STUDIES ON CHOLESTEROL ESTER HYDROLASE

by

ALI M.S. GORBAN

Thesis submitted for the degree of
Doctor of Philosophy in the
University of Edinburgh

Department of Biochemistry
University of Edinburgh

June, 1980
This thesis was composed by myself and the results therein are the product of my own work.
TO

MY PARENTS
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ABSTRACT OF THE THESIS

The adrenal cortical cytosol was shown previously to contain an ACTH-activatable cholesterol ester hydrolase. This enzymic activity was purified from bovine adrenal cortex, and was shown to be activated in vitro by a cAMP-dependent protein kinase and the activation was shown to occur through a protein phosphorylation reaction. The site of the phosphorylation of cholesterol ester hydrolase and its specificity towards substrates other than cholesterol esters was not known.

The aim of the present study was to investigate the specificity of adrenal cholesterol ester hydrolase towards esters other than cholesterol esters especially glycerol esters. The object was to purify adrenal cholesterol ester hydrolase to a degree that might permit the localisation of the phosphorylation site on the enzyme. From these studies it was hoped to assess the distribution of activatable cholesterol ester hydrolase in several mammalian tissues that utilise cholesterol as an important metabolite.

In rat adrenal an ACTH-activatable triacylglycerol lipase was found, and this enzymic activity exhibited a remarkable similarity to cholesterol ester hydrolase in term of being activated and inactivated in vitro by cAMP and Mg++ catalysed reactions respectively. Purification of cholesterol ester hydrolase from bovine adrenocortical cytosol resulted in a parallel purification of triacylglycerol lipase. These two hydrolytic activities were not separated by the purification methods used nor were they differentiated by inhibition studies. The phosphorylation of the purified preparation of cholesterol ester hydrolase and triacylglycerol lipase by cAMP-dependent protein kinase was accompanied by activation of both hydrolytic activities. However, the site of phosphorylation of the enzyme complex was hampered by finding several different phosphopeptides which complicated the interpretation of the results. From these studies
it was concluded that either cholesterol ester hydrolase and triacylglycerol lipase activities represent two enzymes that are related to each other and have similar binding properties or these enzymic activities reside in a single enzyme.

Rat adipose tissue cytosol was found to contain an ACTH-activatable cholesterol ester hydrolase which was similar to triacylglycerol lipase in term of being both sensitive to lipolytic hormones, epinephrine and ACTH, and this hormonal stimulation was blocked both in vivo and in vitro by prior administration of nicotinic acid, a known in vivo inhibitor of lipolysis.

The high cholesterol ester content of ovarian tissue encouraged investigation also. Immature female rats injected with Pregnant Mare's Serum gonadotrophin (PMSG) respond by ovulation and the production of luteinised ovaries. LH administration in vivo to such PMSG-primed rats resulted in the stimulation of progesterone synthesis and secretion from the ovaries of these rats. However, this progesterone stimulation was not correlated with a decrease of ovarian free and esterified cholesterol. Moreover, cytosolic cholesterol ester hydrolase in the ovaries of the LH treated rats was not stimulated. However, cholesterol ester hydrolase was activated in vitro by cAMP by a process possibly involving a cAMP-dependent protein kinase. In this case a phosphorylation-dephosphorylation mechanism did not seem to be involved in the regulation of ovarian cholesterol ester hydrolase in this animal model, which was used to simulate the mature female rats ovulation system.

The rat responds to a dietary load of cholesterol by storing some of this dietary supplement as cholesterol esters in the liver. Studies on rat liver revealed that the cytosolic cholesterol ester hydrolase is apparently not hormone-activatable in vivo nor protein kinase-activatable in vitro. The cytosolic cholesterol ester hydrolase was not affected
also by feeding rats a high-cholesterol diet a situation that stimulates bile acid production in the liver. These results ruled out the phosphorylation of liver cytosolic cholesterol ester hydrolase as a mechanism of its regulation and cast doubt about the importance of this enzyme in regulating liver esterified cholesterol.
ABBREVIATIONS

The following abbreviations are used throughout the text:

ACTH - Adrenocorticotropic hormone
ATP - Adenosine-5'-triphosphate
Cholesterol - 5-Cholesten-3β-ol
cAMP or cyclic AMP - Cyclic adenosine-3',5'-monophosphate
cGMP or cyclic GMP - Cyclic guanosine-3',5'-monophosphate
EDTA - Ethylenediamine tetra-acetic acid
h - Hour(s)
hCG - Human chorionic gonadotrophin
KRB - Krebs-Ringer bicarbonate buffer pH 7.4
KRBB - Krebs-Ringer bicarbonate buffer pH 7.4, containing bovine serum albumin (10 mg/ml)
LH - Luteotrophic hormone, Luteinising hormone
min - Minute(s)
NAD - Nicotinamide adenine dinucleotide
NADH - Reduced nicotinamide adenine dinucleotide
NADP - Nicotinamide adenine dinucleotide phosphate
NADPH - Reduced nicotinamide adenine dinucleotide phosphate
Progesterone - Pregn-4-ene-3,20-dione
Pregnenol - Pregn-5-ene-3β-ol
Pregnenolone - 3β-Hydroxy-pregn-5-ene-20-one
PMSG - Pregnant mare's serum gonadotrophin (follicle stimulating hormone)
S.D. - Standard deviation
S.E.M. - Standard error of the mean
S.D.S. - Sodium dodecylsulphate
Enzymes

-Acyl-CoA-cholesterol acyltransferase (ACAT) (EC 2.3.1.26).

-Adenylate cyclase or adenyl cyclase (EC 4.6.1.1).

-Alkaline phosphatase (EC 3.1.31).

-Cholesterol ester hydrolase or sterol ester acylhydrolase (EC 3.1.1.13).

-Cholesterol 7α-hydroxylase (EC 1.14.13.17).

-Cyclic nucleotide phosphodiesterase or 3':5'-cyclic-nucleotide 5'-nucleotidohydrolase (EC 3.1.4.17).

-Hydroxymethylglutaryl-CoA reductase (HMG-CoA reductase) (EC 1.1.1.34).

-Phosphoprotein phosphatase or phosphoprotein phosphohydrolase (EC 3.1.3.16).

-Phosphorylase kinase or ATP:phosphorylase phosphotransferase (EC 2.7.1.38).

-Phosphorylase kinase phosphatase (EC 3.1.3.-).

-Protein kinase or ATP:protein phosphotransferase (EC 2.7.1.37).

-Triacylglycerol lipase (hormone-sensitive lipase) or triacylglycerol acylhydrolase (EC 3.1.1.3).

Statistical analysis

Where indicated, the statistical significance of the difference was tested using the Student t test. Unless otherwise indicated, the difference between groups was significant p<0.05.
ACKNOWLEDGEMENTS

I would like to thank Professor G.S. Boyd for his advice and enthusiastic supervision throughout my study in the Department of Biochemistry.

My thanks are due to my past and present colleagues in the MRC sterol metabolism group for the discussion and criticism of my work. I would like to thank also Miss M.E. Lawson for her technical assistance and for typing most of the first draft of this thesis.

The work on ovarian cholesterol ester hydrolase described in Section VII was carried out in collaboration with Dr. K.M. Henderson. My thanks are due to Dr. G.W. Pettigrew for advice and the facilities he provided during the peptide mapping experiments described in Section V. The work of this thesis was supported by an MRC Programme Grant. My thanks are due to Miss H.M. Scott for her excellent typing of the final draft of this thesis.

I am grateful to my wife Khairah, whose patience and encouragement throughout my study in Edinburgh University made this work possible.

This work was performed while I was in receipt of a studentship from Riyadh University, Riyadh, Saudi Arabia.
## SECTION I

### GENERAL INTRODUCTION

1.1 The action of ACTH on the adrenal cell

1.2 Initial mechanism of cAMP

1.3 Protein kinase

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1.5 Cholesterol ester hydrolase

1.6 Deactivation of cholesterol ester hydrolase and the role of phosphoprotein phosphatase

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SECTION I: GENERAL INTRODUCTION

Steroid hormones are produced in the adrenals, gonads and placenta. In the process of steroidogenesis, cholesterol plays an important part as the sole precursor of the steroid hormones (Sandor, Fazekas and Robinson (1976), Simpson and Mason (1979), Dufau and Catt (1978)).

The tissues involved in steroidogenesis contain mitochondria capable of effecting a transformation of cholesterol into the key intermediate in steroid hormone production, pregnenolone (Schulster, Burstein and Cooke (1976), Simpson (1979)). This reaction is termed the sterol side chain cleavage reaction and it occurs in the inner cristae of the mitochondria. In the adrenal, the cholesterol side chain cleavage reaction occurs as a result of sequential hydroxylations in the sterol side chain at C\textsubscript{22}, followed by hydroxylation at C\textsubscript{20} and finally a splitting of the cholesterol side chain between C\textsubscript{20} and C\textsubscript{22} to yield pregnenolone and isocaproic aldehyde (McKerns (1968), Gower (1975), Burstein, Kimball and Gut (1970)).

In the adrenal cortex, pregnenolone produced in the mitochondria is subsequently modified in the endoplasmic reticulum by 17α-hydroxylation and or 21-hydroxylation and by oxidation of the 3β-hydroxysteroid Δ-5 structure to 3-keto Δ-4 structure. It is therefore possible within the endoplasmic reticulum of the adrenal cortex to convert pregnenolone into deoxycortisol or deoxycorticosterone, the production of these steroids is in part dependent upon the species (Sandor, Fazekas and Robinson (1976)). Deoxycortisol and deoxycorticosterone may be then transported back into the mitochondria for 11β-hydroxylation or in some cases for 11β-hydroxylation and 18-hydroxylation. The role of steroid hormones in the control of carbohydrate, protein and lipid metabolism as well as in electrolytes and water balance has been the subject of recent reviews (Martin (1976), Gower (1979), Finkelstein and Shafer (1979)).
1.1 THE ACTION OF ACTH ON THE ADRENAL CELL

It is now well established that the process of steroidogenesis in the adrenal cortex is markedly stimulated by ACTH (for reviews see Schulster (1974), Halkerston (1975), Haynes (1975), Urquhart (1974) and Gill (1979)). The link in the chain, between the interaction of ACTH with the plasma membrane of the adrenal cell and the ultimate increase in output of steroid hormones by the adrenal cells has been the subject of intensive research in the past two decades. This subject was further stimulated by the discovery that cyclic AMP, a nucleotide subsequently found to be ubiquitous, was also involved in steroidogenesis. Haynes (1958) found that upon the incubation of beef adrenocortical slices with ACTH there was a marked increase in cAMP concentration in this tissue. Subsequently, it was shown that the addition of cAMP in vitro to rat adrenal slices resulted in an increase of corticoid production in this tissue which was similar to the effect of ACTH on these slices (Haynes, Koritz and Peron (1959)). These observations which were confirmed by several studies (see Halkerston (1975)) laid down the foundation of the role of cAMP as the intracellular second messenger for the interpretation of the ACTH signal and its transmission to the remainder of the steroid hormone synthesis machinery in the adrenal cell.

The stimulation of adrenal steroidogenesis by ACTH does not require the entrance of the ACTH-polypeptide molecule into the adrenal cell. The coupling of the ACTH molecule to inert large molecular weight polymers such as agarose, cellulose and polyacrylamide resulted in a molecule incapable of entering the adrenal cell but retaining the steroidogenic potency of the free ACTH molecule (Schimmer, Ueda and Sato (1968), Selinger and Civen (1971), Richardson and Schulster (1972)). These studies emphasised the existence of a specific receptor or receptors on the external surface of the adrenal cell plasma membrane capable of
identifying specific aspect of the ACTH molecule. Moreover, of the 39 amino acids that constitute the single polypeptide molecule of ACTH (Li (1962), Schwyzer (1977)) only the N-terminal 24 amino acids are required for the production of the adrenal cell steroidogenesis response (Grahamme-Smith, Butcher, Ney and Sutherland (1967), Seelig and Sayers (1973), Seelig, Kumar and Sayers (1973)). The interaction of ACTH with the plasma membrane receptor on the adrenal cell may involve activation of membrane phospholipase, alteration to the membrane phospholipids and changes in the sequestration of membrane calcium (Rubin and Laychock (1978)). This hormone-receptor interaction from the outside of the adrenal cell is transmitted to the inner aspects of the plasma membrane, by a process which may involve GTP and a GTP-binding regulatory unit similar to those described by Rodbell (1978), resulting in the activation of adenylate cyclase located in the inside of the plasma membrane (Lefkowitz, Roth, Pricer and Pastan (1970), Schlegel and Schwyzer (1977), Ontjes, Ways, Mahafee, Zimmerman and Gwynne (1977)).

The exposure of the adrenal cell to ACTH either in vivo or in vitro results in a rapid increase of the intracellular concentration of cAMP (Grahamme-Smith, Butcher, Ney and Sutherland (1967); Beall and Sayers (1972); Mackie, Richardson and Schulster (1972)). Within 5 min there may be a 5 fold increase in the concentration of cAMP in the ACTH-activated adrenal cell. By contrast, changes in the concentration of cyclic GMP in the adrenal cells is very slight (Laychock and Hardman (1978); Saez, Evain and Gallet (1978); Podesta, Milani, Steffen and Neher (1979)). This observation supports the generally accepted concept that it is cAMP rather than cGMP which is the key secondary messenger in the adrenal cell (Lincoln and Corbin (1978)).
1.2 INITIAL MECHANISM OF CAMP ACTION

The stereospecific binding of ACTH to the receptor of the adrenal cell plasma membrane with the resultant activation of adenylate cyclase is associated with the increase of intracellular concentration of cAMP. The rise of cAMP concentration in response to ACTH action on the adrenal cell was found to precede the rise in steroid hormone output (Beall and Sayers (1972); Podesta, Milani, Steffen and Neher (1979)). The stimulation of adrenal steroidogenesis by cAMP (Haynes, Koritz and Peron (1959)) promoted research towards finding the mechanism by which cAMP may stimulate adrenal steroidogenesis (see Halkerston (1975)). In this respect, studies on the possible receptor(s) for cAMP in the adrenal cell revealed the presence of a protein receptor which binds cAMP, and the bulk of this receptor was found in the cell cytosol and the microsomes. Moreover, studies on the binding of various nucleotides to the protein receptor showed that the binding was relatively specific for cAMP (Gill and Garren (1969)). The cAMP-protein receptor interaction does not involve any modification of cAMP, and it was postulated that cAMP may modify the protein receptor via nucleotide interaction.

1.3 PROTEIN KINASES

The finding that epinephrine, through increased cAMP concentration in the rat skeletal muscle, resulted in activation of phosphorylase kinase led to the discovery of the rabbit skeletal muscle cAMP-dependent protein kinase (Walsh, Perkins and Krebs (1968)). This kinase was shown to activate and phosphorylate purified phosphorylase kinase in vitro in a reaction dependent on cAMP. Similarly, a protein kinase from bovine adrenocortical cytosol was identified and it was shown that its activity was enhanced by cAMP (Gill and Garren (1970)). Although other cyclic nucleotides mimicked cAMP in activating the adrenocortical protein kinase, the concentration of these nucleotides was required to be supraphysiological
at about $2 \times 10^{-4}$ M, in contrast to the concentration of cAMP required
($1 \times 10^{-7}$ M) to affect maximal stimulation of the protein kinase.
The finding of this cAMP activatable protein kinase in the adrenal cortex
initiated the attempt to separate this kinase from the previously
identified cAMP-binding protein from the adrenal cortical cytosol.
After extensive purification, it was clear that the cAMP-dependent protein
kinase retained significant cAMP-binding activity, and both activities
from the adrenal cytosol were enriched in parallel (Gill and Garren (1970)).

The association of the cAMP-binding protein with the catalytic unit of
protein kinase resulted in the suppression of the protein kinase activity.
However, the addition of cAMP to the suppressed protein kinase activity
resulted in an activation of the enzyme and a partial separation of the
cAMP-binding protein from the enzyme (Gill and Garren (1971)). Thus,
the activation of protein kinase by cAMP was due to the binding of cAMP
to its binding protein; the interaction of cAMP with its receptor
results in the removal of the suppression of protein kinase. Subsequently,
the cAMP-binding protein was referred to as the receptor subunit, while
the protein kinase was referred to as the catalytic subunit of the
holo cAMP-dependent protein kinase complex. This complex, as identified
by ultracentrifugal and electrophoresis studies, contains both the
receptor and the catalytic subunits. Addition of cAMP to the complex
results in the binding of cAMP to the receptor, which in turn causes the
dissociation of the receptor from the catalytic subunit. Consequently,
the liberated kinase is more active when it is freed from the receptor
subunit (Gill and Garren (1971)).

The free catalytic subunit of cAMP-dependent protein kinase, catalyses
the transfer of the terminal phosphate group of ATP to various protein
acceptors. Such a theory is consistent with the evidence that the
diverse manifestations of cAMP in different tissues result from tissue-
specific proteins phosphorylated by ATP in the presence of the activated
protein kinase (see Lincoln and Corbin (1978)). Therefore, there is a
need to identify which protein or proteins are phosphorylated in the
adrenal cortical cells in the chain of events linking the interaction of
ACTH with the plasma membrane of the cell to the final step of steroid
hormones secretion from the adrenal cell. In this respect, it was shown
in the bovine adrenal cortex that a cAMP-dependent protein kinase was
capable of phosphorylating ribosomes. Assuming that cAMP-dependent
protein kinase plays a key role in the response of adrenal cortical cell
to ACTH action and hence to elevated cAMP concentrations, theories were
advanced to link the requirement for protein synthesis associated with
the mode of action of ACTH with this protein kinase reaction. It was
suggested that cAMP-dependent protein kinase might be involved in the
phosphorylation of ribosomes which, in turn, might affect the translation
of messenger RNA (reviewed by Gill (1979)).

Beside the cAMP-dependent protein kinase, the bovine adrenal cortex
also possesses a cAMP-independent protein kinase with casein as a better
phosphate acceptor rather than histone (Cochet, Job and Chambaz (1977a)).
There is also in the adrenal cortex another cAMP-independent protein kinase
which utilizes either ATP or GTP as a phosphate donor (Job, Cochet,
Pirollet and Chambaz (1979)). The role of these kinases thus far, is not
known.

1.4 PROTEIN PHOSPHORYLATION

Although protein modification by phosphorylation has been known for
a long time, the subject gained momentum after the discovery of cAMP-
derpendent protein kinase. The momentum in this aspect of biochemistry
is such that even the most recent reviews on the subject are out of
date before publication (Weller (1979), Cohen (1980)). A considerable
number of enzymes are known to be modified by phosphorylation such as
phosphorylase b kinase, glycogen synthase, hormone-sensitive lipase,
pyruvate kinase and others. In addition, there is evidence for the
phosphorylation of histone, troponin and various protein substrate of
unknown function (for reviews see Nimmo and Cohen (1977), Weller (1979), Krebs and Beavo (1979)). The growing number of enzymes that are reported to undergo phosphorylation reactions catalysed by cAMP-dependent protein kinase called for attempts to define criteria that should be satisfied to establish that a given enzyme undergoes physiologically significant phosphorylation-dephosphorylation. Of these attempts, Krebs (1972) formulated several criteria which he later modified (Krebs and Beavo (1979)). The modified criteria can be summarised as follows. The enzyme in question should be phosphorylated in vitro at a significant rate in a protein kinase mediated reaction; the enzyme should be dephosphorylated by a phosphoprotein phosphatase; the enzyme should also show significant changes in its functional properties and these should correlate with the degree of phosphorylation. Furthermore, the most difficult criterion which only has been met by very few enzymes, so far, demands that the phosphorylation-dephosphorylation of the particular enzyme can occur in vivo and correlate with functional changes. The phosphorylation-dephosphorylation of the enzyme should also correlate with the cellular concentration of protein kinase and phosphoprotein phosphatase.

The widespread distribution of cAMP-dependent protein kinases has suggested that such a protein kinase may mediate all the actions of cyclic nucleotides in the living cell (Lincoln and Corbin (1978)). Such a concept implies that important control events in the living cells are exerted through phosphorylation reactions. In the adrenal cortex, in attempts to interpret how cAMP may modify the production of steroid hormones via protein phosphorylating events, none of the known enzymes modified by phosphorylation was deduced to be a possible candidate as an important key controlling enzyme in steroidogenesis. This prompted the studies on the cholesterol ester hydrolase and its possible phosphorylation (Simpson, Trzeciak, McCarthy, Jefcoate and Boyd (1972)).
1.5 CHOLESTEROL ESTER HYDROLASE

It is known that when animals are subjected to stressful situations, or when ACTH is administered to animals, there is a reduction in the cholesterol content of the adrenal cortex (Sayers, Sayers, Liang and Long (1946), Davis and Garren (1966), Boyd and Trzeciak (1973)). This drop in the cholesterol content of the adrenal is almost entirely due to a decrease in the esterified cholesterol (Davis and Garren (1966)). In the cytosol of the rat adrenal cells, 75% of the total adrenal cholesterol was found in the lipid droplets, and more than 85% of this cholesterol was found to be esterified to long chain unsaturated fatty acids (Sand, Fruhling, Penasse and Calude (1972), Beckett and Boyd (1975)). In these cells, the cholesterol esters appear to be mobilised as a result of ACTH stimulation of the adrenal cell (Davis and Garren (1966)). Moreover, the mobilisation of esterified cholesterol by ACTH does not seem to be affected by prior injection of the animal with cycloheximide, a protein synthesis inhibitor which blocks the ACTH stimulation of adrenal steroidogenesis. Subsequently, it was shown that upon ACTH administration to rats, 15 min before the adrenals were removed, there was an activation of cholesterol ester hydrolase in the adrenal homogenate (Shima, Mitsunaga and Nakao (1972)). Examination of the adrenal cholesterol ester hydrolase in the cell fractions revealed that the activity of the enzyme was associated with the mitochondrial, lysosomal, microsomal and cytosolic fractions. However, more than 60% of the total cholesterol ester hydrolase in the adrenal cell was found in the cell cytosol (Trzeciak and Boyd (1973)). This soluble cholesterol ester hydrolase in the adrenal cortex is interesting because it is associated with the cell fraction that also contains the cholesterol ester-laden lipid droplets described above.

