and Mg-ATP will counteract the cAMP-dependent protein kinase/cAMP and Mg-ATP promoted activation in a similar order of potency reducing CEH activity to the control level or even completely inactivating the basal CEH activity (Khan, 1999). The combined results demonstrate that enzymatic activity of newly identified low (45 kDa) molecular form of CEH is not subject to \textit{in vitro} activation/inactivation cycle involving phosphorylation/dephosphorylation.

It has been well established that mevalonolactone, 7-keto-cholesterol, 25-hydroxycholesterol, 3-hydroxy-3-methylglutaric acid and \(\gamma\)-tocotrienol modulate
in vivo HMG-CoA reductase activity and in turn cholesterol production, but these modulators failed to exert any in vitro effect on cytosolic CEH.

PMSF, known for its inhibitory effects towards proteins with active site serine residue, inhibited (~95%) the activities of cytosolic CEH, partially purified and highly purified 69 kDa CEH and 45 kDa CEH, in a concentration-dependent manner when preincubated for 16 h at 4°C (Yeaman et al., 1980). These results indicate the presence of serine esterase active site in both the molecular forms of CEH. The results are consistent with the PMSF-mediated inhibition of purified rat liver cytosolic 66 kDa CEH previously reported by Natarajan et al. (1996a). These results are also consistent with the identification of a consensus serine esterase active site in 66 kDa CEH (Ghosh et al., 1995). Inhibition of 69 kDa CEH by PMSF is similar to that reported for other cholesteryl esterases (Hyun et al., 1972; Sonnenborn et al., 1982) and microsomal pl 6.1 esterase (Hernandez et al., 1993). Our results indicate that 69 kDa CEH and 45 kDa CEH of rat liver cytosol are similar in terms of serine esterase active site.

We examined cross-reactivity of anti-rat liver CEH (66 kDa) anti serum and anti rat pancreatic CEH IgG with cytosolic fraction of rat liver containing 69 kDa CEH (0-40% ASF) and 45 kDa CEH (40-80% ASF), pancreas, brain and purified, commercial CE hydrolases from rabbit liver, porcine liver, bovine pancreas and pseudomonas by western blot analysis and ELISA. The results demonstrate that antibodies raised against rat liver cytosolic 66 kDa CEH were specific to cytosolic high (69 kDa) molecular form of CEH, whereas, a week cross-reactivity was seen with 45 kDa molecular form of cytosolic CEH. These results suggest that rat liver cytosolic low (45 kDa) molecular form of CEH is immunologically different from 69 kDa CEH. Similar to rat liver cytosolic 45 kDa CEH in 40-80% ASF, a positive but weak reaction with cytosolic CEH of pancreas was observed, whereas no reaction was seen with brain cytosolic CEH. Consistent with the results obtained for cross-reactivity of cytosolic 69 kDa and 45 kDa CEH, the magnitude of cross-reactivity of anti-rat liver CEH anti serum with purified rat liver cytosolic 45 kDa CEH
CEH was approximately 9% in comparison to that with 69 kDa CEH. The combined results provide further evidence that the predominant CEH isoforms of rat liver (69 kDa), rabbit liver (62 kDa) and porcine liver (59 kDa) are similar enzymes, whereas, the newly identified (45 kDa) minor CEH isoform of rat liver cytosol and bovine pancreatic cytosolic CEH (56 kDa) are different enzymes in comparison to 69 kDa CEH of rat liver cytosol.