Ether anaesthesia stress, which is known to increase the ACTH concentration in the blood (Matsayama, Ruhmann-Wennhold and Nelson (1971),
Cook, Greer and Kendall (1972)), has been shown to produce cholesterol ester depletion in the rat adrenal and an increase in the activity of soluble cholesterol ester hydrolase (Boyd and Trzeciak (1973)). Injection of cycloheximide to rats did not change significantly the adrenal free and esterified cholesterol stored in the lipid droplets. However, administration of cycloheximide to rats prior to ether anaesthesia stress resulted in an increase of free cholesterol with a concomitant decrease of cholesterol ester concentration within the adrenal lipid droplets (Boyd and Trzeciak (1973)). In both cases, cycloheximide did not affect cytosolic cholesterol ester hydrolase neither did it prevent the ether-induced enhancement of the activity of the cholesterol ester hydrolase by the ether-activated cAMP-dependent protein kinase. These findings were consistent with the previous observations that ACTH-induced accumulation of cAMP in rat adrenal was not affected by prior administration of cycloheximide (Grahamme-Smith, Butcher, Ney and Sutherland (1967)), and also in accordance with that of Davis and Garren (1966) that cycloheximide did not prevent the ACTH-induced depletion of esterified cholesterol in rat adrenals. It seemed from these studies that the interaction of ACTH with rat adrenal cells stimulated cAMP accumulation in these cells and this was associated with the activation of cAMP-dependent protein kinase and cholesterol ester hydrolase. The role of cAMP-dependent protein kinase in the activation of cytosolic cholesterol ester hydrolase in vivo by ACTH was deduced also from the finding that pre-incubation in vitro of adrenal cytosol with cAMP resulted in the activation of cholesterol ester hydrolase (Boyd and Trzeciak (1973)). From these observations and others on bovine adrenocortical enzyme studies (Trzeciak and Boyd (1974)) it was established that activation of adrenal cytosolic cholesterol ester hydrolase was mediated by cAMP-dependent protein kinase. The presence of cAMP and $^{32}P$-ATP resulted in the
activation of cholesterol ester hydrolase concomitant with the association of \( ^{32}P \) radioactivity with a fraction which exhibited cholesterol ester hydrolase activity on subsequent desalting of the activation mixture in a Sephadex G-25 column. Therefore, it was postulated that the activation of cholesterol ester hydrolase by cAMP-dependent protein kinase involved \( ^{32}P \) transfer from ATP (Trzeciak and Boyd (1974)). This transfer of the terminal phosphate of ATP could have been to cholesterol ester hydrolase or to other proteins. However, it was shown subsequently that this \( ^{32}P \) transfer was associated with cholesterol ester hydrolase when Beckett and Boyd (1977) showed that \( ^{32}P \) radioactivity paralleled the cholesterol ester hydrolase activity in all the purification procedure used for the enzyme. Even after polyacrylamide gel electrophoresis, \( ^{32}P \) radioactivity was associated with a protein band which also exhibited an esterase reactive band. The phosphorylation of bovine adrenocortical cholesterol ester hydrolase was suggested as a mechanism that regulates the hydrolysis of adrenal cholesterol esters in response to the interaction of ACTH with the adrenal cells (Beckett and Boyd (1977)). The phosphorylation of cholesterol ester hydrolase in bovine adrenal was also confirmed by Wallat and Kunau (1976) and Naghshineh, Treadwell, Gallo and Vahouny (1978)).

Using a purified preparation of cholesterol ester hydrolase which was shown to be devoid of cAMP-dependent protein kinase it was possible to show that the activation by cAMP of cholesterol ester hydrolase was dependent on the presence of exogenous protein kinase. It was also shown in the same purified preparation of the hydrolase that the activation rate of the enzyme, by cAMP in the presence of the protein kinase and \( \gamma^{32}P \)ATP, was parallel to the rate of transfer of \( ^{32}P \) to the enzyme preparation; both the phosphorylation and activation of cholesterol ester hydrolase was almost complete in less than 10 min.
1.6 DEACTIVATION OF CHOLESTEROL ESTER HYDROLASE AND THE ROLE OF PHOSPHOPROTEIN PHOSPHATASE

When cytosolic cholesterol ester hydrolase from bovine adrenal cortex was incubated with millimolar concentration of Mg$^{++}$ ions for 30 min, it was shown that such an incubation resulted in a deactivation of cholesterol ester hydrolase (Trzeciak and Boyd (1974)). It was postulated that the deactivation of cholesterol ester hydrolase may have been catalysed by phosphoprotein phosphatase, the latter had been found in the adrenocortical cytosol (Merelevede and Riley (1966)) and was known to be activated by Mg$^{++}$ ions. However the role of phosphoprotein phosphatase in the deactivation of adrenal cholesterol ester hydrolase was not confirmed until a purified preparation of the enzyme was obtained. It was shown that the purified cholesterol ester hydrolase was not deactivated when Mg$^{++}$ ions were added to enzyme preparation as opposed to the deactivation of the cholesterol ester hydrolase in crude adrenal preparation (Beckett and Boyd (1977)). On the other hand, the addition of liver alkaline phosphatase or α and β phosphorylase kinase phosphatase of rabbit skeletal muscle to the purified cholesterol ester hydrolase preparation resulted in deactivation of the hydrolase activity, and this reaction was enhanced by Mn$^{++}$ ions in the case of α phosphorylase kinase phosphatase. If however the purified cholesterol ester hydrolase was previously activated and phosphorylated by $\gamma^32P$ATP, and the preparation was desalted then subjected to the phosphatase incubation, there was a simple relationship between the rate of deactivation of cholesterol ester hydrolase and the rate of $^32P$ release from the phosphorylated protein (Beckett (1975)). These findings established a role for phosphoprotein phosphatase as a regulator of cholesterol ester hydrolase. Although the phosphatases used in these studies had not originated from the adrenal cortex it is possible that the Mg$^{++}$-dependent phosphatase found in the
adrenocortical cytosol (Merelevede and Riley (1966)) and which catalysed the deactivation of phosphorylase a, also catalyses the deactivation of cholesterol ester hydrolase. This suggestion is also supported by the reported low specificity of phosphoprotein phosphatases in general (Ullman and Perlman (1975), Burchell, Foulkes, Cohen, Condon and Cohen (1978)) which are defined as multifunctional phosphatases (Krebs and Beavo (1979)).

The activation and phosphorylation of cholesterol ester hydrolase by cAMP-dependent protein kinase, and its deactivation and dephosphorylation by phosphoprotein phosphatase prompted the proposal that cholesterol ester hydrolase through its regulation plays an important role in supplying substrate cholesterol, through the hydrolysis of cholesterol esters which are stored in lipid droplets, to the rate limiting sterol side chain cleavage complex in the adrenal mitochondria (Beckett and Boyd (1977)). This proposal stemmed from the fact that adrenal mitochondria contain only a limited supply of free cholesterol (Arthur and Boyd (1974)) and addition of exogenous substrate to mitochondrial preparation in vitro sustained and prevented the decline of pregnenolone formation from endogenous cholesterol (Boyd, Arthur, Beckett, Mason and Trzeciak (1975)).

1.7 ADIPOSE TISSUE TRIACYLGLYCEROL LIPASE HORMONE-SENSITIVE LI PASE

The effect of ACTH on adipose tissue free fatty acid mobilisation (Hollenberg, Raben and Astwood (1960)) has been long recognised and attention has focussed on triacylglycerol lipase as an important locus in the mobilisation of adipose tissue triglycerides (see review of Hales, Luzio and Siddle (1978)). The role of cAMP as an intracellular agent for transmitting the hormonal signal to the adipose tissue triacylglycerol lipase was known soon after the discovery of cAMP-dependent protein kinase, and it was demonstrated that rat triacylglycerol lipase activation
was linked to this protein kinase (reviewed by Steinberg (1976), Fain (1977)). Later studies showed that a 100 fold purified triacylglycerol lipase from rat adipose tissue cytosol could be activated in vitro by a cAMP-dependent protein kinase. Again such an enzyme activation paralleled the transfer of $^{32}$P from $^{32}$P-ATP to the protein (Huttunen, Steinberg and Mayer (1970)). Pre-incubation of rat epididymal fat pads with epinephrine, followed by subsequent isolation of triacylglycerol lipase from the tissue cytosol, resulted in a sharp reduction of the degree of triacylglycerol lipase activation even when exogenous cAMP-dependent protein kinase was added. The observation was interpreted as due to the in vivo epinephrine-stimulation of triacylglycerol lipase in intact fat pads and was equivalent to the stimulation of the basal triacylglycerol lipase by cAMP-dependent protein kinase in the preparation (Huttunen and Steinberg (1971)).

Studies on chicken adipose tissue triacylglycerol lipase showed that in this tissue, a Mg$^{++}$-dependent phosphoprotein phosphatase was present which catalyses the reversible deactivation of triacylglycerol lipase (Severson, Khoo and Steinberg (1977)). Thus, the hormonal regulation of rat and chicken adipose tissue triacylglycerol lipase (Steinberg (1978)) seems to involve the phosphorylation and dephosphorylation of the lipase by a cAMP-dependent protein kinase and a phosphoprotein phosphatase, the multifunctional phosphatase respectively.

1.8 PROTEIN INHIBITORS AS MODULATORS OF CYCLIC AMP-DEPENDENT PROTEIN KINASE AND PHOSPHOPROTEIN PHOSPHATASE

It was shown that crude rabbit skeletal muscle cAMP-dependent protein kinase was contaminated with a tryptic-labile and heat-stable protein. Upon the separation and purification of this factor it was shown to inhibit cAMP-dependent protein kinase preparations from several tissues (Walsh, Ashby, Gonzalez, Calkins, Fischer and Krebs (1971)). This heat-stable protein, when it was purified to homogeneity from rabbit skeletal
muscle and several other tissues was shown to have a molecular weight of about 11,300, and many tissues contain this inhibitor (Krebs and Beavo (1979)). This inhibitor binds to the catalytic subunit of cAMP-dependent protein kinase with a $K_i$ of about $2 \times 10^{-9} \text{M}$, and it competes with various protein substrates for the active protein kinase. The role of this inhibitor, under physiological conditions is not known but it is thought to inhibit the catalytically active protein kinase when the concentration of cAMP in the tissue is low. However, this inhibitor proved to be useful in the laboratory in distinguishing the cAMP-dependent protein kinases from the cAMP-independent protein kinases.

The cAMP-dependent protein kinase inhibitor was shown to be present in bovine adrenal cortex (Cochet, Job and Chambaz (1977a)). In association with this inhibitor, there was in the crude preparation, a phosphorylatable protein substrate which is phosphorylated by adrenocortical cAMP-dependent protein kinase. It is not known yet what role this phosphorylatable protein from bovine adrenal cortex plays, neither has its identity been established (Cochet, Job and Chambaz (1977b)). From the same group of workers, it was reported that there is, as yet, another heat-stable protein inhibitor in the adrenal cortex, and this inhibitor was shown to be specific for cAMP-independent protein kinase. The latter kinase has been isolated from the adrenal cortex and uses GTP and ATP as phosphate donors while casein serves as a good phosphate acceptor (Job, Cochet, Pirollet and Chambaz (1979)).

In beef adrenal cortex there exists a heat-stable inhibitor of rabbit muscle phosphorylase phosphatase which separated into two forms; inhibitor-1 and inhibitor-2 (Huang, Tao and Glinsmann (1977)). Inhibitor-1 was found to be activated by cAMP-dependent protein kinase, an activation which was accomplished through the phosphorylation of the inhibitor. Upon its activation and phosphorylation, inhibitor-1 inhibited
phosphorylase phosphatase. Moreover, using a perfusion procedure on rat hind limb, it was shown that epinephrine perfusion resulted in the activation of this phosphatase inhibitor concomitant with the inhibition by epinephrine of the phosphorylase phosphatase (Tao, Huang, Lynch and Glinsmann (1978)). Similar inhibitors were isolated from rabbit skeletal muscle, and it was shown that inhibitor-1, but not inhibitor-2 was phosphorylatable by a cAMP-dependent protein kinase. Upon its phosphorylation, inhibitor-1 becomes active and inhibits phosphoprotein phosphatase-1 (formally known as phosphatase-III (Cohen (1978))). This phosphatase, as described earlier in this section, has a low specificity towards phosphorylated proteins and was described by Krebs and Beavo (1979) as a multifunctional phosphatase.

In chicken adipose tissue there is a heat-stable protein inhibitor, that prevents the deactivation of triacylglycerol lipase by Mg\(^{++}\)-dependent lipase phosphatase (Severson and Sloan (1977)). This inhibitor of the lipase phosphatase was isolated from chicken adipose tissue by a procedure similar to that used for the isolation of beef adrenal phosphatase inhibitor (Huang and Glinsmann (1976)) and described above. However, it is not known whether the adipose tissue phosphatase inhibitor is a phosphorylatable protein.

It was shown that inhibitor-1 from rabbit skeletal muscle can be deactivated and dephosphorylated by its own substrate phosphatase-1 (Cohen (1978)). However, beef adrenal phosphatase inhibitor-1 has not so far been studied to find out whether it is under regulation by its own substrate.

1.9. OVARIAN STEROIDOGENESIS AND THE ROLE OF LH IN CHOLESTEROL ESTER MOBILISATION

The stimulatory effect of LH on ovarian steroidogenesis is well documented (see reviews of Savard, Marsh and Rice (1965), Marsh (1975),
Dufau and Catt (1978)). The mechanism by which LH affects ovarian steroidogenesis is thought to be mediated through an increase in intracellular concentration of cAMP (Marsh (1975)), and the rise of the cAMP concentration precedes the rise of progesterone concentration in rat luteal cell dispersions (Sala, Dufau and Catt (1979)). Progesterone synthesis was enhanced when cAMP was added *in vitro* to rat and bovine corpus luteum slices (Hermier and Jutisz (1969), Marsh (1975)).

The administration of LH *in vivo* to pseudo-pregnant immature female rats (Parlow (1958) preparation) resulted in the stimulation of progesterone synthesis in the ovaries of these rats, and this stimulation was concomitant with a decrease of ovarian cholesterol ester concentration (Flint, Grinwich and Armstrong (1973)). The decrease of esterified cholesterol by LH could be due to either an inhibition of cholesterol ester synthase or stimulation of cholesterol ester hydrolase. However, using this experimental animal model, Beckett (1975) showed that LH administration *in vivo* resulted in a stimulation of cytosolic cholesterol ester hydrolase in the ovaries of these PMSG-hCG pre-treated immature female rats. By analogy to the events occurring in the adrenal, and discussed earlier in this section, Beckett (1975) postulated that LH administration *in vivo* stimulated cytosolic cholesterol ester hydrolase in the ovarian tissue of these rats. It was proposed that the activation could be via a mechanism mediated by cAMP-dependent protein kinase and which might involve protein phosphorylation.

By contrast, cholesterol ester hydrolase from bovine corpus luteum slices was not responsive to either LH or cAMP addition to the incubation medium of the slices. Even the addition of luteal cAMP-dependent protein kinase to cytosolic cholesterol ester hydrolase preparation from the bovine corpus luteum did not produce consistent activation of the hydrolase (Goldstein and Marsh (1973)).
Ovine corpus luteum cholesterol ester hydrolase responded to LH and dibutyryl cAMP by increased hydrolysis of emulsified cholesteryl oleate when LH and cAMP were added in vitro to the medium containing the corpus luteum slices (Caffrey, Fletcher, Diekman, O'Callaghan and Niswender (1979)). Moreover, these authors showed that addition of millimolar concentrations of ATP, Mg++ and dibutyryl cAMP to ovine luteal cytosol did not stimulate cholesterol ester hydrolase. However, when l-methyl-3-isobutyl-xanthine, a phosphodiesterase inhibitor, was added to the mixture, a two fold stimulation of cholesterol ester hydrolase was observed.

Finally, a recent report indicates that cytosolic cholesterol ester hydrolase from bovine corpus luteum was stimulated two fold when dibutyryl cAMP, Mg++ and ATP were added to the luteal cytosol in the presence of theophylline (Bisgaier, Treadwell and Vahouny (1979)). This group postulated that cAMP-dependent protein kinase may be involved in the activation of cholesterol ester hydrolase.

1.10 HEPATIC CHOLESTEROL AND CHOLESTEROL ESTER HYDROLASE

The role of cholesterol as the primary precursor of bile salts synthesis and the concerted reactions that occur exclusively in the liver is well documented (Boyd, Hattersley, Mason, Arthur and Beckett (1977)), Danielsson and Sjövall (1975)). The activity of cholesterol-7α-hydroxylase of rat liver, the enzyme responsible for the conversion of cholesterol to 7α-hydroxycholesterol in a reaction considered to be the major rate-limiting step in bile acid production has been shown to be stimulated upon feeding rats a high-cholesterol diet (Boyd, Scholan and Mitton (1969), Danielsson and Sjövall (1975)). Although normal rat liver contains cholesterol mostly in the free form (85% free and 15% esterified), feeding rats cholesterol, results in an increase of the hepatic esterified cholesterol fraction (Gould (1977)). The efficacy of the liver in the rapid uptake of esterified cholesterol from plasma is
well established (Goodman (1962)). This uptake of esterified cholesterol in chylomicron remnants occurs under many physiological states of the liver (reviewed by Sherril (1978)). The rapid uptake and accumulation of esterified cholesterol in the liver initiated the search for the means by which the liver disposes of the excess of cholesterol esters. Cholesterol ester hydrolase in the liver was one of these means, and it was reported that the liver hydrolase is located mainly in the cell cytosol (Deykin and Goodman (1962)). This cholesterol ester hydrolase was reported to be active at neutral pH and preferentially hydrolyses long chain unsaturated fatty acids ester of cholesterol. These authors suggested from their calculations of the hydrolysis of cholesterol ester given to rats as chylomicrons, that the rate of cholesterol ester hydrolysis in vivo agreed with the activity of the cytosolic cholesterol ester hydrolase measured (Deykin and Goodman (1962)).

Because of the water-insoluble nature of the substrate cholesteryl ester, the accurate determination of liver cholesterol ester hydrolase was difficult. Furthermore, the liver was reported to contain cytosolic (Deykin and Goodman (1962)), microsomal (Sakamoto, Okuda and Fujii (1974)) and lysosomal cholesterol ester hydrolases (Stokke (1972), Nilsson, Norden and Wilhelmsson (1973)). These findings on the distribution of liver cholesterol ester hydrolases are complicated by the employment of various substrate presentation methods, which affects the determination of the activity of cholesterol ester hydrolase (Lundberg, Klemets and Lovgren (1979)). Regardless of possible discrepancies about the possible localisation of cholesterol ester hydrolase in liver, none of these reports discussed the regulation of the enzyme. Although the concept of cAMP as the secondary messenger of hormonal signals acting on a tissue was first recognised in the liver (reviewed by Sutherland and Park (1973)), the implication of cyclic nucleotide in the regulation
of cholesterol ester hydrolase in the liver was not investigated. By analogy to the adrenal, all the agents that catalyse protein phosphorylation-dephosphorylation and discussed earlier with reference to the adrenal cortex are also present in the liver, such as cAMP-dependent protein kinases (Yamamura, Takeda, Kumon and Nishizuka (1970)) and phosphoprotein phosphatases (Khandelwal, Vandenheede and Krebs (1976)). There was a possibility that liver cholesterol ester hydrolase might be under hormonal regulation and could involve the phosphorylation-dephosphorylation mechanism similar to the adrenal cholesterol ester hydrolase regulation.

1.11 THE RELATIONSHIP OF TRIACYLGlycerol LIPASE - CHOLESTEROL ESTER HYDROLASE

Adipose tissue triacylglycerol lipase (hormone-sensitive lipase) was purified 100-fold from rat adipose tissue cytosol (Huttunen and Steinberg (1971)) and the enzyme was shown to be under hormonal control through a cAMP-dependent protein kinase reaction. Because of the difficulty in obtaining a homogenous preparation of adipose tissue triacylglycerol lipase, its molecular weight and its subunit structure could not be determined (Steinberg (1978)). The inhomogeneity of the lipase was complicated further when cholesterol ester hydrolase was detected in adipose tissue cytosol, and this enzyme activity apparently co-purified with the triacylglycerol lipase up to the final stage of the purification (Pittman, Khoo and Steinberg (1975)). Furthermore, that group showed in adipose tissue that both hydrolytic activities were stimulated by cAMP-dependent protein kinases, and also stimulated by epinephrine addition to isolated rat adipocytes. However, neither the role of cholesterol ester hydrolase in adipose tissue was characterised nor was the relationship between triacylglycerol lipase and cholesterol ester hydrolase established.
1.12 THE AIMS OF THE PRESENT STUDY

Cholesterol ester hydrolase was found in rat adipose tissue in association with triacylglycerol lipase (Pittman, Khoo and Steinberg (1975)). Cholesterol ester hydrolase of bovine adrenal was purified 57-fold, but it was not known if it was specific towards cholesteryl esters only (Beckett and Boyd (1977)). ACTH was shown to mobilise rat adrenal triglycerides (Tudman and Garcia (1966)). Adrenal cholesterol ester hydrolase was shown to be regulated by ACTH, and this regulation was mediated by cAMP in a phosphorylation-dephosphorylation mechanism catalysed by cAMP-dependent protein kinase and phosphoprotein phosphatase respectively (Beckett and Boyd (1977)). The site of cholesterol ester hydrolase phosphorylation however was not known because of the difficulties in obtaining a homogenous cholesterol ester hydrolase. The regulation of cholesterol ester hydrolase in rat ovary and rat liver was not known. It remained to be clarified whether the control was similar to adrenal hydrolase regulation. From these outlined observations, it was hoped to investigate the following:

(i) To study triacylglycerol lipase in adrenal, and to separate it from cholesterol ester hydrolase.

(ii) To further purify cholesterol ester hydrolase of the adrenal and localise the phosphorylation site of the enzyme if possible.

(iii) To study cholesterol ester hydrolases in the liver and ovary contrasting these enzyme activities with the corresponding enzyme found in the adrenal, with emphasis on the possible role of cAMP in the regulation of the hydrolases in these tissues.
### SECTION II
**MATERIALS AND METHODS**

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SECTION II: MATERIALS AND METHODS

2.1 ANIMALS AND ANIMAL PRETREATMENT

Experimental animals were rats of the Wistar strain, obtained from the Small Animal Breeding Centre, Edinburgh University. Unless otherwise indicated, they were maintained on stock diet consisting of 25% skimmed milk powder, 5% dried yeast and 70% wholemeal flour. They were given water ad libitum.

2.1.1 ACTH Administration

Female Wistar rats (150-200 g) were divided randomly into two groups. One group received an injection of 0.2 ml saline, while the other group received 4 i.u. ACTH (Acthar Corticotrophin (sterile)) dissolved in 0.2 ml water. The animals were killed by decapitation 12-15 min after the injection. The adrenals and peri-renal adipose tissue were excised and quickly immersed in ice-cold 0.25M sucrose pH 7.0. The adrenals (10-12) were homogenised in 12-14 ml 0.25M sucrose in a motor driven glass/teflon Potter-Elvehjem homogeniser. Adipose tissue was chopped and homogenised in 0.25M sucrose in the same way. The homogenates from both tissues were centrifuged and the 105,000 x g supernatant was prepared as in 2.3.