The level of cross-reactivity of anti-rat pancreatic CEH IgG with rat liver cytosolic 45 kDa CEH was more than 5 fold higher than cross-reactivity with cytosolic 69 kDa CEH. Cross-reactivity of anti-rat pancreatic CEH IgG revealed a five fold higher reaction with cytosolic CEH from pancreas, in comparison to 45 kDa CEH associated with 40-80% ASF of rat liver cytosol. No reaction was observed with cytosolic CEH of rat brain. The combined results demonstrate that rat liver cytosolic 69 kDa CEH and 45 kDa CEH are different enzymes. In addition, a strong cross-reactivity of rat liver cytosolic 45 kDa CEH with anti-rat pancreatic CEH IgG suggests a structural similarity of this enzyme with 69 kDa rat pancreatic CEH in cytosol. In contrast, a relatively week immuno-reaction of rat liver cytosolic high (69 kDa) molecular form of CEH with anti-pancreatic CEH IgG indicates its immunological dissimilarity with pancreatic CEH. The combined results demonstrate that predominant CEH isoforms of rat liver and pancreas are different proteins, whereas, newly identified 45 kDa CEH from rat liver cytosol and 69 kDa CEH from rat pancreatic cytosol are similar proteins. These results also indicate high degree of homology between rat and bovine pancreatic CEH proteins. The level of cross-reactivity with esterases from rat liver cytosol (69 kDa), rabbit liver and porcine liver was below 10% in comparison to cross-reactivity with bovine pancreatic CEH indicating a high degree of structural dissimilarity of these between predominant liver CE hydrolases and pancreatic CEH. The above results are supported by previous findings that the predominant CEH isoforms of liver and pancreas are different enzymes (Gallo et al., 1978; Ghosh and Grogan, 1991a). In contrast, our results are inconsistent with other published reports indicating
complete homology between predominant CEH isoforms of rat liver and pancreas (Harrison, 1988; Camulli et al., 1989).

The in vivo regulation of rat liver cytosolic 69 kDa CEH and 45 kDa CEH associated with 0-40% and 40-80% ASF, respectively, was investigated under different physiological conditions. Previous studies have suggested that hepatic free cholesterol levels, which are tightly regulated over a wide range of dietary influx, may play a role in the regulation of cytosolic CEH. Feeding of cholesterol and saturated fat rich diet for three weeks was associated with severely increased hepatic level of total cholesterol (TC), esterified cholesterol (EC) and free cholesterol (FC) and a significant decline of microsomal HMG-CoA reductase and cytosolic, and partially purified 69 kDa CEH activities, whereas, the activity of partially purified 45 kDa CEH remained unaffected. The results provide evidence that reduction in the protein mass of 69 kDa CEH may be responsible for cholesterol-mediated down-regulation of enzyme activity. In dietary induced hyperlipidemia, increased cellular FC pool would trigger compensatory responses for the suppression of CEH and HMG-CoA reductase, and activation of acyl-CoA cholesterol: acyltransferase (ACAT). These results are consistent with previously published report that feeding of cholesterol and saturated fat to rats, down-regulates the enzyme activity of cytosolic high molecular form of CEH (Grogan et al., 1991). Eight days after the withdrawal of atherogenic diet (AD), hepatic TC, EC and FC levels were normalized as a consequence of which the inhibition of HMG-CoA reductase and CEH was relieved and enzyme activities and protein mass were restored to normal levels.

Feeding of tocotrienols as TRF to normal rats for one week was associated with a significant decline in the activities and protein mass of HMG-CoA reductase and 69 kDa CEH. The enzyme activity and protein mass of low (45 kDa) molecular form of CEH remained unaffected in TRF-treated rats. The results provide evidence that tocotrienols being powerful hypocholesterolemic agents, inhibit HMG-CoA reductase and CEH activities by reducing the protein mass.
As discussed above, feeding of AD for 3 weeks to rats induced hyperlipidemia. However, feeding of TRF along with AD was associated with further down-regulation of the activity of 69 kDa CEH (0-40% ASF). The results are consistent with the known anticholesterol property of tocotrienols. The results demonstrate that 8 days after the withdrawal of AD, the 69 kDa CEH activity and protein mass were restored to normal levels, whereas, the HMG-CoA reductase activity and protein mass remained inhibited, though to a lower extent. The TC, EC and FC levels remained elevated in comparison to normal levels after 8 days of withdrawal of AD. TRF treatment to HLP rats for 8 days was associated with further reduction in the activities and protein mass of both 69 kDa CEH and HMG-CoA reductase, in comparison to HLP-control rats. In addition, the levels of TC, EC and FC were reduced to normal values. Feeding of AD tends to load the liver with excess cholesterol stored as CE, and decreases the activity of HMG-CoA reductase and cytosolic CEH. The increase in the CE content of the liver in the rats shows that this occurred in the present experiments. Since there is such a substantial cholesterol supply to the liver, esterification of cholesterol dominates over the hydrolysis. The results are consistent with a substantial increase in EC fraction of TC in the liver. Eight days after the withdrawal of AD, the cellular cholesterol content was significantly reduced but remained elevated (~40%) in comparison to normal control. Consistent with this observation, HMG-CoA reductase activity and protein mass remained inhibited (25%) in comparison to normal control rats. In response to above situation, CEH, being a secondary regulatory enzyme in the overall maintenance of cellular cholesterol homeostasis, its activity and protein mass were restored to normal levels. TRF treatment to HLP rats for 8 days, after the withdrawal of AD, was associated with a decline (60%) in HMG-CoA reductase activity and protein mass. CEH activity and protein mass was also reduced which appears to be in response to elevated (25%) FC content in TRF treated HLP rats. The combined results provide evidence that tocotrienols act as potent hypocholesterolemic agent by inhibiting both HMG-CoA reductase and CEH activities in HLP
rats. The TRF-mediated down-regulation of these two enzymes is partly due to reduction in their protein mass.

The results demonstrate that fasting in rats is associated with a significant elevation in the enzyme activity of cytosolic and 69 kDa CEH (0-40% ASF) which may be due to increase in protein mass. Since there is no increase in the protein mass of 45 kDa CEH (40-80% ASF), a significant increase in 18% in the enzymatic activity in fasted rats remains unexplained. A substantial activation (2 fold) of 69 kDa CEH in response to fasting is due to severe inhibition of HMG-CoA reductase activity (Mitropoulous, 1983) thus causing a severe depletion of cellular FC content as a consequence of which, the cellular FC demand is met by the activation of cytosolic CEH, which continues to contribute to the increased cellular need.

Lovastatin (mevinolin) administration also substantially stimulated the induction of cytosolic 69 kDa and 45 kDa CEH, thus mobilizing EC to enter the FC pool. The results demonstrate that part of activation of 69 kDa CEH is due to lovastatin-mediated increase in its protein mass. These results are consistent with the earlier report where simvastatin, an analogue of lovastatin, administration to rats enhanced HMG-CoA reductase and CEH activities while inhibiting ACAT. The combined effect of increased CEH and decreased ACAT activity caused a significant increase in FC content of the liver (Shand and West, 1995).

Cholestyramine administration, by promoting fecal loss of bile acids (Goldfarb and Pitot, 1972; Hardgrave et al., 1979), creates a cellular need for FC to replace cholesterol utilized for bile acid synthesis (Spady et al., 1985) which is apparently met by the induction of microsomal HMG-CoA reductase (Spady et al., 1985; Goldstein and Brown, 1984; Tanaka et al., 1982) and by inhibition of its degradation (Edwards et al., 1983b). However, our data indicate that this FC requirement could also be partially met by an increase in CE hydrolysis via the enhancement of cytosolic 69 kDa and 45 kDa CEH activities. The up-regulation of CEH activity can be explained by a significant increase in 69 kDa CEH protein
mass. These results are in agreement with earlier report where cholestyramine administration to rats was shown to increase the CEH activity and reduce ACAT activity (Shand and West, 1995). As discussed above, administration of cholestyramine together with lovastatin would deplete the cholesterol level to a greater extent than observed in response to individual drugs. As a consequence of this, a substantial increase in the cytosolic 69 kDa and 45 kDa CEH activity and protein mass was observed, the net result of which would be the increased FC levels in the cell. Our results, indicating an increase in CEH activity, are supported by previously published report where the administration of cholestyramine and simvastatin caused a marked induction of HMG-CoA reductase and cytosolic CEH activities. However, the mechanism of CEH activation was not investigated (Shand and West, 1995). The reduction in ACAT activity following this treatment, which was even greater than that observed in the response to the individual drugs, ensured that newly synthesized cholesterol arising from the induced HMG-CoA reductase was not converted to the ester. This strategy was supported by greater than five fold increase in cytosolic CEH activity, the net result of which should have been increased FC levels in the cell (Shand and West, 1995).