2.1.2 LH Administration

Immature female rats (21-24 days old) were given a subcutaneous injection of 4 i.u. Pregnant Mare Serum Gonadotrophin (PMSG) (Gestyl, Organon) dissolved in 0.2 ml sterile saline-phosphate buffered solution. This dosage was found to induce ovulation similar to spontaneous ovulation in the normal mature female rats (Guillet and Rennels, 1964). Two days later, the animals were divided into two groups. One group received, in the tail vein, an injection of 10 μg LH(NIH) in 0.2 ml sterile saline-phosphate buffered solution. The injection was carried out under ether anaesthesia. The control animals were anaesthetised for a similar time. After 3, 6, 24 and 48 hours, the animals from both groups were killed by
decapitation under ether anaesthesia. Blood was collected for serum progesterone determination. The ovaries were excised and weighed, and some were homogenised directly in chloroform/methanol (2:1) for cholesterol and cholesterol ester determination. The remainder of the ovaries were homogenised in 25 ml 0.25M sucrose containing 10 mM potassium phosphate buffer pH 7.4 and 1 mM EDTA, using a glass/teflon homogeniser. The 105,000 x g supernatant was prepared as described in 2.3.

2.2. BOVINE ADRENAL CORTEX

Bovine adrenals were obtained from the local abattoir and transported in ice, to the laboratory arriving there within one hour of the animals' death. The adrenal glands were trimmed of adhering fat, cut longitudinally and the medulla removed and discarded. The cortex was removed from the adrenal capsule as thin slices which were immersed in ice-cold 0.25M sucrose containing 1 mM EDTA and 10 mM potassium phosphate buffer pH 7.4. A homogenate 20% (w/v) was prepared using a Potter-Elvehjem glass/teflon homogeniser. The homogenate was centrifuged and the 105,000 x g supernatant was prepared as described in 2.3.

2.3 THE PREPARATION OF 105,000 x g SUPERNATANT

The 105,000 x g supernatant from rat adrenal, rat adipose tissue, rat ovary, rat liver, bovine adrenal cortex and bovine corpus luteum was prepared from the homogenate of the appropriate tissue according to the procedure shown in Fig. 2.1.

The homogenate was first centrifuged at 10,000 g for 10 min to remove cell debris, nuclei, mitochondria and the floating lipid layer or fat cake. The 10,000 x g clear infranatant was centrifuged at 105,000 x g for 1 hour. After the lipid droplets were aspirated off, the clear infranatant was pooled leaving the microsomal pellet. The delipidated 105,000 x g infranatant was used as the enzyme source
Tissue homogenate was prepared by homogenising the tissue in 10 mM potassium phosphate buffer pH 7.4, 250 mM sucrose and 1 mM EDTA in a motorised Potter-Elvehjem glass-teflon homogeniser.

The 20% (w/v) homogenate was centrifuged at 10,000 x g for 10 min at 4°C in a fixed angle rotor, MSE centrifuge.

Cell debris, nuclei and mitochondrial pellet discarded

Infranatant

Lipid droplet or fat cake was aspirated away and discarded

Centrifuged at 105,000 x g for 1 h in Beckman ultracentrifuge at 4°C.

Microsomal and light mitochondrial pellet were discarded

Infranatant

Lipid droplet aspirated away and discarded

Pooled for enzymic assay and/or for further purification and designated

Tissue Cytosol

Fig. 2.1. The flow diagram for the preparation of the soluble tissue fraction (CYTOSOL) from rat adrenal, adipose tissue, ovary, liver and bovine adrenal cortex.
and for the purification of cholesterol ester hydrolase and triacylglycerol lipase. This cell fraction will be referred to as the tissue cytosol.

2.4 THE PREPARATION OF EMULSIFIED CHOLESTEROL-\textsuperscript{14}C OLEATE AND GLYCEROL TRI-\textsuperscript{14}C OLEATE

The emulsified substrate used for the assay of cholesterol ester hydrolase and triacylglycerol hydrolase was prepared according to Khoo, Steinberg, Huang and Vagelos (1976) with some modifications.

To the unlabelled cholesterol oleate in benzene, was added a known amount of cholesteryl\textsuperscript{14}C oleate in the same solvent. The solvent was evaporated in a stream of nitrogen, and the dried pellet was dissolved in warm absolute ethanol. This solution was added dropwise, with stirring, to a cold mixture of fatty acid-free bovine serum albumin, EDTA and potassium phosphate buffer of pH 7.2. The mixture was left to stir for 15-30 min before storing at 4\textdegree{}C. Before use the solution was again stirred for 2-3 min. Thus the final constitution of the mixture was: cholesteryl\textsuperscript{14}C oleate, 720 \mu{}M; (specific activity 0.3-0.6 \mu{}Ci/\mu{}mole); EDTA, 10 mM; ethanol, 50 \mu{}l/ml and potassium phosphate buffer, 100 mM. The final pH was 7.2-7.4.

Glycerol tri\textsuperscript{14}C oleate was prepared for use in the same way and in the same concentration of 720 \mu{}M.

Unless otherwise indicated, the substrates were used at these concentrations. In some preliminary experiments the concentrations of bovine serum albumin and EDTA were double those stated but no difference was observed on alteration to the lower concentrations.

When cholesteryl\textsuperscript{14}C linoleate was used as a substrate in comparative experiments, it was prepared in the manner described.
2.5 THE ASSAY OF CHOLESTEROL ESTER HYDROLASE AND TRIACYLGLYCEROL LIPASE

To a protein solution in 50 mM Tris-HCl buffer pH 7.4 in a final volume of 0.2 ml, was added 0.2 ml of emulsified cholesterol\(^{\text{14}C}\)oleate or glycerol tri\(^{\text{14}C}\)oleate emulsion. The incubation was carried out at 37°C for 30 min. Final concentrations in the incubation mixture were: cholesteryl\(^{\text{14}C}\)oleate 144 nmoles; bovine serum albumin 25 mg/ml; ethanol 25 µl/ml; EDTA 2.5 µmole; Tris-HCl buffer 10 µmole and potassium phosphate buffer, 20 µmole. The enzyme protein concentration did not exceed 0.5 mg/ml incubation. The final pH of the incubation was routinely checked and found to be 7.2-7.4.

Control incubations, omitting the enzyme protein were always run in parallel.

The enzymic hydrolysis of \(^{\text{14}C}\)oleic acid ester was terminated by the addition of 1.5 ml of the fatty acid extraction mixture (Khoo and Steinberg (1975)) which contained chloroform:methanol:benzene (2:2:4:1 v/v/v) with 0.29 mM unlabelled oleic acid as a carrier. After the addition of 50 µl 1N NaOH, the incubation tubes were vortexed for 15 seconds then centrifuged at 1,500 x g for 30 min. Aliquots from the upper aqueous phase (1 ml total) were taken into 10 ml Triton-X100-Toluene scintillation cocktail (0.331 Triton-X100, 0.671 Toluene, 4 g/l 2'5'-diphenyloxazole (PPO) and 0.03 g/l 1'4'-bis-5-phenyloxazly-2-benzene (POPOP).

The estimation of radioactive \(^{\text{14}C}\)oleate was carried out in a Packard Tri Carb liquid scintillation spectrometer. Emulsified \(^{\text{14}C}\)oleic acid was prepared by the method described for the ester and the recovery of \(^{\text{14}C}\)oleate from the extraction was found to be 76-80%. Counting efficiency for \(^{\text{14}C}\)oleate was 80%, and quenching was monitored by the addition of an external standard. Table 2.1 shows the recovery of \(^{\text{14}C}\)oleate from emulsified \(^{\text{14}C}\)oleic acid processed as for cholesterol\(^{\text{14}C}\)oleate and glycerol tri\(^{\text{14}C}\)oleate hydrolysis assays.
<table>
<thead>
<tr>
<th>Added $^{14}$C Oleic acid CPM</th>
<th>Recovered $^{14}$C Oleic acid CPM</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>$16,255$</td>
<td>$12,743$</td>
<td>$78.5$</td>
</tr>
<tr>
<td>$32,639$</td>
<td>$24,636$</td>
<td>$75.5$</td>
</tr>
<tr>
<td>$50,957$</td>
<td>$37,391$</td>
<td>$73.1$</td>
</tr>
<tr>
<td>$65,344$</td>
<td>$50,385$</td>
<td>$77.1$</td>
</tr>
<tr>
<td>$96,196$</td>
<td>$72,756$</td>
<td>$75.6$</td>
</tr>
</tbody>
</table>

Table 2.1. Recovery of $^{14}$C oleic acid after liquid-liquid phase partition.
Fig. 2.2. The calibration curve of progesterone determination by the radioimmunoassay procedure.
2.6 THE IN VITRO ACTIVATION AND INACTIVATION OF CHOLESTEROL ESTER HYDROLASE AND TRIACYLGLYCEROL HYDROLASE

To the enzyme protein solution in 50 mM Tris-HCl buffer pH 7.4 was added a mixture of magnesium acetate, ATP and cAMP giving concentrations in a final volume of 200 μl of: protein 200 μg; magnesium acetate, ATP, 400 nmoles; cAMP 2.5 nmoles and Tris-HCl buffer pH 7.4 10 μmoles.

The activation reaction was carried out at 37°C for 10 min after which it was terminated by the addition of the substrate-buffer emulsion of cholesterol ester hydrolase or of triglyceride hydrolase containing an excess of EDTA. Inactivation experiments were carried out in exactly the same way as activation experiments but with the omission of cAMP and ATP. The incubation mixture for the basal activity assay contained no magnesium acetate, no ATP and no cAMP.

2.7 MEASUREMENT OF PROGESTERONE IN RAT SERUM AND OVARIES

Serum (0.1-0.2 ml) from individual immature female rats was extracted with 2 ml petroleum ether (40°-60°). After freezing in a dry ice/methanol bath, the upper petroleum ether extract was decanted into incubation tubes and dried under nitrogen. The dry residue was taken up in 0.5 ml saline-phosphate buffer containing 1% gelatin (Henderson (1977)). Aliquots were taken for progesterone measurement by radioimmunoassay (Neal, Baker, McNatty and Scaramuzzi (1975)). Progesterone was calculated from a calibration curve of known progesterone standards run in parallel. A typical calibration curve is shown in Fig. 2.2.

Individual ovaries were homogenised in 3 ml chloroform:methanol mixture (2:1). After the removal of the protein pellet, the chloroform/methanol extract was dried under nitrogen. The dry residue was assayed for progesterone content as described for serum progesterone determination.
2.8 THE MEASUREMENT OF OVARIAN MITOCHONDRIAL CHOLESTEROL SIDE CHAIN CLEAVAGE ACTIVITY

The ovarian mitochondrial sterol side chain cleavage activity was estimated from measurements of total pregnenolone and progesterone produced. The ovarian mitochondria were prepared by homogenising the ovaries in 0.25M sucrose pH 7.0, followed by the removal of the nuclei and cell debris by centrifugation for 10 min at 650 x g. The post-nuclei supernatant was centrifuged for 15 min at 8,500 x g, followed by washing the mitochondrial pellet and final centrifugation at 8,500 x g for 15 min.

The sterol side chain cleavage assay was carried out after preincubating the mitochondria in a buffer of 10 mM potassium phosphate buffer pH 7.4, 5 mM MgCl₂, 100 mM sucrose, 0.2 mM NADP⁺ and 100 μM cholesterol (or other sterol as indicated) in 10 μl ethanol. After the 15 min preincubation of the mitochondria at 37°C, the sterol side chain cleavage reaction was initiated by the addition of 10 mM DL-iso-citrate. At indicated time intervals, the reaction was stopped by the addition of chloroform:methanol mixture and pregnenolone and progesterone were extracted into the organic phase. Pregnenolone was measured by the radioimmunoassay of Abraham, Buster, Kyle, Corrales and Teller (1973) as described and validated by Mason, Arthur and Boyd (1978). Progesterone was measured also by the radioimmunoassay as described in 2.7.

2.9 PREPARATION OF ISOLATED RAT ADIPOCYTES

Isolated rat adipocytes were prepared by the method of Rodbell (1964) as modified by Langslow and Hales (1969). Male Wistar rats (150-200 g) were killed by decapitation, and the epididymal fat bodies from twelve rats were pooled and washed with Krebs-Ringer bicarbonate buffer pH 7.4. After cleaning, the fat bodies were chopped into small pieces of 5-10 mg and pooled in 2 g portions. Each 2 g portion was
transferred to a 1 x 3 in. capped polythene tube containing 50 mg bovine albumin and 25 mg collagenase in 5 ml Krebs-Ringer bicarbonate pH 7.4. Dispersion of adipocytes was achieved by incubation at 37°C for 30-45 min in a metabolic shaker at 140 oscillations/min. The cell suspension was then centrifuged at 1,000 r.p.m. for 1 min in a bench centrifuge. Using a glass Pasteur pipette, the medium, containing red blood cells and fragments of adipose tissue, was carefully withdrawn from below the floating adipocyte cake. Any lipid droplets were also removed and the cell layer was washed with 2 x 10 ml of Krebs-Ringer bicarbonate buffer containing 10 mg/ml of bovine serum albumin (KRBB). After the final wash, the cells were suspended in the desired volume of KRBB and poured into a silanised conical flask. Cell preparations from different tubes were gently mixed in the flask. This final suspension was then incubated with the desired additions as described in the relevant section.

2.10 DETERMINATION OF CHOLESTEROL AND CHOLESTEROL ESTER CONTENT OF RAT OVARY

Ovaries from rats pretreated with 4 i.u. PMSG were removed, dried on filter paper, weighed and individually immersed in 3 ml chloroform-methanol (2:1). \(^{4,14}C\)cholesterol (5,000 c.p.m.) and \(^{4,14}C\)cholesteryl oleate in 50 \(\mu\)l acetone were added in order to check the recoveries of cholesterol and cholesterol ester during the procedure. After homogenisation in an all glass homogeniser, the chloroform:methanol mixture was taken to dryness under a stream of nitrogen. The lipid residue was suspended in 3 ml absolute ethanol and left overnight to dissolve. One ml of the ethanolic extract was taken for the estimation of free cholesterol, the rest was taken for the determination of esterified cholesterol and for the recovery determination. The esterified cholesterol fraction was hydrolysed by refluxing with 5 ml 10% ethanolic
KOH for 30 min at 60°C. After the addition of 5 ml water the non-
saponifiable lipids were extracted with 3 x 3 ml petroleum ether
(60°-80°). The petroleum ether was removed in a stream of nitrogen
and the lipid residue was dissolved in 1 ml ethanol. Aliquots were
taken for the determination of free cholesterol. Cholesterol recoveries
were also checked in the same fraction. Determination of cholesterol
as free cholesterol was carried out according to the procedure of
Gamble, Vaughan, Kruth and Avigan (1978) and was as follows:

To 50 μl cholesterol ethanolic solution in culture test tubes was
added 3 ml of the following mixture:

- 2 parts cholesterol oxidase (1 units/ml)
- 2 parts Horse radish peroxidase (10 units/ml)
- 1 part Triton X100 (0.5% v/v)
- 1 part sodium deoxycholate (20 mM)
- 3 parts p-hydroxyphenylacetic acid (4 mg/ml)
- 36.4 parts potassium phosphate buffer pH 7.4 (50 mM).

After mixing, the tubes were incubated for 30 min at 37°C. The
fluorescence was then measured in a Perkin-Elmer fluorimeter, using an
emission wavelength of 415 nm and an excitation wavelength of 325 nm.
The cholesterol concentration was calculated from a standard curve of
known cholesterol solutions (50-500 ng/assay) which were run at the same
time of each assay.

2.11 PROTEIN DETERMINATION

Protein concentrations were estimated by the method of Lowry,
Rosebrough, Farr and Randall (1951), and the modified procedure of
Peterson (1977) was used in later experiments. Crystalline bovine albumin
was the protein standard in every determination.

2.12 MATERIALS

All common reagents used for these studies were obtained from either
Sigma (London) Chemical Co. Ltd., or B.D.H. and were of analytical grade.
Cholesterol oleate (99% pure), triolein (glycerol trioleate) (98% pure), cholesteryl linoleate (99% pure), linoleic acid, oleic acid, essentially fatty acid-free bovine albumin, chloroquine, adenosine 3'5'-cyclic monophosphoric acid, guanosine 3'5'-cyclic monophosphoric acid, crystalline adenosine 5'-triphosphate (ATP) disodium salt, reduced nicotinamide adenine dinucleotide (NADH), nicotinic acid (free-acid), rabbit skeletal muscle 3'5'-cyclic AMP-dependent protein kinase, rabbit skeletal muscle protein kinase inhibitor, bovine heart 3'5'-cyclic nucleotide phosphodiesterase and horse radish peroxidase were all from Sigma (London) Chemical Co. Ltd. (Poole).

Cholesteryl\(^{14}\)C\(\text{oleate} (21.5-34 \text{ mCi/mmol})\), glycerol tri-\(^{14}\)C\(\text{oleate} (60 \text{ mCi/mmol})\), \(^{14}\)C\(\text{cholesterol} (35.6 \text{ mCi/mmol})\), \(^{14}\)C\(\text{oleic acid}\), \(^{32}\)P\(\text{phosphorus and cholesteryl}^{14}\)C\(\text{linoleate were obtained from The Radiochemical Centre, Amersham.}\)

\(^{14}\)C\(\text{Cholesteryl oleate and cholesteryl oleoyl ether were from NEN GmbH, West Germany.}\)

Calcium phosphate gel, cholesterol oxidase and cholesterol clinical assay kit were from BDH (Poole).

Chloropyrifos oxone was a gift from DOW Chemical Co., Holland.

Sepharose 4B, Sephadex G25, Percoll, Octyl Sepharose-CL were from Pharmacia Fine Chemicals, London.

Biogel-A 150M, Biogel-A 0.5M, hydroxylapatite HTP were from Bio-Rad Laboratories Ltd., Bromley, Kent.

Collagenase type I (Worthington) was obtained from Cambrian Chemicals Ltd., Croydon.

Phosphoglycerate kinase, glyceraldehyde-3-phosphate dehydrogenase, glycerol kinase and lactate dehydrogenase were obtained from Boehringer Corporation (London) Ltd., Lewes.

Crystalline bovine albumin was a product of Miles Biochemicals.
\(^{32}\text{P}}\text{ATP was prepared according to Glynn and Chappell (1964) as modified by Walsh, Perkins, Bromstrom, Ho and Krebs (1971).}
SECTION III

STUDIES ON RAT ADRENAL SOLUBLE CHOLESTEROL ESTER HYDROLASE AND TRIACYLGlycerOL LIPASE

3.1 Introduction

3.2 The basal activities of triacylglycerol lipase and cholesterol ester hydrolase in rat adrenal cytosol

3.3 In vivo stimulation of cholesterol ester hydrolase and triacylglycerol lipase of rat adrenal cytosol by ACTH administration

3.4 The effect of nicotinic acid administration in vivo on rat adrenal cholesterol ester hydrolase and triacylglycerol lipase

3.4.1 Nicotinic acid effect on the basal activities of adrenal hydrolases

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3.5 The hydrolysis of cholesteryl\(\text{\textsuperscript{14}C}\)oleate, cholesteryl \(\text{\textsuperscript{14}C}\)linoleate and glycerol tri-\(\text{\textsuperscript{14}C}\)oleate by rat adrenal cytosol

3.6 Summary
SECTION III: STUDIES ON RAT ADRENAL CYTOSOLIC CHOLESTEROL ESTER HYDROLASE AND TRIACYLGlycerol LIPase

3.1 INTRODUCTION

The observation of Sayers, Sayers, Fry, White and Long (1944) that crude preparations of adrenocorticotropic hormone (ACTH) on injection into hypophysectomised rats, caused a marked depletion of adrenal cholesterol, led these authors to postulate that ACTH might play a role in the formation and secretion of adrenal cortical steroids. Soon after, Pincus, Hechter and Zaffaroni (1951) and Saffran, Grad and Bayliss (1952) found that ACTH increased the output of corticoids from perfused cow adrenals and bisected rat adrenals when these tissues were treated with ACTH in vitro. After the discovery of cAMP production in response to hormonal signals in rat liver (Sutherland and Rail (1957)), Haynes (1958) reported a marked increase in the intracellular concentration of cAMP in the adrenal slices incubated in vitro with ACTH. Haynes, Koritz and Peron (1959) showed that bovine adrenocortical slices incubated in vitro with ACTH responded by increased corticoid production, and this stimulation could be simulated by the addition of cAMP alone.

In the work of Davis and Garren (1966), adrenal cholesterol ester was depleted in response to ACTH injections to hypophysectomised rats, and a build up of free cholesterol in the adrenals was found when adrenal steroidogenesis was blocked by cycloheximide, a protein synthesis inhibitor, given to the animals prior to ACTH administration. Davis and Garren (1966) concluded that ACTH not only affects adrenal steroidogenesis but it has an effect on the formation of free cholesterol from the stored esterified cholesterol. Since then, research has focussed on the relationship between ACTH and the depletion of adrenal cholesterol esters.
The discovery of a cAMP-dependent protein kinase in the bovine adrenal cortical cytosol (Gill and Garren (1970)) and the finding of a cAMP-binding protein in the same tissue proved to be significant observations (Gill and Garren (1969)). A relationship between ACTH and cholesterol ester hydrolysis was proposed by Trzeciak and Boyd (1973) and Boyd and Trzeciak (1973) for the hydrolysis of rat adrenal cholesterol ester. This work was extended to the bovine adrenal cytosol (Trzeciak and Boyd (1974)), Naghshineh, Treadwell, Gallo and Vahouny (1974) and the proposed scheme envisaged that upon the interaction of ACTH and the rise of intracellular concentration of cAMP, an inactive protein kinase was released from the suppressing cAMP-binding protein to become a fully active protein kinase which in turn activated an inactive cholesterol ester hydrolase possibly through phosphorylation of the enzyme. In recent years, the phosphorylation of the cholesterol ester hydrolase was fully established by the work of Beckett and Boyd (1977), Wallat and Kanau (1976) and Naghshineh, Treadwell, Gallo and Vahouny (1978), although the site of phosphorylation is yet not known. However, although much attention has been devoted to adrenal cholesterol esters, triglyceride hydrolysis has not been studied in much detail in the adrenal cortex.

There are early observations of Rudman and Garcia (1966) and Palkovic, Macho and Mosinger (1965) that ACTH causes a marked depletion of total adrenal triglycerides in hypophysectomised rats, and the stimulation of a lipase activity in rat adrenal homogenate by ACTH was noted. A study on rat adrenal cytosolic triacylglycerol lipase showed this enzyme to be sensitive to ACTH treatment in vivo (this section). This was confirmed by Pittman and Steinberg (1977) who showed the activation of cytosolic triacylglycerol lipase by cAMP-dependent protein kinase. Pittman and Steinberg (1977) also found that the triacylglycerol lipase co-purified
with the cholesterol ester hydrolase in the rat adrenal cytosol. These workers had previously found that these two enzymic activities co-purified from the cytosol of rat adipose tissue (Pittman, Khoo and Steinberg (1975)). The experiments in this section were designed to investigate the role of ACTH in the activation of the adrenal cytosolic triacylglycerol lipase, contrasting this activation with that of cholesterol ester hydrolase previously studied by Trzeciak and Boyd (1973) and Beckett (1975).