Four h after the administration of mevalonolactone (MVL) to rats was associated with a 40% increase in enzymatic activity of both cytosolic and partially purified 69 kDa CEH. However, the protein mass of 69 kDa CEH showed a significant but small increase of 11%. No significant increase was seen in activity and protein mass of 45 kDa CEH. The 40% activation of 69 kDa CEH can be explained in part by an 11% increase in protein mass, whereas, bulk of the increase in CEH activity is due to interconversion of inactive to active form of CEH involving reversible phosphorylation (Khan, 1999). It has been previously established that 1-4 h administration of MVL to rats was associated with long-term regulation of HMG-CoA reductase activity involving the inhibition of its synthesis (Beg et al., 1987). In contrast to HMG-CoA reductase, delayed effect of MVL on CEH activity, involving both short and long-term control, is due to secondary effect
because, increased CEH-mediated hydrolysis of CE is dependent on increased cellular FC demand. These results represent an initial demonstration of MVL-mediated \textit{in vivo} modulation of cytosolic CEH activity.

In summary, identification, characterization, \textit{in vitro} and \textit{in vivo} regulation of a new rat liver cytosolic minor low (45 kDa) molecular form of CEH represents an initial demonstration. As discussed earlier, the specificities for substrates and kinetic properties including $K_m$, temperature and pH dependency, bile salt-mediated activation and PMSF-mediated inhibition of 45 kDa CEH are quite similar in comparison to that of well established predominant 69 kDa CEH activity. However, based on \textit{in vitro} results, it can be concluded that in contrast to cytosolic high (69 kDa) molecular form of CEH, cytosolic low (45 kDa) molecular form of CEH is not subject to modulation involving phosphorylation and dephosphorylation reaction sequence. Immuno-characterization of cytosolic 45 kDa and 69 kDa molecular forms of CEH revealed that 45 kDa CEH is structurally and immunologically different from 69 kDa CEH. On the contrary, the results suggest a high degree of similarity between rat liver cytosolic 45 kDa and CEH of rat and bovine pancreas. The combined \textit{in vivo} results demonstrate that under extreme physiological stress such as prolonged fasting and administration of cholestyramine, lovastatin and cholestyramine plus lovastatin to rats, newly identified minor 45 kDa cytosolic CEH becomes regulatory. The mechanisms of up-regulation of rat liver cytosolic 45 kDa CEH in the above manipulations remain to be investigated.

In earlier reports, particularly in the rat, it had been thought that CE hydrolysis plays a relatively insignificant role in the balance of cholesterol in the liver. However, the present study shows that a variety of dietary manipulations which alter the flux of cholesterol across the liver, have significant effects on the activity of CE hydrolases. These CEH enzyme activities must, therefore, be taken into account when considering the response of the liver to the changes in cellular cholesterol pool.
Based on the combined results, presented in the thesis and other studies conducted in our laboratory (Khan, 1999), it can be concluded enzymatic activity of predominant cytosolic 69 kDa CEH is subject to regulation by both reversible phosphorylation and long-term control involving the modulation of CEH activity by changes in enzyme concentration through alteration in synthesis and/or degradation. In addition, under increased physiological stress, when the cellular cholesterol need is extremely high, the enzymatic activity of newly discovered, cytosolic minor 45 kDa CEH isoform is also regulated involving long term control only. In the overall regulation of cholesterol metabolism, the esterification of cholesterol is catalyzed by microsomal ACAT, the conversion of cholesterol to bile acids by cholesterol 7α-hydroxylase (C7αH), the hydrolysis of esterified cholesterol by CEH and de novo synthesis of cholesterol by HMG-CoA reductase. There is substantial evidence for long-term regulation of HMG-CoA reductase and C7αH by changes in enzyme concentration through transcriptional and post-transcriptional modifications as well as degradation (Faust et al., 1982; Mitropoulous, 1983; et al., 1983; Gibbons, 1983; Edwards et al., 1983c; Sinensky and Logel, 1983; Liscum et al., 1983b; Clarke et al., 1983; Jelinek et al., 1990; Ness et al., 1990; Li et al., 1990; Pandak et al., 1991). Although, in vivo long-term regulation of enzymatic activities of ACAT and cytosolic CEH has been reported (Drevon et al., 1980; Stange et al., 1983; Grogan et al., 1991; Shand and West, 1995), little data is available on the mechanisms of regulation of rat liver ACAT and CEH (Natarajan et al., 1996b; Natarajan et al., 1997). Our results provide evidence for the in vivo long-term control involving modulation of CEH activity by alteration in its protein mass under variety of physiological conditions. Based on these observations, it appears that there is a coordinate control of these four enzymes in cholesterol metabolism by changes in their protein synthesis and/or degradation. Under conditions of decreased cholesterol need such as diet induced lipidemia, HMG-CoA reductase protein mass and activity would be inhibited and the free cholesterol pool would be depleted due to increased protein mass and activities of C7αH and
ACAT. CEH activity would then be reduced due to reduction in its protein mass which in turn would maintain the free cholesterol level in the cell (Figure 32). In contrast, under increased cholesterol need, the long-term control would trigger an increase in HMG-CoA reductase activity due to increase in protein mass. On the other hand, both C7αH and ACAT activities will remain inhibited due to reduction in their protein mass. CEH enzyme will then be activated due to increased protein mass and would become important in maintaining the pool of metabolically active free cholesterol in the cell (Figure 32). However, the mechanism(s) by which cellular cholesterol levels might affect the enzymatic activities by alteration in protein mass of these enzymes in a coordinated fashion remains to be investigated. It is possible that the long-term regulation responds to the nutritional status or other humoral factors affecting hormone levels, rather than to cholesterol levels per se.

Knowledge of the molecular mechanisms involved in the regulation of cytosolic CE hydrolases may provide new insight into the coordinate regulation of cholesterol homeostasis. This will provide an enhanced capability for the rationale design of potential drugs in the therapy and management of hypercholesterolemia and premature cardiovascular diseases.
A. Cholesterol Excess (Decreased Cholesterol Need)

1. ↓ HMG-CoA Reductase Activity and Mass
2. ↑ 7α-Hydroxylase Activity and Mass
3. ↑ ACAT Activity and Mass
4. ↓ CEH Activity and Mass

B. Cholesterol Deprivation (Increased Cholesterol Need)

1. ↑ HMG-CoA Reductase Activity and Mass
2. ↓ 7α-Hydroxylase Activity and Mass
3. ↓ ACAT Activity and Mass
4. ↑ CEH Activity and Mass

FIG. 32. PROPOSED LONG-TERM REGULATORY ADJUSTMENTS (ARROWS) INVOLVING MODULATION OF ENZYME CONCENTRATION BY CHANGES IN PROTEIN SYNTHESIS AND/OR DEGRADATION OF RAT LIVER CYTOSOLIC CHOLESTERYL ESTER HYDROLASES IN CONCERT WITH OTHER KEY ENZYMES IN CHOLESTEROL METABOLISM, IN CHOLESTEROL EXCESS (A) OR CHOLESTEROL DEPRIVATION (B).