3.2 THE BASAL ACTIVITY OF TRIACYLGlyCEROL LIPASE AND CHOLESTEROL ESTER HYDROLASE IN THE RAT ADRENA L CYTOSOL

Triacylglycerol lipase was measured in the 105,000 x g supernatant of rat adrenal homogenate. It had been shown by Trzeciak and Boyd (1973) that more than 60% of bovine adrenal cholesterol ester hydrolase activity is associated with this fraction, and it was decided to compare these two enzymic activities in this cell fraction. Rat adrenal supernatant was prepared as described in Section II. Triacylglycerol lipase and cholesterol ester hydrolase were assayed after pre-incubation with or without cAMP and ATP as described in the previous section. Table 3.1 shows the results from this experiment in which triacylglycerol lipase and cholesterol ester hydrolase activities were compared.

The activity of triacylglycerol lipase in the rat adrenal cytosol varied from one batch of animals to another, the mean being 427±59 pmoles/min/mg protein, (Mean±S.E.M. of 12 different experiments carried out in several months of the year), and each observation being made at least in triplicate. This variability was not surprising since other lipolytic enzymes such as lipoprotein lipase in rat adipose tissue is reported to vary considerably (Ashby, Bennett, Spencer and Robinson (1978)). Similarly triacylglycerol lipase in the rat adrenal cytosol has been found to be quite variable (Pittman and Steinberg (1977)). In this
<table>
<thead>
<tr>
<th>Incubation</th>
<th>Cholesterol ester hydrolase</th>
<th>Activation %</th>
<th>Triacylglycerol lipase</th>
<th>Activation %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>957±48</td>
<td>100</td>
<td>754±23</td>
<td>100</td>
</tr>
<tr>
<td>+cAMP/ATP</td>
<td>1572±18</td>
<td>164</td>
<td>1167±16</td>
<td>154</td>
</tr>
</tbody>
</table>

Table 3.1. The activity of triacylglycerol lipase and cholesterol hydrolase in rat adrenal 105,000 x g supernatant. Results are expressed as the mean of pmole oleic acid released/min/mg protein ± S.E.M. of five replicates.
laboratory, the activity of triacylglycerol lipase was markedly different from that reported by Pittman and Steinberg (1977). This might be due to the different strains of rat used. However in later studies from that group (Khoo, Sperry, Gill and Steinberg (1977)) comparable activities of cholesterol ester hydrolase in the rat adrenal and triacylglycerol lipase in rat adipose were reported to those obtained in this work. Cholesterol ester hydrolase, on the other hand, in rat adrenal cytosol has a basal activity, under the assay conditions used, which varies from day to day but averages at 907±119 pmol/min/mg protein, (Mean± S.E.M. of 12 experiments). This activity was always higher than that of the triacylglycerol lipase, and the ratio of the two activities was about 2. This value is close to the ratio obtained by Pittman and Steinberg (1977) which varied from 1.3-2.5 depending upon the preparation.

The activation of rat adrenal cytosolic cholesterol ester hydrolase by a cAMP-dependent protein kinase was reported by Trzeciak and Boyd (1973), Beckett and Boyd (1975)). In these studies cholesterol ester hydrolase was activated by pre-incubation of adrenal cytosol with cAMP and Mg++/ATP for 10 min before the standard assay was carried out. Exogenous protein kinase was not added, since the activity of protein kinase is high in the adrenal cytosol (Trzeciak and Boyd (1973)). The average activation of cholesterol ester hydrolase in rat adrenal cytosol was found to be 30-40%, compared to the activity of the enzyme incubated with no cAMP. In a similar fashion, triacylglycerol lipase was found to be activated to the same extent as cholesterol ester hydrolase. These findings were compatible with other workers, and several questions have since been posed regarding the cholesterol ester hydrolase. These questions include: How specific is cholesterol ester hydrolase? What relationship has cholesterol ester hydrolase to triacylglycerol lipase? Can these two enzyme activities be differentiated?
Experiments in this section and others, were performed in an attempt to answer these questions using in vivo and in vitro experiments.

3.3 *IN VIVO STIMULATION OF CHOLESTEROL ESTER HYDROLASE AND TRIACYLGLYCEROL LIPASE OF RAT ADRENAL CYTOSOL BY ACTH ADMINISTRATION*

Cholesterol ester hydrolase in the rat adrenal cytosol was reported to be stimulated in vivo by ether anaesthesia, a condition known to elevate plasma ACTH (Matsayuna, Ruhmann-Wennhold and Nelson (1971)), and this stimulation was attributed to the phosphorylation of the enzyme protein by a cAMP-dependent protein kinase (Boyd and Trzeciak (1973)). Since it was found that adrenal triacylglycerol lipase can be activated in vitro by pre-incubating the adrenal cytosol with cAMP (see Table 3.1 this section), it was of interest to test whether this activation could be matched in vivo by exposing rats to hormonal stimuli. It was also important to try to establish whether triacylglycerol lipase could be differentiated from cholesterol ester hydrolase by this treatment.

Adrenals were obtained from saline or ACTH injected rats as described in Section II. The adrenal cytosol was prepared and assays for cholesterol ester hydrolase and triacylglycerol lipase were performed as described in Section II.

In a series of four experiments, cholesterol ester hydrolase and triacylglycerol lipase in the adrenal cytosol of ACTH injected rats were both stimulated, their activities were significantly higher than in the adrenal cytosol of saline injected control animals. The degree of stimulation of cholesterol ester hydrolase was +38% while that of triacylglycerol lipase was +31%. Table 3.2 shows the actual values of the activities of cholesterol ester hydrolase and triacylglycerol lipase in preparations of the cytosol prepared from both saline and ACTH injected rats. Although both enzymes were stimulated with ACTH, further stimulation was observed when the cytosol was pre-incubated in vitro for
<table>
<thead>
<tr>
<th>Experiment</th>
<th>No. of rats</th>
<th>Cholesterol ester hydrolase</th>
<th>Triacylglycerol lipase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Saline injected</td>
<td>ACTH injected</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(pmole oleic acid/min/mg protein)</td>
<td>(pmole oleic acid/min/mg protein)</td>
</tr>
<tr>
<td>1</td>
<td>12</td>
<td>877±31</td>
<td>1264±41</td>
</tr>
<tr>
<td>2</td>
<td>12</td>
<td>1506±40</td>
<td>1818±75</td>
</tr>
<tr>
<td>3</td>
<td>12</td>
<td>993±23</td>
<td>1399±29</td>
</tr>
<tr>
<td>4</td>
<td>12</td>
<td>841±14</td>
<td>1260±8</td>
</tr>
</tbody>
</table>

Table 3.2. *In vivo* stimulation by 4 i.u. ACTH of cholesterol ester hydrolase and triacylglycerol lipase in rat adrenal cytosol. Each figure represents the mean ± S.E.M. of five replicates.
10 min with cAMP. This further activation might indicate that ACTH stimulation in vivo of both cholesterol ester hydrolase and triacylglycerol lipase was not complete. Alternatively, it may be that a complete stimulation of both enzymes was achieved by the in vivo action of ACTH but, upon processing the tissue and assaying the enzymic activity, significant de-activation of both enzymes occurs. This de-activation could be mediated through a phosphoprotein phosphatase known to be present in adrenal cytosol (Merlevede and Riley (1966)). The effect of this enzyme on bovine adrenal cholesterol ester hydrolase has been observed (Trzeciak and Boyd (1974), Beckett and Boyd (1977)). Table 3.3 summarises the activation in vivo and in vitro of both cholesterol ester hydrolase and triacylglycerol lipase. The extent of activation is expressed as the percentage of the control activity found in the adrenal cytosol from saline injected rats.

3.4 THE EFFECT OF NICOTINIC ACID ADMINISTRATION IN VIVO ON RAT ADRENAL CHOLESTEROL ESTER HYDROLASE AND TRIACYLGlycerol LIPASE

The antilipolytic action of nicotinic acid on hormone-stimulated lipolysis of adipose tissue is well documented, although the precise mechanism of action of nicotinic acid is not yet settled. Most reports however, cite adenylate cyclase as the site of inhibition, with the consequent decrease of intracellular concentration of cAMP (for reviews see Gey and Carlson (1971) and Fain (1973, 1977). It is also reported that nicotinic acid treatment decreased plasma cholesterol and triglycerides concentration in human subjects (Froberg, Boberg, Carlson and Eriksson (1971)). These pieces of evidence together with the finding that adrenal cholesterol ester hydrolase and triacylglycerol lipase are under hormonal control, prompted the investigation of the effect of nicotinic acid on the activities of both enzymes.
<table>
<thead>
<tr>
<th>Experiment</th>
<th>Cholesterol ester hydrolase %</th>
<th>Triacylglycerol lipase %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Saline injected rats</td>
<td>ACTH injected rats</td>
</tr>
<tr>
<td></td>
<td>control activated</td>
<td>control activated</td>
</tr>
<tr>
<td>1</td>
<td>100±3.5 140±2.7 144±4.7 187±6.7</td>
<td>100±8.4 144±5.6 156±1.9 183±6.9</td>
</tr>
<tr>
<td>2</td>
<td>100±2.7 175±7.9 121±4.9 182±2.8</td>
<td>100±5.2 145±3.7 120±4.9 151±4.5</td>
</tr>
<tr>
<td>3</td>
<td>100±2.3 144±4.9 140±2.9 158±3.9</td>
<td>100±8.5 167±3.8 134±4.6 182±3.8</td>
</tr>
<tr>
<td>4</td>
<td>100±1.7 153±2.6 150±0.9 197±5.9</td>
<td>100±1.9 127±2.2 115±1.9 124±4.4</td>
</tr>
</tbody>
</table>

Table 3.3. The percentage stimulation by 4 i.u. ACTH in vivo of cholesterol ester hydrolase and triacylglycerol lipase in rat adrenal cytosol and the stimulation in vitro by preincubation with cAMP. Control represents cytosol pre-incubated with 2 mM magnesium, while activated represents cytosol pre-incubated with 10 μM cAMP, 2 mM ATP and 2 mM magnesium for 10 min. The mean release of oleic acid in the saline injected control rat adrenal cytosol was considered as 100% for each enzyme and each experiment individually. All figures + S.E.M. are referred to that figure.
3.4.1 Nicotinic acid effect on the basal activities of rat adrenal cholesterol ester hydrolase and triacylglycerol lipase

When nicotinic acid was administered *in vivo* to quiescent rats prior to their sacrifice, adrenal cholesterol ester hydrolase was not affected as compared to the enzyme activity in adrenals of rats injected with saline solution. Although the triacylglycerol lipase was depressed in the adrenals of nicotinic acid injected rats, this slight inhibition was not significant. This was reinforced by the fact that triacylglycerol lipase from the saline injected rats was not activated at a higher level of activity in the saline injected animals thus showing a difference between the nicotinic acid and the saline injected rats. Table 3.4 illustrates the comparison of the effect of nicotinic acid on the rat adrenal cholesterol ester hydrolase and triacylglycerol lipase, both of control and cAMP pre-incubated adrenal cytosol preparations. It seems that nicotinic acid injected to quiescent animals did not alter the activities of either enzyme. The nicotinic acid effect blocking the *in vitro* activation by cAMP of cholesterol ester hydrolase might be through an activated cyclic nucleotide phosphodiesterase. Cytosolic rat adipose tissue cyclic nucleotide phosphodiesterase is known to be activated by pre-treatment of the adipose tissue with nicotinic acid *in vitro*. This activation is enhanced when the adipose tissue cytosol fraction is incubated with ATP (Schwabe (1971)). Thus, in the activation assay, ATP was present and the decrease of percentage activation of cholesterol ester hydrolase seen in this experiment could be due to this phosphodiesterase effect.

3.4.2 Nicotinic acid effect on the stimulation of rat adrenal cholesterol ester hydrolase and triacylglycerol lipase

The observation that nicotinic acid has no effect on the basal activities of rat adrenal cholesterol ester hydrolase described in
Table 3.4, argues strongly that the antilipolytic action of nicotinic acid lies primarily in blocking the stimulation of lipolysis by hormones through a depression of the intracellular cAMP concentration. Accordingly, a series of four experiments were performed on rat adrenal cholesterol ester hydrolase and triacylglycerol lipase previously stimulated in vivo by subjecting the rat to acute ether anaesthesia. This is known to elevate the plasma ACTH concentration (Matsayuna, Ruhmann-Wennhold and Nelson (1971), Cook, Greer and Kendall (1972)). A comparison was then made of the activities of the two enzyme activities in the adrenals of nicotinic acid injected rats prior to and after exposure to ether anaesthesia.

Table 3.5 summarises the result of the effect of nicotinic acid on stimulated rat adrenal cholesterol ester hydrolase and triacylglycerol lipase. Administering nicotinic acid to rats 15 min before they were acutely stressed resulted in the adrenal cholesterol ester hydrolase activity being significantly lower than the activity found in saline treated animals. Likewise, adrenal triacylglycerol lipase activity was lower in the nicotinic acid treated rats but this was not seen in all experiments. This variation could not be explained in terms of the drug not affecting the target tissue, since cholesterol ester hydrolase was affected in this tissue. However, activation of triacylglycerol lipase in vivo might be affected by either a de-activation process during tissue processing or due to a lower percentage stimulation of the enzyme by endogenous ACTH. This could obscure the inhibitory effect of nicotinic acid on the stimulation of the triacylglycerol lipase.

When the results of the four experiments shown in Table 3.5 were pooled and the adrenal cholesterol ester hydrolase and triacylglycerol lipase activities of the saline injected rats were considered as 100%,
<table>
<thead>
<tr>
<th>Group</th>
<th>Cholesterol ester hydrolase pmole oleic/min/mg protein*</th>
<th>Triacylglycerol lipase pmole oleic/min/mg protein*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control +cAMP</td>
<td>Control +cAMP</td>
</tr>
<tr>
<td>Saline</td>
<td>868±15</td>
<td>1220±9</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>887±17</td>
<td>1054±11</td>
</tr>
</tbody>
</table>

Table 3.4. The effect of nicotinic acid administration *in vivo* on the basal activities of rat adrenal cytosolic cholesterol ester hydrolase and triacylglycerol lipase. Nicotinic acid (30 mg) in 0.2 ml saline was injected intraperitoneally 15 min before animals were sacrificed. Control animals received saline only and were killed 15 min later.

*Results are expressed as the mean ± S.E.M. of triplicate determinations.
<table>
<thead>
<tr>
<th>Experiment</th>
<th>No. of rats</th>
<th>Cholesterol ester hydrolase</th>
<th>Triacylglycerol lipase*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Saline</td>
<td>Nicotinic acid**</td>
</tr>
<tr>
<td>1</td>
<td>12</td>
<td>1142+14</td>
<td>894+12&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>12</td>
<td>1014+10</td>
<td>1049+10&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>12</td>
<td>433+3</td>
<td>309+1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>4</td>
<td>12</td>
<td>1737+14</td>
<td>1435+11&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Table 3.5. The effect of nicotinic acid administration in vivo on the in vivo stimulated adrenal cholesterol ester hydrolase and triacylglycerol lipase.

*Results are expressed as the mean value of pmole oleic acid released/min/mg protein ± S.E.M. (n=3).

**30 mg nicotinic acid in 0.2 ml saline.

<sup>a</sup>Significantly different from saline control value at P<0.05 level.

<sup>b</sup>Not significantly different from saline control value.
The effect of nicotinic acid administration in vivo on in vivo stimulated rat Adrenal Cholesterol Ester Hydrolase and Triacylglycerol lipase.

Fig. 3.1. The inhibition in vivo by nicotinic acid of in vivo stimulated rat adrenal cholesterol ester hydrolase and triacylglycerol lipase. Nicotinic acid (30mg in 0.2ml saline solution) was injected into rats before the animals were subjected to acute ether anaesthesia. Adrenals were collected from control animals (injected with saline) and nicotinic acid injected rats. Cholesterol ester hydrolase and triacylglycerol lipase were assayed in the adrenal cytosol as described in the methods section. Results are shown as the percentage change from control, and are the mean values of four different experiments ± S.E.M.
nicotinic acid inhibited both cholesterol ester hydrolase and triacylglycerol lipase significantly $p<0.0005$ and $p<0.005$ respectively as it is shown in Fig. 3.1. From the above experimental evidence, it seems that nicotinic acid by itself has no effect on rat adrenal cholesterol ester hydrolase or triacylglycerol lipase when administered in vivo shortly before the animal was sacrificed. However, an acute affect of nicotinic acid on cholesterol ester hydrolase and triacylglycerol lipase was noted when these enzymes were stimulated in vivo. This action was interpreted as preventing stimulation through a mechanism which might involve a decrease of the intracellular concentration of cAMP and the consequent blockade of the release of active protein kinase from its receptor-catalytic subunit complex. Thus both cholesterol ester hydrolase and triacylglycerol lipase may be rendered insensitive to hormonal stimulus, a situation which was also found in isolated adipocytes as will be discussed in a later section.

3.5 THE HYDROLYSIS OF CHOLESTERYL-$[^{14}C]$OLEATE, CHOLESTERYL-$[^{14}C]$ LINOLEATE AND GLYCERYLTRI-$[^{14}C]$OLEATE BY RAT ADRENAL CYTOSOL

The rat adrenal cell contains most of its esterified cholesterol stored in lipid droplets which float in the cell cytosol (Boyd and Trzeciak (1973)). Upon exposure of rats to ether anaesthesia, a condition shown to stimulate cholesterol ester hydrolase in the adrenal cytosol (Trzeciak and Boyd (1973)), adrenal cholesterol esters in the lipid droplets undergo rapid hydrolysis in vivo (Beckett and Boyd (1975)). Moreover, it appears that there is a selective hydrolysis of these stored cholesterol esters in that the esters with saturated fatty acid are less susceptible to enzymic hydrolysis than the esters found from unsaturated fatty acids. By examination of the hydrolysis of unsaturated fatty acid cholesterol esters, it appears that cholesteryl linoleate was hydrolysed at a faster rate than the oleate or palmitoleate cholesteryl
esters (Beckett and Boyd (1975)). These observations were also deduced from the recent studies on rat adrenal cells challenged with dibutyryl cAMP (Vahouny, Hodges and Treadwell (1979)). It follows that since cholesteryl linoleate is apparently more susceptible to enzymic hydrolysis in vivo than cholesteryl oleate, then addition of these substrates to rat adrenal cytosol should show a differential hydrolysis in vitro. To examine the validity of this argument, rat adrenal cytosol was assayed against cholesteryl$\alpha$-$^{14}C$oleate, cholesteryl$\alpha$-$^{14}C$linoleate and glycerol tri-$\alpha$-$^{14}C$oleate. All three substrates were prepared in an identical manner as described in Section II, and assayed under similar conditions in a single experiment.

Table 3.6 illustrates the hydrolysis of these esters in control and cAMP activated rat adrenal cytosol preparation. Cholesteryl linoleate was hydrolysed faster than cholesteryl oleate in both control and cAMP-activated adrenal cytosol (150% and 254% respectively). By contrast glycerol trioleate was hydrolysed at a slower rate than cholesteryl oleate as found in previous experiments described in this section.

From this evidence, it seems that there is a preferential hydrolysis by rat adrenal cytosol of the different cholesterol esters having the same number of carbon atoms in their fatty acid moiety, but with different degrees of unsaturation. The possibility can not be ignored that the in vitro might reflect structural differences in the emulsion difference in hydrolysis. If the in vitro hydrolysis reflects the true enzymic hydrolysis occurring in vivo, it is possible that linoleic acid could be elongated and desaturated to be used for prostaglandins biosynthesis. This is in agreement with the report of Riley (1963) who found that when human adrenals were stimulated with ACTH, adrenal linoleic acid of the cholesterol esters fraction was decreased and arachidonic acid was increased. Recently it was shown that if adrenal cortical cells of rats were pre-labelled with radioactive arachidonic acid,
<table>
<thead>
<tr>
<th>Substrate</th>
<th>Enzymic Specific Activity (pmole fatty acid released/min/mg protein)</th>
<th>Control</th>
<th>+CAMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesteryl $\text{I}^{-14}C$ linoleate</td>
<td></td>
<td>1856±67</td>
<td>3156±68</td>
</tr>
<tr>
<td>Cholesteryl $\text{I}^{-14}C$ oleate</td>
<td></td>
<td>1238±39</td>
<td>2765±6</td>
</tr>
<tr>
<td>Glyceryl tri-$\text{I}^{-14}C$ oleate</td>
<td></td>
<td>212±43</td>
<td>395±21</td>
</tr>
</tbody>
</table>

Table 3.6. The hydrolysis of cholesteryl oleate, linoleate and glyceryl trioleate by rat adrenal cytosol. All three substrates were prepared in an identical manner as described in Section II. Each substrate was assayed in one experiment under identical conditions using rat adrenal cytosol pre-incubated with or without cAMP as described in the activation procedure in Section II. Results are mean values ± S.D.
ACTH stimulated the synthesis and the release of radioactive prostaglandins (Chanderbhan, Hodges, Treadwell and Vahouny (1979)). These observations lend support to the postulate of Beckett (1975) that cholesterol ester hydrolase does not show great specificity towards the sterol moiety of the sterol ester, but rather it has specificity towards the fatty acid of the sterol ester, since altering the side-chain length of the sterol did not inhibit cholesterol ester hydrolase and a faster rate was actually observed in the hydrolysis of pregnenol oleate and norcholesteryl oleate compared to the hydrolysis of cholesterol oleate. However, if the hydrolysis of glycerol trioleate is performed by the same enzyme, this substrate should be hydrolysed at a similar rate to that of cholesteryl oleate. This does not appear to be occurring in these studies.

The possibility is thus raised that another enzyme, similar to cholesterol ester hydrolase in terms of regulatory mechanism, may have responsibility for the hydrolysis of adrenal triglycerides. To further investigate this possibility, studies in the following sections were aimed at the differentiation of these enzymes using physical and chemical approaches.

3.6 SUMMARY

1. In the rat adrenal cytosol there is a triacylglycerol lipase enzyme which is activatable by a cAMP-dependent protein kinase in vitro.

2. The triacylglycerol lipase activity is stimulated in vivo by ACTH administration, and this stimulation is similar to the in vivo stimulation of cholesterol ester hydrolase.

3. Nicotinic acid administration in vivo to quiescent rats did not alter the basal activity of adrenal triacylglycerol lipase or cholesterol ester hydrolase.

4. Nicotinic acid administration prior to ACTH stimulation in vivo of adrenal cholesterol ester hydrolase and triacylglycerol lipase, blocked this ACTH stimulation.
5. Adrenal cholesterol ester hydrolase was found to hydrolyse cholesteryl linoleate at a faster rate than it hydrolysed cholesteryl oleate.

6. The possibility that cholesterol ester hydrolase and triacylglycerol lipase are one and the same enzyme or two different enzymes is discussed.
SECTION IV

PURIFICATION OF BOVINE ADRENOCORTICAL
CHOLESTEROL ESTER HYDROLASE AND
TRIACYLGLYCEROL LIPASE

4.1 Introduction

4.2 Triacylglycerol lipase in the bovine adrenal cortical
105,000 x g supernatant

4.3 Purification of triacylglycerol lipase and cholesterol
ester hydrolase from bovine adrenocortical cytosol

4.3.1 pH 5.0 precipitate preparation

4.3.2 Gel filtration of cholesterol ester hydrolase and
triacylglycerol lipase

4.3.3 Floatation of cholesterol ester hydrolase and
triacylglycerol lipase

4.4 Summary
SECTION IV: PURIFICATION OF BOVINE ADRENAL CORTICAL CHOLESTEROL ESTER HYDROLASE AND TRIACYLGLYCEROL LIPASE

4.1 INTRODUCTION

Cholesterol ester hydrolase in the bovine adrenal cytosol was purified by Beckett and Boyd (1977) to an apparent purification of 57 fold. They showed that the enzyme was excluded from Sepharose-4B column, and estimated the molecular weight to be about 330,000, with a molecular weight of the eight sub-units to be 41,000 as estimated from SDS-gel electrophoresis of the phosphorylated enzyme. The void volume fraction of cholesterol ester hydrolase eluted from the Sepharose-4B column has a turbid appearance and Beckett (1975) suggested that the enzyme was heavily aggregated and associated with lipids giving the enzyme an apparently high molecular weight. The lipids could be removed by the application of adsorption chromatography using a hydroxylapatite matrix. Because of the lipoprotein nature of cholesterol ester hydrolase, the enzyme may be aggregated to the lipid droplets found in the cytosol of the adrenal and which were characterised by Trzeciak and Boyd (1973) and shown to contain high concentrations of cholesterol ester. It was very difficult to delipidate this enzyme without loss of enzymic activity. The lipid droplets associated with the enzyme caused it to float during centrifugation and a considerable loss of enzyme activity occurred on removing the lipid droplets. However, this flotation property was used to isolate and purify the enzyme from other heavier proteins using gradient centrifugation.

The purified preparation of bovine adrenal ester hydrolase reported by Beckett and Boyd (1977) had not been shown to be specific for cholesteryl oleate hydrolysis, although Beckett (1975) showed that rat adrenal cholesterol ester hydrolase was able to hydrolyse norcholesteryl oleate and pregnenol oleate, and Beckett (1975) concluded that the side-
chain of the cholesterol moiety did not influence the binding of the substrate to the active site of cholesterol ester hydrolase. However, Pittman, Khoo and Steinberg (1975) showed that a 100 fold purified hormone-sensitive lipase isolated from rat adipose tissue catalysed the hydrolysis of cholesteryl oleate, and this activity co-purified with the hydrolysing activity of triacylglycerol with a constant ratio between the two enzymic activities in all fractions assayed. They did not comment on the relationship between the two activities in their fraction.

The experiments described in Section III showed that there is an activatable triacylglycerol hydrolase in the rat adrenal cytosol which is sensitive to ACTH pre-treatment in vivo, and to cAMP preincubation in vitro. These findings were confirmed by the report of Pittman and Steinberg (1977) when they found a cAMP-dependent protein kinase activatable triacylglycerol hydrolase in the rat adrenal cytosol. These findings prompted the investigation on the presence of a cAMP activatable triacylglycerol lipase in the bovine adrenal cortical cytosol.

The experiments in this section were designed to investigate the presence of an activatable triacylglycerol hydrolase in the bovine adrenal cortical cytosol and, to try to isolate it from that source. The relationship between the two activities of cholesterol ester hydrolase and triacylglycerol lipase were explored in the bovine adrenal cytosol.

4.2 TRIACYLGLYCEROL HYDROLASE IN THE BOVINE ADRENAL CORTICAL 105,000 x g SUPERNATANT

The presence of triacylglycerol lipase in the bovine adrenal cortical cytosol was investigated by assaying the 105,000 x g delipidated infranatant, isolated by the procedure described in Section II, using glycerol tri-[^14C]oleate. The assay was performed as described in Section II. Table 4.1 shows there is a triacylglycerol lipase in the
<table>
<thead>
<tr>
<th>Addition to Incubation</th>
<th>Cholesterol ester hydrolase (%)</th>
<th>Triacylglycerol lipase (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (Control)</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>2 mM Mg^{++} (Inactivated)</td>
<td>95</td>
<td>95</td>
</tr>
<tr>
<td>10 μM CAMP + 2 mM ATP/Mg^{++} (Activated)</td>
<td>119</td>
<td>127</td>
</tr>
</tbody>
</table>

Table 4.1. The effect of magnesium ions and cAMP on the activities of bovine adrenocortical cytosolic cholesterol ester hydrolase and triacylglycerol lipase. The results are the percentage value changes from the specific activities of both hydrolytic enzymes (considered as 100%) in control incubations. For details of the pre-incubation with Mg^{++} or cAMP see the methods section.
bovine adrenal cytosol. This activity was compared with the activity of cholesterol ester hydrolase in the same fraction and in the same experiment. Table 4.1 also shows that upon preincubation of the 105,000 x g supernatant with magnesium ions or with cAMP and ATP, triacylglycerol lipase activity was altered by deactivation and activation respectively. This indicates that a cAMP-dependent protein kinase mediated activation of triacylglycerol lipase appears to operate in the bovine adrenal cytosol as it does in the activation of cholesterol ester hydrolase (Beckett and Boyd (1977)). The basal activity of cholesterol ester hydrolase and triacylglycerol lipase varied from one batch of adrenals to another, ranging between 100-300 pmole/min/mg protein and 40-120 pmole/min/mg protein for cholesterol ester hydrolase and triacylglycerol lipase respectively. This fluctuation of the basal activity does not reflect a change of substrate emulsion condition, but rather reflects the state of activation and inactivation of both enzymes in a particular preparation of bovine adrenocortical cytosol. This was deduced from the finding that the activation and inactivation percentage of both enzymes varies from batch to batch and ranges from 20-80%. For this reason, during purification, the degree of purification of either enzyme could not be estimated correctly if the purification was expressed in terms of the specific activity of a particular fraction compared to the specific activity of the starting fraction. The cytosolic endogenous substrate effect on the estimation of the release of the $[^{14}C]$oleic acid was minimised since the amount of protein added per assay did not exceed 200 $\mu$g. This gave an endogenous substrate concentration of cholesterol ester not more than 2-4 $\mu$g, which was only 2-3% of the added substrate concentration.
4.3 PURIFICATION OF TRIACYLGLYCEROL LIPASE AND CHOLESTEROL ESTER HYDROLASE FROM THE BOVINE ADRENOCORTICAL CYTOSOL

The procedure for the purification of bovine adrenal cortical cytosolic triacylglycerol lipase and cholesterol ester hydrolase is summarised in Fig. 4.1.

4.3.1 pH 5.0 preparation

Beckett (1975) reported that cholesterol ester hydrolase was precipitated between 25-35% ammonium sulphate, with a recovery of 40-50% of the specific activity. This procedure however proved to be costly and time consuming regarding the volume of the bovine adrenocortical cytosol as a starting material. Wallat and Kanau (1976) reported that cholesterol ester hydrolase was precipitated between pH 5.8 and 5.0 from bovine adrenal cytosol, with a recovery of 50% which is comparable to that observed using the ammonium sulphate precipitation. In practice the acid titration proved to be much easier with a good recovery of enzymic activity and an apparent purification of between 2 and 3 fold over the original specific activity of the starting solution.

Table 4.2 shows the recovery of cholesterol ester hydrolase and triglyceride lipase in different fractions of the acid titration step. It can be seen that most of the specific activity was recovered between pH 5.8 and pH 5.0. The loss of about half the total activity seemed unavoidable and several attempts to improve the recovery, by decreasing the temperature, activating the enzyme or by addition of mercaptoethanol, were unsuccessful. The recovery of the enzyme activity was similar to the recovery of cholesterol ester hydrolase reported by Wallat and Kanau (1976). When the most active fraction precipitating between pH 5.0 and pH 5.8 had been established, subsequent preparations collecting the total precipitate were carried out acidifying the cytosol from pH7 to pH5 and . This fraction was generally concentrated to about 15-25 ml by ultrafiltration in an Amicon 200 system with Diaflow PM30 membrane. The filtration was carried
Bovine adrenocortical cytosol prepared as described in Fig. 2.1. (Adrenal cytosol)

Titrated to pH 5.0 with 0.1M acetic acid at 4°C and centrifuged for 20 min at 10,000 x g

PELLET SUPERNATANT DISCARDED

Dissolved in 20 mM phosphate buffer pH 7.2, centrifuged at 100,000 x g for 1 h.

PELLET DISCARDED INFRANATANT LIPID DROPLET ASPIRATED (pH 5.0 precipitate) AWAY

Concentrated by ultrafiltration in Amicon system using Diaflow membrane PX 30 (Concentrated pH 5.0 precipitate)

Concentrated pH 5.0 precipitate was applied to a Bio-gel A 150 m, the protein was eluted with the equilibrating buffer which was 20 mM phosphate buffer pH 7.2.

Protein eluted in the void volume of the column was pooled and concentrated by ultrafiltration (Concentrated void volume fraction)

The concentrated void volume fraction was layered carefully on top of a 12 ml pre-formed linear gradient of sucrose (10-40%, w/v) in 20 mM potassium phosphate buffer pH 7.2. The gradient tube was then centrifuged for 40 h at 40,000 rpm in Beckman SW 41 rotor.

Tubes were punctured and fractionated. Approximately the top half of the tube contents were pooled and assayed (10-25% sucrose gradient fraction)

Fig. 4.1. The flow diagram of the purification of bovine adrenocortical cytosolic cholesterol ester hydrolase and triacylglycerol lipase.
<table>
<thead>
<tr>
<th>Fraction from acid titration</th>
<th>Cholesterol Ester Hydrolase (pmole oleic acid/min/mg)</th>
<th>Triacylglycerol Lipase (pmole oleic acid/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adrenal cytosol</td>
<td>None 354 +Mg++ 229 +cAMP 423</td>
<td>None 88 +Mg++ 24 +cAMP 111</td>
</tr>
<tr>
<td>pH 7.4-pH 5.8 precipitate</td>
<td>pH 7.4-pH 5.8 precipitate 589 +Mg++ 278 +cAMP 704</td>
<td>pH 7.4-pH 5.8 precipitate 142 +Mg++ 105 +cAMP 145</td>
</tr>
<tr>
<td>pH 5.8-pH 5.4 precipitate</td>
<td>pH 5.8-pH 5.4 precipitate 735 +Mg++ 359 +cAMP 1024</td>
<td>pH 5.8-pH 5.4 precipitate 234 +Mg++ 208 +cAMP 230</td>
</tr>
<tr>
<td>pH 5.4-pH 5.0 precipitate</td>
<td>pH 5.4-pH 5.0 precipitate 625 +Mg++ 443 +cAMP 672</td>
<td>pH 5.4-pH 5.0 precipitate 152 +Mg++ 134 +cAMP 136</td>
</tr>
<tr>
<td>post pH 5.0 supernatant</td>
<td>post pH 5.0 supernatant 64 +Mg++ 45 +cAMP 77</td>
<td>post pH 5.0 supernatant n.d. n.d. n.d.</td>
</tr>
</tbody>
</table>

Table 4.2. The recovery of bovine adrenocortical cytosolic cholesterol ester hydrolase and triacylglycerol lipase through the acid titration of the adrenal cytosol with 0.1M acetic acid. Each fraction was assayed after pre-incubation (10 min) with buffer, 2 mM Mg++ or 10 uM cAMP/2 mM ATP. Acid titration precipitate fractions were collected by centrifugation and were suspended in potassium phosphate buffer pH 7.4.
out at 4°C under nitrogen gas, and the eluted filtrate was checked for activity but none was detected. The concentrated fraction was then centrifuged at 100,000 x g for 1 hour and the floating lipid droplets were removed. The clear infranatant was pooled and designated concentrated pH 5.0 fraction.

4.3.2 Gel filtration of cholesterol ester hydrolase and triacylglycerol lipase

The concentrated pH 5.0 fraction was further purified on a Biogel A150m column (46 cm x 3 cm). A volume of the pH 5.0 precipitated fraction equivalent to 4-6% of the total column volume, was layered on top of a Biogel column which had been previously equilibrated with 20 mM phosphate buffer pH 7.4. The column was then eluted with the same buffer and 5 ml fractions were collected. These fractions were assayed for cholesterol ester hydrolase, triacylglycerol lipase and protein concentration. Fig. 4.2 shows the elution profile of a typical experiment. It may be noted that about 75% of the total applied enzymic activity was retained on the column and 20-25% was excluded. The protein content of the void volume fraction was very little compared to the amount retained on the column. The similarities in the elution patterns of cholesterol ester hydrolase and triacylglycerol lipase were consistent in all experiments and agreed with the findings of Pittman and Steinberg (1977) on the elution from 6% Agarose Gel of these two enzymes obtained from rat adrenal cytosol.

The void volume fractions could not be retarded by the column even after a mild delipidation process had been carried out on the concentrated pH 5.0 fraction before application to the Biogel column. Fig. 4.3 shows the elution profile of cholesterol ester hydrolase and triglyceride lipase from a lyophilised concentrated pH 5.0 fraction which had been delipidised by homogenisation of the powder with butanol and acetone followed by further homogenisation of the delipidised material in buffer.
Fig. 4.2. The elution profile of bovine adrenocortical cytosolic cholesterol ester hydrolase and triacylglycerol lipase in Biogel A 150m column (46 x 3cm). Concentrated pH 5.0 precipitate of bovine adrenocortical cytosol, prepared as described in Fig. 4.1, was applied to the column which was pre-equilibrated with potassium phosphate buffer pH 7.4. The column was eluted with the same buffer and fractions of 5ml each were assayed for cholesterol ester hydrolase, triacylglycerol lipase and protein concentration as described in the methods section.
Fig. 4.3. The elution profile of bovine adrenocortical cytosolic cholesterol ester hydrolase and triacylglycerol lipase using a Biogel A 150m column. Butanol extract of bovine adrenal cortical cytosol, prepared as described in Fig. 4.4, was eluted through the column under identical conditions to that described in the legend of Fig. 4.2.
It appears that this protein is firmly bound to lipid and is not easily removed by this mild delipidation process already shown to be effective in the removal of cholesterol from bovine mitochondria (R. Hume, Personal Communication). On the other hand no improvement was achieved regarding the recovery of the enzyme in this fraction neither was the retained fraction significantly altered. Although no kinetic experiments were carried out on those two fractions, it appears that the retarded peak might be just another form of enzymic aggregate in which the ratio of lipid-protein may be different. This could also be explained by the fact that this fraction had a very high protein content per ml offering it a good hydrophobic environment and a higher generated protein-protein stabilising force. Huttunen and Steinberg (1971) reported that the addition of bovine albumin to purified hormone-sensitive lipase prolonged the time of stabilisation of the enzyme. The relationship between the two peaks from the Biogel column was not investigated thoroughly. However Pittman, Golanty and Steinberg (1972) showed that hormone-sensitive lipase from rat adipose tissue was eluted from a 4% Biogel column in two peaks, the retarded peak having a density greater than that of the excluded peak, the latter's density being less than $d_{1.12}$. These workers also showed that both fractions were sensitive to cAMP activation. Recently Pittman and Steinberg (1977) found that rat adrenal cytosol contains triacylglycerol lipase which was eluted from the 6% agarose column in two peaks. The fraction excluded from the gel had a lower $K_m$ for glycerol trioleate than the retained fraction which was also more dense, being $d_{1.12}$, in sucrose solution.

The void volume fraction from the Biogel A-150m column of cholesterol ester hydrolase and triacylglycerol lipase was thought to be a large aggregate of the enzyme with lipid. It was decided to test whether the enzyme's void volume could be retarded on the column if a mild delipidation
process was carried out on the protein prior to its application to the column. Before testing this theory, the effect of the delipidation process was determined on a pH 5.0 precipitate prepared as described earlier in this section. The pH 5.0 precipitate was dialysed against 1 mM potassium phosphate buffer overnight, then dialysed against water for three hours before lyophilisation. The lyophilised material was then homogenised with cold butanol at -18°C, quickly centrifuged at 10,000 x g for 1 min at -18°C, and the pellet was resuspended, by homogenisation, in cold acetone at -18°C and filtered under vacuum. The protein was then quickly dried in a vacuum desiccator for 30 min, after which it was homogenised in 20 mM phosphate buffer and centrifuged at 100,000 x g for 1 hour. The clear infranatant was designated as the Butanol powder extract. Fig. 4.4 summarises the steps used to prepare such an extract.

This extracted fraction was assayed for cholesterol ester hydrolase and triacylglycerol lipase. Table 4.3 shows the activity of these enzymes in different fractions together with the enzymatic specific activity recovered in each fraction. As can be seen this mild delipidation did not significantly affect the enzyme activity. There was a slight loss which may be due to loss of denatured protein and labile protein. It was shown that this procedure was very effective in reducing the amount of cholesterol in the mitochondria of the bovine adrenal cortex without affecting the cytochrome P450 enzyme system of the cholesterol side-chain cleavage reaction (R. Hume, Personal Communication). However after storing the butanol powder extract at -18°C for four days, almost 50% of the cholesterol ester hydrolase and triacylglycerol lipase was lost. This may be due to the reduction of a stabilisation effect due to the lipid content of these two enzymes. Huttunen and Steinberg (1971) found that the addition of minute amounts of glycerol trioleate to a 100-fold purified hormone-sensitive lipase isolated from rat adipose tissue cytosol, prolonged the stabilisation time of the enzyme activity.
Concentrated pH 5.0 precipitate of bovine adrenal cytosol prepared as described in Fig. 4.1.

The fraction was dialysed overnight against potassium phosphate buffer pH 7.4, then dialysed for three hours against distilled water at 4°C.

The dialysed fraction was lyophilised overnight.

The dried material was homogenised in 25 volumes of cold butanol (-18°C) in dry glass homogeniser. The homogenate was centrifuged for 1 min at 10,000 x g in MSE centrifuge at -30°C.

The supernatant was discarded, the pellet was homogenised briefly in cold acetone (redistilled).

The acetone-protein homogenate was filtered and the protein cake was washed quickly several times with cold acetone.

The protein cake was dried in a vacuum desiccator for 30 min, then homogenised in potassium phosphate buffer pH 7.4 and centrifuged for 1 h at 100,000 x g at 4°C.

Any pellet sedimented after centrifugation was discarded and the clear infranatant was carefully collected and designated BUTANOL EXTRACT.

Fig. 4.4. The delipidation of bovine adrenocortical cytosol by butanol/acetone extraction.
<table>
<thead>
<tr>
<th>Fraction</th>
<th>Cholesterol ester hydrolase*</th>
<th>Recovery (%)</th>
<th>Triacylglycerol lipase*</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adrenal cytosol</td>
<td>499±23</td>
<td>100</td>
<td>70±14</td>
<td>100</td>
</tr>
<tr>
<td>pH 5.0 precipitate</td>
<td>1193±13</td>
<td>56</td>
<td>173±31</td>
<td>58</td>
</tr>
<tr>
<td>Butanol powder extract</td>
<td>1142±22</td>
<td>50</td>
<td>135±13</td>
<td>42</td>
</tr>
</tbody>
</table>

Table 4.3. The effect of delipidation and lyophilisation of bovine adrenocortical cytosolic cholesterol ester hydrolase and triacylglycerol lipase. For the details of the delipidation process see Fig. 4.1 and Fig. 4.4 respectively. The total recovery of each fraction are expressed in percentage value of the total activities of both hydrolytic enzymes in the adrenal cytosol.

*Enzymic activities are pmole oleic acid produced/min/mg protein.
After the effect of the mild delipidation process on cholesterol ester hydrolase and triacylglycerol lipase activity had been established, a purification experiment was carried out using the butanol extract instead of the concentrated pH 5.0 precipitate fraction. A fresh batch of bovine adrenal supernatant was used, the butanol extract was prepared as in Fig. 4.4, and applied to the same Biogel column. The butanol extract was eluted with 20 mM potassium phosphate buffer pH 7.4 and fractions eluted from the column were assayed for cholesterol ester hydrolase and triacylglycerol lipase. In Fig. 4.3 the elution profile of the butanol extract is shown. As can be seen from the figure, cholesterol ester hydrolase and triacylglycerol lipase both gave two peaks, one emerging at the void volume. Although the peaks seem to be eluted about two fractions earlier than in the pH 5.0 elution pattern, the overall elution pattern is identical in terms of protein curve and the very slight shift may be due to the differences in protein content in the two experiments. However, the aim of the experiment was not achieved, in that the void volume peak could not be retained on the column by this mild delipidation process showing that the enzymic activities have a very strong protein-lipid interaction which could not be freed without losing the enzyme activity. Also from this experiment little had been gained in terms of recovery of the enzyme activity, and the ratio between the two peaks remained identical in both the pH 5.0 and the butanol extracted material. For this reason, in subsequent experiments, the butanol extract stage was not included in the purification scheme.

The emergence of the void volume fraction of cholesterol ester hydrolase and triacylglycerol lipase coincided with very low protein contents. For this reason, this fraction was used for the subsequent purification step or for routine assays. The lower contaminating protein content and the ease by which this fraction could be identified spectrophotometrically at 280 nm made it suitable for these purposes.
4.3.3 Flo-tation of cholesterol ester hydrolase and triacylglycerol lipase in sucrase gradient ultracentrifugation

In the report of Huttunen and Steinberg (1971), hormone-sensitive lipase was purified by ultracentrifugation of a pH 5.2 precipitate, prepared from rat adipose tissue cytosol, in a dense medium of sucrose. It was reported that the active enzyme was less dense than d 1.12. From that report, it was decided to carry the purification of bovine adrenal cytosolic cholesterol ester hydrolase and triacylglycerol lipase one step further than the Biogel A column. A linear gradient of sucrase solution was prepared in an ultracentrifuge tube to give a 10-40% (w/v) sucrase concentration. This gradient was then topped very carefully with a 5% total volume of concentrated Biogel A150m void volume fraction. The sucrase gradient, with the material on top of it was centrifuged at 40,000 rpm in a Beckman SW41 rotor for 43 hour at 4°C. The gradient tubes were then punctured at the bottom, and fractions were pumped out by a peristaltic pump. Each fraction was analysed for the cholesterol ester hydrolase, triacylglycerol lipase and protein concentration. In Fig. 4.5 the result of such an experiment is shown. It can be observed that, no separation of the two enzymic activities occurred. It can also be noted that the point of the gradient at which cholesterol ester hydrolase and triacylglycerol lipase were floated was about 15% (w/v) sucrase solution which is similar to the flotation pattern of hormone sensitive lipase isolated from rat adipose tissue (Huttunen, Ellingboe, Pittman and Steinberg (1970)).

By this purification stage the amount of enzymic protein was so low that it was impractical to carry out any further purification steps, although a trial stepwise sucrase gradient was attempted, but no further apparent purification was achieved. The usual purification achieved at this stage was 22-25 fold of the supernatant enzymic activity.
Fig. 4.5. Distribution of cholesterol ester hydrolase and triacylglycerol lipase of bovine adrenocortical cytosol in linear sucrose gradient.
Concentrated void volume fraction of both enzymes activities, prepared as described in Fig. 4.1, was layered on top of pre-formed sucrose gradient (10-40%, w/v) in 20mM potassium phosphate buffer pH 7.4 and the gradient tubes were centrifuged in Beckman ultracentrifuge using SW 41 rotor at 40,000 rpm for 40h at 4°C. After centrifugation, tubes were punctured and fractions were assayed for cholesterol ester hydrolase and triacylglycerol lipase as described in the methods section.
Table 4.4. The purification of bovine adrenocortical cytosolic cholesterol ester hydrolase and triacylglycerol lipase.

<table>
<thead>
<tr>
<th>pH</th>
<th>Grad 10-25% Sucrose</th>
<th>Gradient 0.5</th>
<th>Gradient 4.4</th>
<th>Gradient 2.2</th>
<th>pH 5.0 ppt</th>
<th>Recovery 100</th>
<th>Recovery T</th>
<th>Recovery 74</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.6</td>
<td>8.6 1890 7.25 21.5 5</td>
<td>3060</td>
<td>5</td>
<td>21.5</td>
<td>705</td>
<td>0.5</td>
<td>74</td>
<td>100</td>
</tr>
<tr>
<td>1.4</td>
<td>14.4 6.8 506</td>
<td>4.4</td>
<td>183</td>
<td>4.4</td>
<td>368</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1.4 2.4</td>
<td>2.4</td>
<td>2.4</td>
<td>14.4</td>
<td>8.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1.4 2.4</td>
<td>1.4</td>
<td>1.4</td>
<td>1.4</td>
<td>1.4</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Specific activities are mmole oleic acid produced/min/mg protein.

The ratio of activity of cholesterol ester hydrolase to triacylglycerol lipase.
Through all the purification steps both cholesterol ester hydrolase and triacylglycerol lipase were enriched at a constant ratio. In Table 4.4 the purification of cholesterol ester hydrolase and triacylglycerol lipase is described in a typical experiment together with the recoveries of both enzymes.

4.4 SUMMARY

1. Bovine adrenocortical cytosol has the ability to hydrolyse glycerol trioleate by a triacylglycerol lipase.

2. The enzymic hydrolysis of glycerol trioleate is controlled in vitro by activation by cAMP, and inactivation by a process involving magnesium ion.

3. Cholesterol ester hydrolase and triacylglycerol lipase were co-precipitated by acid titration and the precipitate was shown to be sensitive to activation by cAMP preincubation in the case of the cholesterol ester hydrolase but not the triacylglycerol lipase.

4. Cholesterol ester hydrolase and triacylglycerol lipase were co-chromatographed in an agarose gel yielding two fractions, one being eluted at the void volume of the column and the other at twice the void volume. The former fraction could not be retained in the gel by a mild delipidation process.

5. Cholesterol ester hydrolase and triacylglycerol lipase floated at a density of $d \approx 1.2$ in a sucrose gradient.

6. Apparent purification of both enzymes were 22 fold, and their ratio in each purification step was constant.
SECTION V

STUDIES ON THE PARTIALLY PURIFIED BOVINE ADRENOCORTICAL CYTOSOLIC CHOLESTEROL ESTER HYDROLASE AND TRIACYLGLYCEROL LIPASE

5.1 Introduction

5.2 The protein concentration

5.3 The time course of the assay of bovine adrenal hydrolases

5.4 The comparative effects of cAMP and cGMP on the stimulation in vitro of partially purified cholesterol ester hydrolase

5.5 The activation and phosphorylation of partially purified bovine adrenocortical cholesterol ester hydrolase and triacylglycerol lipase

5.6 The effect of organophosphate insecticide chloropyrifos oxone on the activities of bovine adrenocortical cholesterol ester hydrolase and triacylglycerol lipase

5.7 Chloropyrifos oxone and the processes of activation and inactivation of bovine adrenal cholesterol ester hydrolase

5.8 The comparative hydrolysis of cholesteryl oleate and cholesteryl linoleate by partially purified bovine adrenocortical cholesterol ester hydrolase

5.9 An attempt to the study of the site and number of phosphorylation of bovine adrenocortical cholesterol ester hydrolase

5.10 The effect of cholesteryl oleyl ether on bovine adrenocortical cholesterol ester hydrolase and triacylglycerol lipase

5.11 Summary
SECTION V: STUDIES ON THE PARTIALLY PURIFIED BOVINE ADRENOCORTICAL CYTOSOLIC CHOLESTEROL ESTER HYDROLASE AND TRIACYLGLYCEROL LIPASE

5.1 INTRODUCTION

The purification scheme of bovine adrenocortical cholesterol ester hydrolase which was described in the previous section, did not differentiate this activity from that of triacylglycerol lipase. This failure of the conventional separating methods is consistent with the finding of Pittman and Steinberg (1977) that rat adrenal cytosol contains cholesterol ester hydrolase which copurifies with triacylglycerol lipase in the same fraction. However these authors found that ammonium sulphate fractionation and organo-phosphate insecticide inhibition studies resulted in the differentiation between the cholesterol ester hydrolase and triacylglycerol lipase activities in rat adrenal cytosol (Pittman and Steinberg (1977)). From the experiments described in Section IV, acid titration of bovine adrenal cytosol did not produce a clear cut separation between cholesterol ester hydrolase and triacylglycerol lipase activities, and in the best purified fraction described in the previous section, these two activities remained associated in a manner which was difficult to resolve. Accordingly, experiments were designed to test the chemical approach to the problem using the bovine adrenal cytosol which was purified to and including the Biogel A column chromatography step as described in the previous section. Studies were made on this fraction because it has a high specific activity of both cholesterol ester hydrolase and triacylglycerol lipase. It is also easily obtainable in large quantities and relatively free from a number of other proteins.

5.2 THE PROTEIN CONCENTRATION

The effect of the protein concentration on the assay of cholesterol ester hydrolase and triacylglycerol lipase was investigated using the Biogel A void volume fraction of the partially purified enzyme of bovine
adrenal cortical cytosol. These experiments were performed early in this study when the assay was established as described in Section II. Fig. 5.1 shows that the cholesterol ester hydrolase and triacylglycerol lipase activities assay is linear only up to 200 µg protein per assay which fits well with the original assay devised by Khoo, Steinberg, Huang and Vagelos (1976). The experiments in these studies thereafter were carried out with a protein concentration not exceeding 200 µg per assay. There is another advantage in the use of smaller amounts of protein and that this reduces the endogenous unlabelled substrate to a limit of not more than 2 µg cholesteryl esters in the assay mixture since Trzeciak and Boyd (1974) found that delipidated bovine adrenal cortical cytosol contains cholesterol ester at a concentration less than 10 µg/mg protein. Thus in the assay used in these studies, the added labelled substrate is 94 µg cholesteryl[^1-14]C oleate, about 47 times the maximum endogenous substrate present in the crude cytosol preparation. Hence, the dilution of the labelled substrate was minimised greatly which could affect the estimation of the apparent activity of both enzymes. This is in contrast with the assay of Naghshineh, Treadwell, Gallo and Vahouny (1978), who reported that their assay was linear up to 9 mg protein. This seems unlikely as bovine adrenocortical cytosol has cholesterol ester hydrolase of specific activity 170 nmoles/mg protein/hour and as these workers used 150 nmoles[^1-14]C cholesteryl oleate, the substrate will be depleted in less than 6 min in an assay reported to be linear for up to 2 hours. However, in the assay system used in the studies reported in this thesis, the total substrate used in the 30 min incubation never exceeded 10% of the added substrate even when using the highest purified fraction isolated as described in the previous section.
Fig. 5.1. The effect of protein concentration on the hydrolysis of cholesteryl\(\Delta^{14}\)oleate and glyceryl tri-\(\Delta^{14}\)oleate by purified preparation (Biogel void volume, Fig. 4.1) of bovine adrenal cholesterol ester hydrolase and triacylglycerol lipase respectively. The assay of both enzymic activities was conducted as described in the methods section except various amounts of Biogel void volume fraction protein were used as indicated.
Fig. 5.2. Time course of the hydrolysis of cholesteryl\[^{14}C\]oleate (A) and glyceryl tri-[\(^{14}C\)]oleate (B) by purified adrenocortical cholesterol ester hydrolase and triacylglycerol lipase (Biogel void volume fraction, Fig. 4.1) respectively. The assays of both enzyme activities were performed exactly as described in the methods section except the hydrolysis reaction was terminated at the indicated time.
5.3 THE TIME COURSE OF THE ASSAY OF CHOLESTEROL ESTER HYDROLASE AND
TRIACYLGLYCEROL LIPASE

This experiment was also performed during the early stages of these
studies. The hydrolysis of cholesteryl oleate was measured at intervals
during a 30 minute incubation and the result is shown in Fig. 5.2. It was
found that the rate of hydrolysis was linear over a 30 min incubation
period at 37°C using Biogel A void volume fraction of bovine adreno-
cortical cytosolic cholesterol ester hydrolase and triacylglycerol lipase
isolated as described in Section IV. It was decided therefore to use a
30 min incubation period for the routine analysis of cholesterol ester
hydrolase and triacylglycerol lipase activities.

5.4 THE COMPARATIVE EFFECTS OF CYCLIC AMP AND CYCLIC GMP ON THE
STIMULATION IN VITRO OF PARTIALLY PURIFIED CHOLESTEROL ESTER HYDROLASE

Rat adrenal mitochondrial steriodogenesis is stimulated by ACTH,
and the process is mediated by a cAMP-dependent protein kinase phosphoryl-
ation (for reviews see Halkerston (1976), Schulster (1974) and Gill
(1979)). It is thought that the interaction of ACTH with its receptor
in the adrenal cell membrane results in the stimulation of the membrane
adenylate cyclase with the consequent rise of intracellular concentration
of cAMP (Schimmer, Ueda and Sato (1968), Lefkowitz, Roth, Pricer and
Pastan (1970)). However this view is not shared by Sharma, Ahmed and
Shanker (1976) who reported that low concentrations of ACTH added to
isolated rat adrenal cell preparations increased cGMP concentration
without any detectable changes in the concentration of cAMP when adrenal
steroidogenesis was activated. These authors concluded that cAMP is not
the second messenger of ACTH in the adrenal cell, and cGMP is the
physiological mediator of ACTH (Berchellet, Shanker and Sharma (1978)).
This conclusion is not supported by recent reports that ACTH stimulated
the rise of cAMP which preceded the rise in steroidogenesis in adrenal
Fig. 5.3. The effect of cyclic AMP vs cyclic GMP on the activation in vitro of bovine adrenocortical cytosolic cholesterol ester hydrolase (purified up to and including the Biogel column chromatography). The activation of the enzyme was carried out as described in the activation procedure, detailed in the methods section, except cyclic AMP and cyclic GMP concentrations were varied as indicated. The assay of cholesterol ester hydrolase was started after the 10 min activation period elapsed, by the addition of labelled cholesteryl oleate emulsion as described in the methods section.
cells (Podesta, Milani, Steffen and Neher (1979), Sala, Hayashi, Catt and Duffau (1979) and Laychock and Hardman (1978)). It was of interest to compare the abilities of both cyclic AMP and cGMP in the stimulation of the activity of partially purified bovine adrenocortical cholesterol ester hydrolase, since this enzyme plays a possible role in the response of adrenal steroidogenesis to an ACTH signal. No effect of cGMP by itself on cholesterol ester hydrolase of rat adrenal was observed by Khoo, Sperry, Gill and Steinberg (1977) but the addition of purified bovine lung cGMP-dependent protein kinase plus cGMP resulted in the activation of cholesterol ester hydrolase of rat adrenal cytosol.

Partially purified bovine adrenocortical cholesterol ester hydrolase of the Biogel A void volume fraction was used in these experiments. The protein was pre-incubated for 10 min with the indicated concentration of the cyclic nucleotide in the presence of 2 mM ATP and 2 mM Mg++. The control tubes contained no cyclic nucleotide. After the incubation, the assay of cholesterol ester hydrolase was conducted as described in Section II. Fig. 5.3 shows such an experiment and clearly illustrates that, at the physiological level of cAMP found in the adrenal cell, maximum stimulation was achieved of cholesterol ester hydrolase while cGMP at the same concentration did not affect the enzyme. The apparent Km for cAMP lies between 30-80 nM which is similar to that found for adipose tissue hormone sensitive lipase (Huttunen and Steinberg (1971)). Thus cAMP produced the stimulation in vitro of partially purified bovine adrenal cortical cholesterol ester hydrolase, while cGMP at the same concentration of cAMP did not affect the enzyme. These findings contribute to the overall concept, that cAMP rather than cGMP is the second messenger in the stimulation of adrenal steroidogenesis by ACTH, a process requiring the availability of the precursor cholesterol (Boyd, Arthur, Beckett, Mason and Trzeciak (1975)) which could be furnished by...
the hydrolysis of esterified cholesterol stored in the lipid droplets bathing in the cell cytosol, and catalysed by the cytoplasmic cholesterol ester hydrolase.

5.5 THE ACTIVATION AND PHOSPHORYLATION OF PARTIALLY PURIFIED BOVINE ADRENOCORTICAL CHOLESTEROL ESTER HYDROLASE AND TRIACYLGlycerol LIPASE

The activation of bovine adrenocortical cytosolic cholesterol ester hydrolase was suggested to be through the phosphorylation of the enzyme protein (Trzeciak and Boyd (1974)). This was later confirmed by Beckett and Boyd (1977). In this process the transfer of the terminal phosphate group of ATP to the enzyme protein is accompanied by the activation of the enzyme as reflected by an increase in enzymic specific activity. The process is dependent on cAMP, which activates a less active protein kinase. The latter in turn mediates the transfer of the phosphate group from ATP on to the enzyme protein. The correlation of the activation of cholesterol ester hydrolase and the increased acid precipitable phosphoprotein was considered as evidence for the phosphorylation process. Likewise, rat adipose tissue hormone-sensitive lipase is activated by a phosphorylation reaction mediated by a cAMP-dependent protein kinase, and the correlation between these two parameters was used as evidence for the phosphorylation of the enzyme protein (Huttunen and Steinberg (1971)).

In Sections III and IV, experimental evidence is provided which shows that pre-incubation of rat and bovine adrenocortical cytosol with ATP and cAMP causes a marked increase in the specific activity of both triacylglycerol lipase and cholesterol ester hydrolase, and this activation is proposed to be through a phosphorylation reaction. To confirm such a proposal, the following experiment was performed with partially purified bovine adrenal cholesterol ester hydrolase and triacylglycerol lipase from the Biogel A void volume fraction isolated as described in Section IV.
Fig. 5.4. The in vitro activation and phosphorylation of bovine adrenocortical cholesterol ester hydrolase and triacylglycerol lipase. Purified enzyme preparation (Biogel void volume fraction, Fig. 4.1) was incubated with Mg$^{++}$ ions for 20 min to deactivate both enzymic activities then the activation and phosphorylation reaction was started by the addition of cAMP and $\gamma-^32\text{P}ATP$. For details see text.
In order to measure the phosphorylation and activation of cholesterol ester hydrolase and triacylglycerol lipase, an inactivated enzyme preparation was obtained by incubating the Biogel A void volume fraction of bovine adrenal cytosol with 5 mM Mg\textsuperscript{++} for 20 min. This procedure was shown to reversibly deactivate cholesterol ester hydrolase to a basal level (Beckett 1975). The deactivated enzyme preparation was incubated in duplicate tubes with 200 μg rabbit skeletal muscle cAMP-dependent protein kinase, 10 μM cAMP and 50 μM\[^{32}\text{P}\]ATP (131 μCi/umole). The reaction was started by the addition of\[^{32}\text{P}\]ATP and aliquots of 0.2 ml were withdrawn at timed intervals for subsequent assay of cholesterol ester hydrolase, triacylglycerol lipase and protein bound\[^{32}\text{P}\]radioactivity. The assay of cholesterol ester hydrolase and triacylglycerol lipase were carried out as described in Section II, and determination of\[^{14}\text{C}\]oleic acid was performed after all of the radioactive\[^{32}\text{P}\]phosphate had disintegrated. Protein bound\[^{32}\text{P}\]activity was determined by the method of De Lange, Kemp, Riley, Cooper and Krebs (1968) as described by Beckett (1975), in which 0.2 ml of the incubation mixture was withdrawn into another tube containing 2 ml ice cold 10% trichloroacetic acid followed by the addition of 0.2 ml bovine serum albumin (6.25 mg/ml) as a carrier protein and the mixture was vortexed. After keeping the tube in ice bath for 10 min, it was centrifuged and the protein pellet was dissolved in 0.2 ml 1N NaOH and quickly re-precipitated with 2 ml 10% trichloroacetic acid. After a further 10 min standing in an ice bath the tube was once more centrifuged. The protein pellet was washed twice with 5% trichloroacetic acid and dissolved in 0.2 ml 23M formic acid. The acid dissolved phosphoprotein was poured into a scintillation vial containing 10 ml Triton-Toluene scintillation cocktail (prepared as described in Section II) and the\[^{32}\text{P}\]radioactivity was measured in a Packard Tri Carb scintillation spectrometer.
Fig. 5.4 summarises the results of this experiment in which each point is the mean of duplicate determinations. It can be seen that there is a close correlation between the activation of both cholesterol ester hydrolase and triacylglycerol lipase and the incorporation of radioactive phosphate into acid-precipitable protein, especially in the first 6 min during which time the activation of cholesterol ester hydrolase and triacylglycerol lipase reached their maxima. The similarity in the course of activation of both enzymes was also observed in this experiment although there is a very slight difference between the two enzymes in terms of the percentage activation achieved. The figure also shows that the phosphorylation and activation processes are very rapid, which is consistent with the finding of Beckett and Boyd (1977), and Huttunen and Steinberg (1971) but not of Naghshineh, Treadwell, Gallo and Vahouny (1978). However, the latter group were using a different sampling time intervals of 15 min and this might be the cause of the difference. The assay used for cholesterol ester hydrolase and triacylglycerol lipase in our studies, is more powerful in halting the activation and phosphorylation reaction because of the presence of enough EDTA to chelate Mg$^{++}$ ions, thus the degree of activation of replicate incubation tubes is similar.

5.6 **THE EFFECT OF ORGANOPHOSPHATE INSECTICIDE CHLOROPYRIFOS OXONE ON THE ACTIVITY OF PARTIALLY PURIFIED BOVINE ADRENOCORTICAL CHOLESTEROL ESTER HYDROLASE AND TRIACYLGlycerol LIPASE**

The role of free cholesterol as a precursor of adrenal corticoids was postulated by Sayers, Sayers, Liang and Long (1946). Since most of the adrenal cholesterol is esterified to long chain fatty acids stored in the lipid droplets of the cytosol, a mechanism must exist to mobilise this esterified cholesterol for use in adrenal mitochondrial steroidogenesis. Davis and Garren (1966) found that ACTH administration to hypophysectomised
rats stimulated adrenal steroidogenesis with a simultaneous decrease of the lipid droplet's cholesterol ester stores. Then the report of Trzeciak and Boyd (1973) correlated this decrease of adrenal cholesterol esters in response to ACTH administration *in vivo* with the activation of cytosolic cholesterol ester hydrolase. Subsequent work of Boyd, Arthur, Beckett, Mason and Trzeciak (1975) suggested that the supply of free cholesterol to adrenal mitochondria is one of the factors regulating adrenal steroidogenesis and this supply of free cholesterol is under hormonal control through regulation of cytosolic cholesterol ester hydrolase. That report placed cholesterol ester hydrolase as a possible factor regulating the events between the ACTH interaction with the adrenal cell membrane and corticosterone production from the adrenal. This postulate was supported by the findings of Civen, Brown and Morin (1977) who found that isolated rat adrenal cell preparations responded to ACTH and cAMP by increased production of corticoids. Moreover, this stimulation of adrenal steroidogenesis in isolated cells can be blocked by the addition to the medium of minute amounts of chloropyrifos oxone, an organophosphate insecticide known to be an inhibitor of plasma and erythrocyte acetylcholine esterase (Civen, Lifrack and Brown (1977)). The inhibitory action of organophosphate on adrenal steroidogenesis does not affect the intracellular concentration of cAMP in the adrenal cell, nor does the insecticide affect corticoid production of isolated adrenal cells with added pregnenolone *in vitro* (Civen, Lifrack and Brown (1977)), and it was concluded by these authors that organophosphate insecticides act at a level beyond cAMP formation and before pregnenolone synthesis. It was found that chloropyrifos oxone inhibited the hydrolysis and esterification of rat adrenal cholesterol esters, and cholesterol ester hydrolase was inhibited at a very low concentration of the organophosphate insecticides added to adrenal cytosol (Pittman and Steinberg
Cholesterol ester hydrolase was found to be more sensitive to the organophosphate insecticide than triacylglycerol lipase. However, Pittman and Steinberg (1977) used for this differential inhibition experiment crude rat adrenal cytosol, and they found that upon purification of this fraction by gel chromatography, the activity of cholesterol ester hydrolase coincided with the activity of triacylglycerol lipase. It was therefore decided to assay the effect of organophosphate insecticide chloropyrifos oxone on the partially purified bovine adrenocortical cholesterol ester hydrolase obtained from the Biogel A column chromatography step (prepared as described in Section IV) which also possesses triacylglycerol lipase activity. This fraction has a very high enzymic specific activity of both cholesterol ester hydrolase and triacylglycerol lipase, thus any small change in enzymic specific activity due to the inhibitor, might be detected. This is especially so in the case of triacylglycerol lipase, the specific activity of which is less than that of cholesterol ester hydrolase.

Fig. 5.5 illustrates the effect of chloropyrifos oxone on both cholesterol ester hydrolase and triacylglycerol lipase. Addition of chloropyrifos oxone 1 hour before the assay of enzymic activity resulted in a dose-dependent inhibition of cholesterol ester hydrolase and triacylglycerol lipase with 50\% inhibition occurring between $10^{-8}$-$10^{-7}$ M chloropyrifos oxone. In two identical experiments no dissociation of cholesterol ester hydrolase and triacylglycerol lipase was observed. The failure of this powerful inhibitor to differentiate between the two enzymic activities is not because of different procedures in applying the inhibitor to the enzyme preparation, since the procedure and the time of incubation of the inhibitor was identical to that used by Pittman and Steinberg (1977). The different result could be attributed to species differences but is more likely to be due to different protein fractions.
Inhibition by chloropyrifos oxone of bovine adrenal soluble cholesterol ester hydrolase (●—●) and triglyceride lipase (▲—▲)

![Chemical Structure of Chloropyrifos Oxone](image)

\[ \text{Chloropyrifos oxone} \]

**Fig. 5.5.** The inhibition of bovine adrenocortical cholesterol ester hydrolase and triacylglycerol lipase by chloropyrifos oxone.

Purified preparation of both enzyme activities (Biogel void volume fraction isolated as described in Fig. 4.1) was incubated with the indicated concentration of chloropyrifos oxone (delivered in 5 µl ethanol) for 1 h at 37°C before the enzymic assay was started as described in the methods section. Enzymic specific activities in control incubation (enzyme incubated with ethanol only) were considered 100%, and the activity of both enzymes in the test incubations was referred to that figure.
used in this experiment and in the Pittman et al assays. Recently Pittman and Steinberg (personal communication (1979)) found that chloropyrifos oxone failed to give consistent results in dissociating cholesterol ester hydrolase and triacylglycerol lipase of chicken adipose tissue.

This experiment shows the strong relationship between cholesterol ester hydrolase and triacylglycerol lipase enzymic activities which purified together and were sensitive to this toxic inhibitor to such a similar degree. The results so far described in this section and elsewhere, do not give any clear cut answer as to whether we are dealing with two enzymes having a similar regulatory site or a single enzyme complex that is regulated by one active site.

5.7 CHLOROPYRIFOS OXONE AND THE PROCESSES OF ACTIVATION AND INACTIVATION OF BOVINE ADRENAL CHOLESTEROL ESTER HYDROLASE

Pittman and Steinberg (1977) reported that rat adrenal cytosolic cholesterol ester hydrolase was inhibited to 2% of original specific activity when the enzyme was incubated with 1 μM chloropyrifos oxone, and this inhibition was reversed by incubation for 18 hour at 23°C with 100 mM NaF to 70% of the uninhibited enzyme activity.

From the experiments described in sub-section 5.6, 50% inhibition of cholesterol ester hydrolase was achieved between 10^{-8}-10^{-7} M chloropyrifos oxone, and it was decided to investigate the effect of activation-deactivation on the chloropyrifos oxone-inhibited bovine adrenocortical cholesterol ester hydrolase purified via the Biogel A fraction as described in Section IV. Bovine cholesterol ester hydrolase was incubated with 50 nM chloropyrifos oxone in ethanol for 60 min at 37°C. The excess inhibitor was removed by desalting the incubation mixture on a Sephadex G25 column equilibrated with 20 mM phosphate buffer pH 7.4 and eluted with the same buffer. The desalted protein was then pre-incubated
with cAMP plus ATP/Mg\(^{++}\), or with Mg\(^{++}\) only, for 10 minutes as described in the method section. A control incubation mixture was run in parallel but contained no chloropyrifos oxone and the mixture was processed as for the chloropyrifos oxone mixture. Fig. 5.6 shows that upon the incubation of chloropyrifos oxone-inhibited cholesterol ester hydrolase with cAMP, a reversibility of chloropyrifos oxone inhibition was observed after the 10 minute incubation with cAMP, and cholesterol ester hydrolase specific activity was reversed to a level similar to the Mg\(^{++}\) ion-inactivated cholesterol ester hydrolase not treated with the inhibitor previously.

It was concluded also that chloropyrifos oxone may not interfere with the process that activates cholesterol ester hydrolase, i.e. cyclic AMP-dependent protein kinase mediated phosphorylation and the phosphoprotein phosphatase mediated inactivation (dephosphorylation) process. It is possible that chloropyrifos oxone phosphorylates cholesterol ester hydrolase at site(s) that either decrease the phosphorylation of the enzyme by the cAMP-dependent protein kinase or it accelerates the enzymic dephosphorylation of phosphorylated cholesterol ester hydrolase by phosphoprotein phosphatase. A similar proposal was put forward by Civen, Brown and Morin (1977) based on the inhibition through a possible phosphorylation by organophosphate of serine residue to acetyl cholinesterase (Reiner and Aldridge (1967), Krupka (1964)). It is possible as well that the organophosphate insecticide phosphorylates cholesterol ester hydrolase at a subunit which is different from that subunit which is phosphorylatable by cAMP-dependent protein kinase, like the phosphorylation of the \(\alpha\) and \(\beta\) subunits of phosphorylase kinase in which the phosphorylation of the \(\alpha\) subunit accelerates the dephosphorylation of the phosphorylated \(\beta\) subunit (Cohen (1978)). It seems from this postulate that a phosphorylatable cholesterol ester hydrolase would be prone to the organophosphate insecticide as well as the less phosphorylated.

An alternative explanation is that chloropyrifos oxone at the concentration used simply inhibits 50% of the enzyme activity irreversibly and that subsequent cAMP activation is due to the unmodified enzyme.
Fig. 5.6. The activation and inactivation of chloropyrifos oxone inhibited-cholesterol ester hydrolase. Purified enzyme preparation was incubated with 50 nM chloropyrifos oxone for 1 h at 37°C then the incubation mixture was desalted to remove unbound inhibitor. The inhibited enzyme was then incubated with buffer only, Mg$^{++}$ ions and cAMP/ATP/Mg$^{++}$ to affect the activation and inactivation of the enzyme before the enzymic assay was carried out as described in the methods section. Control enzyme preparation incubated with ethanol only and desalted, was run in parallel and the specific enzyme activity of the enzyme incubated with buffer only considered as 100%, to which all figures of the test incubations in both inhibited and not inhibited enzyme preparations were referred. For details see the text.
Fig. 5.7. The inhibition by chloropyrifos oxone of activated and inactivated bovine adrenocortical cholesterol ester hydrolase. Purified enzyme preparation (Biogel void volume fraction) was activated and inactivated as described in the methods section, then EDTA was added to terminate both the activation and inactivation processes. Both incubated enzyme forms were incubated with chloropyrifos oxone exactly as described in the legend of Fig. 5.5, and the assay of cholesterol ester hydrolase was conducted as described in the methods section.
enzyme, and phosphorylation of the enzyme would not protect it from the inhibitory action of the organophosphate insecticide. To test the validity of this argument, partially purified bovine adrenal ester hydrolase (the Biogel A fraction) was activated by cAMP plus ATP/Mg\(^{++}\) as described in Section II. Control enzyme which was de-activated by incubation with Mg\(^{++}\) ion only was run in parallel. After the 10 min incubation, 5 mM EDTA solution was added to stop both activation and deactivation of cholesterol ester hydrolase. Aliquots (0.2 ml) were then incubated with different concentrations of chloropyrifos oxone added in 5 µl ethanol and incubation was carried out for 1 hour at 37°C before assaying for cholesterol ester hydrolase as described in Section II. Fig. 5.7 shows the inhibitory action of chloropyrifos oxone on active and inactive bovine adrenal cholesterol ester hydrolase. It seems that both forms of cholesterol ester hydrolase were inhibited in a similar manner with 50% inhibition occurring between 10\(^{-8}\)-10\(^{-7}\) M chloropyrifos oxone. Thus unlike the deactivation of cholesterol ester hydrolase by phosphoprotein phosphatase in which the presence of ATP and cAMP prevent such deactivation (Beckett and Boyd (1977)), chloropyrifos oxone interacts and inhibits the active enzyme in the presence of cAMP.

5.8 THE COMPARATIVE HYDROLYSIS OF CHOLESTERYL OLEATE AND CHOLESTERYL LINOLEATE BY PARTIALLY PURIFIED BOVINE ADRENOCORTICAL CHOLESTEROL ESTER HYDROLASE

All the studies on cytosolic adrenal cholesterol ester hydrolase were performed using cholesteryl oleate as substrate, partly because it is the predominant unsaturated fatty acid ester found in the adrenal lipid droplet esterified cholesterol and partly because of the relative stability of this ester compared to esters of other long chain unsaturated fatty acids (Trzeciak and Boyd (1975), Beckett and Boyd (1977) Naghshineh, Treadwell, Gallo and Vahouny (1974), Wallat and Kanau (1976) and Pittman
and Steinberg (1977)) and partly therefore of the ready availability of the labelled substrate. No report so far has described a comparative study on the hydrolysis of different unsaturated fatty acid cholesterol esters by cytosolic adrenal cholesterol ester hydrolase, except indirect studies by Beckett and Boyd (1975) and Vahouny, Hodges and Treadwell (1979) in which the differential hydrolysis of cholesterol esters of rat adrenal lipid droplets and cholesterol esters of rat adrenal cells was measured before and after subjecting the animal to hormonal stimulus such as ether stress (Beckett and Boyd (1975)) or challenging adrenal cell preparations with dibutyryl cAMP (Vahouny, Hodges and Treadwell (1979)). In Section III evidence was presented that under identical conditions and with similar substrate emulsions of cholesteryl\(^{\text{14C}}\)oleate and cholesteryl\(^{\text{14C}}\)linoleate, an apparent preferential hydrolysis of cholesteryl\(^{\text{14C}}\)linoleate by rat adrenal cytosol was observed. Similarly, partially purified bovine adrenocortical cholesterol ester hydrolase was tested against cholesteryl\(^{\text{14C}}\)oleate and cholesterol-\(^{\text{14C}}\)linoleate since there is no information regarding the comparative hydrolysis of these cholesterol esters by the bovine enzyme. Cholesteryl-\(^{\text{14C}}\)linoleate and cholesteryl\(^{\text{14C}}\)oleate were prepared in an identical manner to that described in Section II, while the enzyme source was the Biogel A fraction of bovine adrenal cytosol prepared as described in Section IV. The two substrates were assayed under identical experimental conditions using activated, inactivated and basal cholesterol ester hydrolase prepared as described in Section II.

Table 5.1 illustrates the result of such an experiment, and it seems that cholesteryl\(^{\text{14C}}\)linoleate was a better substrate for bovine adrenal cholesterol esterase than for the rat adrenal enzyme described in Section III. Although the percentage activation of cholesterol ester hydrolase, when assayed against cholesteryl\(^{\text{14C}}\)oleate, was higher,
<table>
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<th>Addition in vitro to incubation</th>
<th>Cholesteryl ester hydrolase Specific Activity</th>
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<td></td>
<td>Cholesteryl(^{14}C)oleate</td>
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<td></td>
<td>(pmole oleic acid produced/min/mg protein)</td>
</tr>
<tr>
<td>None (control)</td>
<td>554±31</td>
</tr>
<tr>
<td>+Mg(^{++}) (partially inactivated)</td>
<td>270±32</td>
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<tr>
<td>+cAMP + ATP/Mg(^{++}) (activated)</td>
<td>860±57</td>
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Table 5.1. The hydrolysis of cholesteryl\(^{14}C\)oleate and cholesteryl \(^{14}C\)linoleate by partially purified bovine adrenocortical cholesterol ester hydrolase. The enzyme was pre-incubated with buffer, 2 mM Mg\(^{++}\) or 10 μM cAMP/2 mM ATP/2 mM Mg\(^{++}\) to get control, inactivated and activated enzyme preparations respectively, under condition similar to those described in the activation section of Section II. Results are the mean values ± S.D.
it did not match the activity of the enzyme assayed against cholesteryl-$\Delta^{14}$-linoleate. This might indicate that the enzyme acts preferentially on cholesteryl linoleate. The apparent higher percentage activation of cholesterol ester hydrolase, when assayed against cholesteryl oleate, was not sufficient to bring the enzyme to a state such that hydrolysis of cholesteryl oleate equals the hydrolysis of cholesteryl linoleate. 

On the other hand, the physical properties of each substrate and consequently the interaction of the different emulsified substrates could influence the apparent rate of hydrolysis of cholesteryl linoleate and cholesteryl oleate. However, these results are consistent with the in vivo studies on rat adrenal cholesterol ester hydrolase of Beckett and Boyd (1975) in which they found a relatively higher depletion of cholesteryl linoleate than of cholesteryl oleate in the adrenal lipid droplets. This was observed when the rat was stressed with ether anaesthesia, a condition known to stimulate adrenal cholesterol ester hydrolase (Trzeciak and Boyd (1973)). The selective hydrolysis of cholesteryl linoleate by cholesterol ester hydrolase could be similar to the reported selectivity of a rabbit heart lipase (Hsuch, Isakson and Needleman (1977)) and of a human platelet phospholipase (Bills, Smith and Silver (1977)). The apparent selectivity of adrenal cholesterol ester hydrolase toward different polyunsaturated fatty acid cholesterol esters described in this section, together with the report of Beckett and Boyd (1975) might be true in vivo, and if this is the case, the liberated linoleic acid could be desaturated and elongated to arachidonic acid in comparison to the events occurring in rat liver. Sprecher (1977) found that liver microsomes rapidly converted linoleic acid to arachidonic acid. The arachidonic acid once produced could serve as precursor for prostaglandin biosynthesis (Lands (1979)). This postulate is consistent with the recent report of Chanderbhan, Hodges, Treadwell
and Vahouny (1979) who found that a rat adrenal cell preparation pre-labelling with $\Delta^1\text{D}^{-14}\text{C}^{}\text{arachidonic}$ acid responded to ACTH by an increased production of prostaglandin $\text{PGE}_2$ and $\text{PGA}_2(B2)$. However the role of these prostaglandins in adrenal steroidogenesis stimulated with ACTH is not clear.

5.9 ATTEMPT TO STUDY THE SITE AND NUMBER OF PHOSPHORYLATIONS IN THE BOVINE ADRENOCORTICAL CHOLESTEROL ESTER HYDROLASE

The site to which the phosphate group is bound to the native cholesterol ester hydrolase after activation and phosphorylation of the enzymes by transfer of the phosphate group from $\text{P}^{-32}\text{P}^{}\text{ATP}$, is still unknown. This is because all efforts directed towards these studies have been hampered by the lipoprotein nature of cholesterol ester hydrolase, that is, it is heavily aggregated with lipid especially phosphatidyl choline (Wallat and Kunau (1976)). The delipidisation of cholesterol ester hydrolase which would effectively remove all lipid (ether/ethanol extraction) leads to a rapid loss of activity of the enzyme activity and possibly denaturation of the enzyme. So far, a homogenous preparation of cholesterol ester hydrolase from bovine adrenal cortical cytosol has not been achieved (Beckett and Boyd (1977), Wallat and Kunau (1976) and Naghshineh, Treadwell, Gallo and Vahouny (1978)), neither did the studies presented in Section IV of this thesis produce such a preparation. Similar difficulties are also being encountered with the hormone-sensitive triacylglycerol lipase of rat adipose tissue (Steinberg (1978)) although a recent report showed that the enzyme could be solubilised by detergent which facilitates the subsequent purification procedures to obtain an apparent near homogenous preparation of triacylglycerol lipase (Belfrage, Jergil, Stralfors and Tornqvist (1977)). However, this preparation was still active toward di- and mono-acylglycerol substrates, and the authors' did not comment
on the strong possibility of any activity toward the hydrolysis of
cholesteryl esters. The foregoing reasons however did not prevent the
speculation about where the phosphate is bound to cholesterol ester
hydrolase. It has been shown by Beckett and Boyd (1977) that the
radioactivity bound to cholesterol ester hydrolase is acid
precipitatable, a property shared by other phosphorylatable enzymes such
as rabbit muscle phosphorylase kinase (Cohen (1978)) and rat adipose
tissue hormone-sensitive lipase (Steinberg (1978)). It is also known
that radioactive phosphate bound to these other two enzyme proteins is
alkali-labile which suggests the phosphorylation of a serine residue in
these proteins (Huttunen and Steinberg (1971)). This was proved for
rabbit muscle phosphorylase kinase (Cohen, Watson and Dixon (1975)).
It was decided to attempt to isolate such a phosphopeptide(s) and if
possible to study the amino acid to which the radioactive phosphate is
bound after the phosphorylation of bovine adrenal cholesterol ester
hydrolase, despite the fact that the enzyme has not been purified to a
homogenous state. Therefore, the following experiments were performed
to obtain further information on the feasibility of such studies.

At the time these experiments were performed, Percoll, a new density
gradient medium was available from Pharmacia Fine Chemicals. This medium
was designed to give within a very short time a self generating density
gradient upon centrifugation. A test experiment was run to see if
Percoll could replace sucrose as a medium for a linear gradient
centrifugation as used for the final step of bovine adrenocortical
cholesterol ester hydrolase described in Section IV. A purified Biogel
void volume fraction of cholesterol ester hydrolase was mixed with
Percoll in a sucrose and potassium phosphate buffer to a final concentration
of 45% Percoll, 0.25M sucrose and 20 mM potassium phosphate buffer.
The final pH was 7.4. The mixture was then centrifuged for 1 hour at
Fig. 5.8. The distribution of cholesterol ester hydrolase in a self-generated density gradient of Percoll. Purified adrenocortical cholesterol ester hydrolase was mixed with a mixture of Percoll in 0.25M sucrose and 20 mM potassium phosphate buffer pH 7.4. The final density of the mixture was 1.09 g/ml. The mixture was centrifuged at 27,000 rpm in Spinco ultracentrifuge using fixed angle rotor 40. Centrifugation was carried out for 1 h at 4°C, after which cholesterol ester hydrolase was assayed as described in the methods section. See text for details.
27,000 rpm in a Spinco ultracentrifuge using Spinco fixed angle rotor 40. After centrifugation, fractions were obtained as described in Section IV and each fraction was analysed for cholesterol ester hydrolase and the refractive index was measured to get the density of the fraction as described in Pharmacia Fine Chemicals booklet (Percoll). Fig. 5.8 shows the result of such an experiment, and clearly it gave a result similar to that obtained by sucrose gradient centrifugation described in Section IV but within a shorter time. Hence, the procedure was adapted for further experiment using a phosphorylated cholesterol ester hydrolase preparation.

A Biogel A void volume fraction of cholesterol ester hydrolase obtained as described in Section IV was incubated for 20 min with Mg$$^{++}$$ to dephosphorylate and deactivate the enzyme as far as possible. The mixture was then incubated with 10 $$\mu$$M cAMP and $$^{32}$$P-ATP under conditions similar to those described earlier in this section. After 15 min the incubation mixture was desalted by passing it through a Sephadex G-25 column (2.5 x 30 cm) to remove the excess $$^{32}$$P-ATP. The desalted phosphorylated cholesterol ester hydrolase was mixed with Percoll in 0.25M sucrose and 20 mM phosphate buffer and processed as described earlier in this subsection. The active cholesterol ester hydrolase was pooled and Percoll was removed by further centrifugation at 40,000 rpm for 2 hour in a Beckman ultracentrifuge using a swing-out rotor SW41. The supernatant containing the purified phosphorylated cholesterol ester hydrolase (5.6 mg protein) was dialysed against water and lyophilised overnight. The lyophilised protein was suspended in 6 ml 0.1M ammonium acetate buffer pH 8.5 and 0.1 mg chymotrypsin containing 0.01 mg soya bean trypsin inhibitor was added in 50 $$\mu$$l ammonium acetate buffer pH 8.5. The mixture was incubated at 37° for four hours, then lyophilised overnight. The time of the incubation was found to be adequate to
release more than 80% of radioactive phosphate as acid soluble phosphopeptide when phosphorylated cholesterol ester hydrolase was incubated with trypsin at 37°C for 3 hour and shown in Fig. 5.9.

After the digestion and lyophilisation of digested cholesterol ester hydrolase, the lyophilised material was solubilised in 1 ml of 5% formic acid and was layered on top of a Sephadex G25 column (90 x 1 cm). The peptides were eluted with 5% formic acid and fractions of 1 ml were collected. Aliquots of 20 µl from each fraction were taken for \(^{32}\)P estimation. Fig. 5.10 shows the radioactivity curve of \(^{32}\)P in fractions 20-58, where fraction 29 represents the void volume of the column. It seems that \(^{32}\)P radioactivity was not associated with a discrete peak, but rather it is spread through many fractions which is quite different to results obtained with rabbit skeletal muscle phosphorylase kinase (Cohen 1978). Only two peaks of \(^{32}\)P radioactivity were found when the phosphorylated protein was subjected to tryptic digestion. However, each fraction was then processed for peptide mapping by a procedure basically described by Ambler (1963) which is as follows: Fractions 20-60 of the eluted peptide digest were dried overnight in a vacuum dessicator. The dry samples were dissolved in 50 µl 0.1M ammonia and aliquots (8 µl) from each fraction were spotted onto Whatman 1MM paper together with amino acid standards and marker. After the applied spots were dried, the paper was subjected to high-voltage paper electrophoresis (Ambler 1963) using pyridine-acetate buffer pH 6.5 for 50 min. The paper was then dried at 60°C and autoradiography of the map was performed by layering Ilford X-Ray film on top of the peptide map for 24 hours. The film was developed and Fig. 5.11 shows a picture of the autoradiogram. Many radioactive spots are seen and fractions 37-45 which were associated with the major peak of radioactivity in Fig. 5.10 gave multiple bands on electrophoresis.
Fig. 5.9. The release of acid-soluble $^{32}$P-phosphopeptides of tryptic-digested $^{32}$P-phosphorylated cholesterol ester hydrolase. Purified phosphorylated cholesterol ester hydrolase prepared as described in the text was incubated with trypsin (45 μg per 2.2 mg hydrolase) in ammonium acetate buffer pH 8.5 at 37°C. At the indicated time intervals, aliquots were withdrawn and acid-precipitable $^{32}$P-phosphoprotein was isolated for bound $^{32}$P determination. Acid-soluble phosphopeptides were calculated from the total radioactivity of the acid-precipitable protein found at zero time.
Elution profile of the chymotrypsin digest of the phosphorylated purified cholesterol ester hydrolase using Sephadex-G25

Fig. 5.10. Elution profile of chymotrypsin-digested $^{32}$P-phosphorylated bovine adrenocortical cholesterol ester hydrolase. Phosphorylated enzyme was digested with chymotrypsin in 0.1M ammonium acetate buffer pH 8.5. Digestion was carried out at $37^\circ$C for 4 h, then the digested material was lyophilised overnight. The dry digest was solubilised in 1 ml 5% formic acid and applied to the Sephadex G-25 column (1 x 90 cm) equilibrated with 5% formic acid. Elution, with the same buffer, of phosphopeptide was followed by total phosphate radioactivity determination in each fraction.
remaining peptide fractions 37-45 and 33-36 were combined and applied, as a single band, to 1 mM Whatman paper and electrophoresis carried out as before but in a pyridine/acetate buffer at pH 3.5 instead of pH 6.5. After 50 min, the paper was dried and an autoradiogram obtained as before. A picture of the autoradiogram is shown in Fig. 5.12. The paper was then stained with a solution of ninhydrin in acetone. From the Fig. 5.12 it can be seen that there are several bands of $^{32}$P radioactivity which made it a difficult task to isolate any specific one band and analyse it for amino acid composition. This result was confirmed by protein staining which showed no specific peptide band and a diffused stain throughout the entire map (not shown).

It is possible that there are several proteins to which $^{32}$P radioactivity could be bound. Until the present time no precise information is available regarding the molecular weight, the subunit structure of the ratio of phosphate per mole cholesterol ester hydrolase, and several reports have given conflicting figures regarding the molecular weight of the enzymes (Beckett (1975), Neghshineh, Treadwell, Gallo and Vanouny (1978)). The association of triacylglycerol lipase activity with cholesterol ester hydrolase in bovine adrenal cytosol, and the similar co-purification pattern obtained during these studies and described in Section IV make it more difficult to decide whether the phosphorylated band of cholesterol ester hydrolase obtained by Beckett and Boyd (1977) in SDS-polyacrylamide gel electrophoresis was a single component.

It is also possible that the digestion produced several peptides of different lengths and that the phosphorylated amino-acid(s) is included within these different peptides with a slight change in the N or/and C terminals. These observations have also been noticed with rabbit muscle phosphorylase kinase but not to the extent found in this experiment.
Fig. 5.11. Autoradiogram of $^{32}$P-phosphopeptides map. Fractions 25-60 which were eluted from the column (Fig. 5.10) were applied to Whatman 1 M paper together with amino acids standard, and the fractions were subjected to high-voltage paper electrophoresis in pyridine/acetate buffer pH 6.5 for 50 min. The peptide map was dried then it was covered with Ilford X-Ray film for 24 h. The film was developed for the localisation of the phosphopeptides. See text for details.
Fig. 5.12. Autoradiogram of $^{32}$P-phosphopeptide map of pooled fractions (33-36) and (37-45) eluted from Sephadex column (Fig. 5.10). The pooled fractions were spotted on Whatman 1 MM paper, and peptide map was obtained as described in the legend of Fig. 5.11, except the electrode buffer was replaced with pyridine/acetate buffer pH 3.5. The dried peptide map was covered with Ilford X-Rays film for 24 h for localising the $^{32}$P-phosphatepeptides. See text for details.
The experiments described above show that although it is possible to study and localise the binding site of $^{32}P$ phosphate, it will be impossible to be certain whether this phosphorylation site is really part of a peptide derived from cholesterol ester hydrolase until the latter has been obtained in a homogenous state. The precise molecular weight of the enzyme, its subunit structure and its relation to triacylglycerol lipase remain to be determined before an insight can be obtained on the chemical modification of cholesterol ester hydrolase due to phosphorylation by protein kinase. This in turn could lead to a better understanding of the hormonal regulation of cholesterol ester metabolism in the adrenal cortex.

5.10 THE EFFECT OF CHOLESTEROL OLEYL ETHER ON BOVINE ADRENOCORTICAL CHOLESTEROL ESTER HYDROLASE AND TRIACYLGLYCEROL LIPASE

There have been reports indicating that glycerol triethers are neither absorbed nor hydrolysed when these compounds are fed to rats (Spencer, Paltauf and Holasek (1968), Morgan and Hoffman (1970)). The structural similarities between these compounds and triglyceride provide a functional characteristic to natural fats and oils when added to food but they have a lesser calorific value (Go and Branen (1975)). Likewise, cholesteryl ethers could be similar to glycerol triether in being resistant to being absorbed and hydrolysed if fed to rats. On this basis, cholesteryl ester hydrolase will not hydrolyse cholesteryl ether, but the ether could affect the hydrolysis of cholesteryl esters. It was presumed therefore that such an effect could be used to differentiate between cholesterol ester hydrolase and triacylglycerol lipase in bovine adrenal cytosol if they are quite different entities, by giving different responses to such compounds. Accordingly, the following experiment was performed in which partially purified bovine adrenocortical cholesterol ester hydrolase and triacylglycerol lipase from the Biogel A
<table>
<thead>
<tr>
<th>Additions</th>
<th>Cholesterol ester hydrolase (%)</th>
<th>Triacylglycerol lipase (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>+ Solvent</td>
<td>82</td>
<td>68</td>
</tr>
<tr>
<td>+ Cholesterol oleyl ether 10:1 ether:substrate</td>
<td>56</td>
<td>39</td>
</tr>
<tr>
<td>+ Cholesterol oleyl ether 50:1 ether:substrate</td>
<td>33</td>
<td>21</td>
</tr>
</tbody>
</table>

Table 5.2. Inhibition of cholesteryl ester hydrolase and triacylglycerol lipase by cholesteryl oleyl ether dissolved in n-hexadecane. Bovine adrenal cholesterol ester hydrolase and triacylglycerol lipase purified up to and including the Biogel column chromatography as described in Section IV were incubated with cholesteryl oleyl ether dissolved in 5 μl n-hexadecane. Cholesteryl\(^{14}\)C-oleate and glyceryl tri-\(^{14}\)C-oleate concentrations in the assay were 144 nmoles.
void volume fraction were incubated with cholesteryl oleyl ether (Analab (N.E.N.), U.S.A.) and the hydrolysis of cholesteryl-\(^{14}C\)oleate and glycerol tri-\(^{14}C\)oleate was measured as described in Section II. Because the cholesteryl oleyl ether is not soluble in a conventional solvent such as acetone or ethanol, and although it is soluble in diethyl ether this solvent was not used since during 10-20 min incubation, the solvent evaporated leaving crystalline cholesteryl oleyl ether. However, n-hexadecane was found suitable and has a less inhibitory effect than other solvents tried. Table 5.2 shows the effect of n-hexadecane by itself and the effect of two concentrations of cholesteryl oleyl ether. In both concentrations, cholesteryl oleyl ether inhibited both cholesterol ester hydrolase and triacylglycerol lipase to the same degree. The low quantity of cholesteryl oleyl ether which was available and its insolubility in convenient solvents prevented further detailed study on the type of inhibition of cholesterol ester hydrolase and triacylglycerol lipase. It does seem however, that these results are similar to those of Pittman and Steinberg (1977) where unlabelled triglyceride was added prior to labelled cholesterol-\(^{14}C\)oleate in the inhibition studies of rat adrenal cholesterol ester hydrolase where the authors concluded a concerted binding of both substrates to the enzyme protein.

5.11 SUMMARY

1. The effect of protein concentration and time of incubation on the assay of bovine adrenocortical cholesterol ester hydrolase and triacylglycerol lipase was studied.

2. The stimulation of cholesterol ester hydrolase in vitro requires cAMP at a physiological concentration and cGMP at the same concentration did not affect the enzyme at all.

3. Inhibition studies of purified cholesterol ester hydrolase and triacylglycerol lipase by chloropyrifos oxone revealed that both enzymes
gave identical response to increased concentrations of the inhibitor with a 50% inhibition occurring between $10^{-8}$-$10^{-7}$ M of chloropyrifos oxone, and no dissociation of enzymic activities could be observed.

4. The inhibition of cholesterol ester hydrolase by micromolar concentrations of chloropyrifos oxone could be partially reversed by incubation with cAMP and ATP/Mg$^{++}$ and it is thought that the deactivation of cholesterol ester hydrolase by chloropyrifos oxone might be through a chemical phosphorylation of specific serine-residue as it occurs in the inhibition of acetylcholine esterase.

4. The substrate analogue cholesteryl oleyl ether inhibited both cholesterol ester hydrolase and triacylglycerol lipase to a similar degree, and the relationship between the two enzymes is discussed.

5. Activation of cholesterol ester hydrolase and triacylglycerol lipase in vitro in the bovine adrenal was accompanied by increased binding of radioactive phosphate to acid precipitable protein, and the percentage activation of both enzymes was similar and was closely parallel to the phosphorylation of the acid-precipitable protein.

6. Chymotryptic digestion of phosphorylated purified cholesterol ester hydrolase from bovine adrenal resulted in the release of acid soluble phosphopeptides indicated the presence of several radioactive phosphate bands none of which could be identified as originating from cholesterol ester hydrolase.

7. The hydrolysis of cholesteryl linoleate and cholesteryl oleate by cholesterol ester hydrolase were compared. Cholesterol linoleate was apparently hydrolysed faster than cholesteryl oleate.
SECTION VI

STUDIES ON RAT ADIPOSE TISSUE SOLUBLE
CHESTEROL ESTER HYDROLASE AND
TRIACYLGlycerol LIPASE

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SECTION VI: STUDIES ON RAT ADIPOSE TISSUE SOLUBLE CHOLESTEROL ESTER HYDROLASE AND TRIACYLGLYCEROL LIPASE

6.1 INTRODUCTION

In the previous sections, attention focussed on the activity of rat and bovine adrenal cytosolic cholesterol ester hydrolase and triacylglycerol lipase and the regulation of these enzymes in vivo by ACTH through the phosphorylation-dephosphorylation mechanism which involves a cAMP-dependent protein kinase. Although this mechanism of hormonal regulation is unique neither for these enzymes nor for adrenals (Krebs and Beavo (1979)), it seemed of interest to investigate whether these hydrolase enzymes are regulated similarly in other tissues of the rat especially adipose tissue, corpus luteum and liver, since these tissues are known to have cholesterol ester hydrolase activity (Arnaud and Boyer (1974), Behrman and Armstrong (1969), Deykein and Goodman (1962)).

In this section studies are reported on the activity of cholesterol ester hydrolase and triacylglycerol lipase of rat adipose tissue cytosol. The modulation of these enzymic activities by lipolytic hormones and nicotinic acid was studied since these agents are known to influence lipolysis in rat adipose tissue (Pain (1973)), and to study the possibility that rat adrenal triacylglycerol lipase may be regulated like cholesterol ester hydrolase (Section III). It was decided to investigate whether agents which affect triacylglycerol lipase in adipose tissue may also affect cholesterol ester hydrolase.

6.2 THE STIMULATION IN VIVO BY ACTH OF RAT ADIPOSE TISSUE CHOLESTEROL ESTER HYDROLASE AND TRIACYLGLYCEROL LIPASE

The in vivo adipokinetic action of ACTH on free fatty acid mobilisation from rat adipose tissue has been reported by Hollenberg, Raben and Astwood (1961). These authors pointed out that ACTH action was associated with the activation of a lipase which is different from
lipoprotein lipase. Subsequent work of Rodbell (1964) showed that incubation of epinephrine with rat epididymal fat pads also mobilises free fatty acids and hence is a lipolytic hormone. It was shown that epinephrine enhanced the concentration of cAMP in this tissue (Butcher, Ho, Meng and Sutherland (1965)). This report was the start of active research on rat adipose tissue, isolated adipocytes and cell-free fractions. The object of much of the research was the understanding of the many steps between the hormonal signal received by the cell, and the end production of free fatty acid. The role of cAMP as a mediator for cellular regulation of these steps had to be clarified (for reviews see Jeanrenaud and Hepp (1970) and Fain (1977)). The availability of partially purified hormone-sensitive lipase of rat adipose tissue made it possible to study the direct effect of cAMP on the activation of the enzyme \textit{in vitro}, and this activation was shown to be dependent on the presence of a cAMP-dependent protein kinase (Huttunen and Steinberg (1971)). The same group of workers showed that a cholesterol ester hydrolase activity was found to co-purify with hormone-sensitive lipase, and this cholesterol ester hydrolase was shown to be stimulated \textit{in vitro} when isolated adipocytes were preincubated with epinephrine before preparation of the cell cytosol (Pittman, Khoo and Steinberg (1975)). The same report showed that this stimulation of isolated adipocyte cholesterol ester hydrolase was mediated by cAMP and catalysed by a cAMP-dependent protein kinase.

Experimental evidence was presented in Section III on the stimulation of adrenal cholesterol ester hydrolase \textit{in vivo} by ACTH. Since studies were being directed toward other systems in which regulation of cytosolic cholesterol ester hydrolase may be under hormonal control, the following experiments were performed in order to compare the stimulation of adipose tissue cholesterol ester hydrolase with that found in the adrenal.
Two groups of adult female rats were divided at random and one group was injected with 0.2 ml saline subcutaneously while the other group received 4 iu ACTH/0.2 ml saline. After 12-15 min, rats of each group were sacrificed by decapitation and perirenal adipose tissue was excised and immersed in a sucrose solution. The cell cytosol was prepared as described in Section II, and the assay of cholesterol ester hydrolase and triacylglycerol lipase was performed within 2 hours of killing the animals. Table 6.1 shows the result of four such experiments on the activity of adipose tissue cholesterol ester hydrolase and triacylglycerol lipase. The Table shows the activities of both enzymes in the inactive and active forms after 10 min pre-incubation with magnesium acetate or cAMP respectively, under conditions previously described in Section II.

The effect of ACTH on adipose tissue cytosolic cholesterol ester hydrolase and triacylglycerol lipase does not seem to be impressive in terms of percentage stimulation in the experiments where activation was observed. The maximum stimulation observed was 41% above control, while in vitro activation by cAMP preincubation of both enzymes from saline injected rats could reach 60%. Moreover in two experiments, stimulation in vivo by ACTH of cholesterol ester hydrolase and triacylglycerol lipase was observed, and in the other two experiments the hormones did not affect the enzyme activity or slightly inhibited both enzymes. However one can explain this variable effect of ACTH in several ways. Firstly, most studies of the adipokinetic effect of ACTH were performed using either epididymal fat pad or isolated adipocytes and the release of glycerol was measured without the problem of re-esterification or allowance for less of substrate during the assay time. On the other hand, working with these lipolytic enzymes requires assay procedures of say 30 minutes duration and in this time the activity of the enzyme could change. In consideration of this, the experiments described in Table 6.1 were
<table>
<thead>
<tr>
<th>Experiment</th>
<th>Group</th>
<th>Cholesterol Ester Hydrolase</th>
<th>Triacylglycerol Lipase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>+Mg&lt;sup&gt;++&lt;/sup&gt;</td>
<td>+CAMP/ATP/Mg&lt;sup&gt;++&lt;/sup&gt;</td>
</tr>
<tr>
<td>1</td>
<td>Saline</td>
<td>2062&lt;sup&gt;±&lt;/sup&gt;36</td>
<td>2619&lt;sup&gt;±&lt;/sup&gt;43</td>
</tr>
<tr>
<td></td>
<td>ACTH</td>
<td>2373&lt;sup&gt;±&lt;/sup&gt;41</td>
<td>2549&lt;sup&gt;±&lt;/sup&gt;96</td>
</tr>
<tr>
<td>2</td>
<td>Saline</td>
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<td>3376&lt;sup&gt;±&lt;/sup&gt;30</td>
</tr>
<tr>
<td></td>
<td>ACTH</td>
<td>2119&lt;sup&gt;±&lt;/sup&gt;18</td>
<td>2554&lt;sup&gt;±&lt;/sup&gt;45</td>
</tr>
<tr>
<td>3</td>
<td>Saline</td>
<td>4651&lt;sup&gt;±&lt;/sup&gt;87</td>
<td>6944&lt;sup&gt;±&lt;/sup&gt;48</td>
</tr>
<tr>
<td></td>
<td>ACTH</td>
<td>4299&lt;sup&gt;±&lt;/sup&gt;48</td>
<td>6213&lt;sup&gt;±&lt;/sup&gt;29</td>
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<tr>
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<td>Saline</td>
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<td>4459&lt;sup&gt;±&lt;/sup&gt;57</td>
</tr>
<tr>
<td></td>
<td>ACTH</td>
<td>4443&lt;sup&gt;±&lt;/sup&gt;183</td>
<td>5441&lt;sup&gt;±&lt;/sup&gt;101</td>
</tr>
</tbody>
</table>

Table 6.1. The stimulation of adipose tissue cytosolic cholesterol ester hydrolase and triacylglycerol lipase in vivo by 4 i.u. ACTH, injected to rats 12-15 minutes before sacrifice. Cytosol was incubated with magnesium ions or cAMP for 10 min prior the enzymic assay to get inactivated and activated form of both enzymes respectively under condition described in the methods section. Results are means value of the released oleic acid/ min/mg protein ± S.D.
carried through within two hours of killing the animals. Allen, Largis, Miller and Ashmore (1973) reported that withdrawal of epinephrine from maximally stimulated lipolysis, in perfused rat adipose tissue, resulted in a rapid decline of lipolysis, the basal activity being reached 15 min after removal of the hormone from the perfusing medium. Similarly, it could be that the time lag between homogenisation of the tissue and assaying of the enzyme activity is a factor which makes it difficult to demonstrate the stimulation of cholesterol ester hydrolase. Secondly, this rapid decline of the activities of cholesterol ester hydrolase and triacylglycerol lipase after being stimulated in vivo by ACTH may be catalysed by a deactivating system such as a phosphoprotein phosphatase, and recent evidence from other laboratories strongly supports this view (Severson, Khoo and Steinberg (1977), and Steinberg (1978)). Thirdly, to sustain a highly active form of cholesterol ester hydrolase and triacylglycerol lipase, cAMP-dependent protein kinase and phosphoprotein phosphatase are involved. Possibly the prime factor for the activity is sufficient cAMP (Wong, Laten and Park (1978)), and the concentration of cAMP is influenced by the activities of cyclic nucleotide phosphodiesterase and adenylate cyclase (reviewed by Fredholum (1978) and Pain (1977)). Recently, a heat-stable protein inhibitor from rat epididymal fat pad has been identified and it has been found that this factor modulates the activity of chicken lipase phosphatase (Severson and Sloan (1977)). The mechanism of this modulation has not been investigated yet, but from other phosphatase inhibitor studies (Huang and Glinsmann (1976), Nimmo and Cohen (1977) it may be possible that adipose tissue lipase phosphatase inhibitor is regulated by phosphorylation-dephosphorylation mechanism which might also involve cAMP-dependent protein kinase.

Table 6.1 also shows that in all four experiments, the brief pre-incubation of adipose tissue cytosol with cAMP resulted in a consistent
<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Saline</th>
<th>ACTH</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>+Mg&lt;sup&gt;++&lt;/sup&gt;</td>
<td>+cAMP/ATP/Mg&lt;sup&gt;++&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cholesterol ester hydrolase</td>
<td>100 (3090)</td>
<td>141 (4357)</td>
</tr>
<tr>
<td>Triacylglycerol lipase</td>
<td>100 (1305)</td>
<td>152 (1984)</td>
</tr>
</tbody>
</table>

Table 6.2. The mean percentage stimulation in vivo by ACTH and percentage activation in vitro by cAMP preincubation of rat adipose tissue cytosolic cholesterol ester hydrolase and triacylglycerol lipase (Four experiments). The figures in parentheses represent the mean percentage value expressed in absolute rate of oleic acid released/min/mg protein.
activation of cholesterol ester hydrolase and triacylglycerol lipase in both control and hormone treated animals. The average percentage activation in vitro of cholesterol ester hydrolase was \( 38.8\% \pm 9.7\% \) (Mean+S.D.) while triacylglycerol lipase showed average percentage activation of \( 51.5\% \pm 8\% \) (Mean+S.D.). These figures were reduced in the studies on the cytosol of ACTH treated animals to \( 23.3\%\pm15.3\% \) and \( 24\%\pm14.8\% \) for cholesterol ester hydrolase and triacylglycerol lipase respectively. This indicates that both enzymes were at least activated partially in vivo and additional cAMP added in vitro slightly increased the activities of both enzymes. These results are in good agreement with the figures reported by Pittman, Khoo and Steinberg (1975) on the activation in vitro of cholesterol ester hydrolase and triacylglycerol lipase of rat adipocyte cytosol. It seems therefore that, although the stimulation in vivo of the adipose tissue soluble lipolytic enzyme was not observed in this study with consistent frequency, the in vitro experiments showed that the hydrolytic enzymes from the adipose tissue of ACTH-treated animals were less activated by cAMP in vitro perhaps because they are already stimulated in vivo. This concept is supported by the results in Table 6.2 in which the activation of cholesterol ester hydrolase and triacylglycerol lipase of adipose tissue cytosol from control animals, was considered as 100% and all other enzyme activities are calculated from that figure. The mean of the four experiments was then calculated in percentage and absolute values.

6.3 THE EFFECT OF NICOTINIC ACID ADMINISTRATION IN VIVO AND IN VITRO ON RAT ADIPOSE TISSUE CYTOSOLIC CHOLESTEROL ESTER HYDROLASE AND TRIACYLGlycerol LIPASE

The antilipolytic effect of nicotinic acid on hormone-stimulated lipolysis was first suggested by Carlson and Ore (1962) when they measured plasma free fatty acid concentration in human subjects treated
with nicotinic acid, and in dogs treated with norepinephrine. They showed that fatty acid mobilisation in these dogs was completely blocked by prior \textit{in vivo} administration of nicotinic acid. However the mechanism by which nicotinic acid exerts its antilipolytic action on hormone-stimulated lipolysis remains obscure. It was reported by Butcher (1971) that nicotinic acid decreased the isolated adipocyte cAMP concentration when these cells were stimulated with epinephrine, ACTH and glucagon in the presence of caffeine. The lowering of cAMP concentration could be either through the activation of the breakdown of cAMP by cyclic nucleotide phosphodiesterase (Schwabe 1971) or the inactivation of cAMP synthesis by adenylate cyclase (Skidmore, Kritchevsky and Schonhofer (1971)). Partially purified hormone-sensitive lipase of adipose tissue cytosol is stimulated through a phosphorylation mechanism mediated by cAMP-dependent protein kinase (Huttunen and Steinberg (1971)) so that a decreased cAMP concentration could result in a lowered lipolytic activities. On the other hand, possibly by a different mechanism, nicotinic acid has been shown to inhibit \textit{in vitro} a cytosolic-microsomal lipase of rat epididymal fat pads (Shafrir (1971)). A subsequent report (Khoo, Janett, Mayer and Steinberg (1972)) showed that adipose tissue hormone-sensitive lipase is located mainly in the cell cytosol, about 80% of the total lipase in the fat cake-free homogenate of adipose tissue. Thus, the observations of the inhibition by nicotinic acid of hormone-stimulated lipase reported by Shafrir (1971) and Chemelar and Chemelarova (1971) might explain the inhibition by nicotinic acid of the cytosolic triacylglycerol lipase described by Huttunen and Steinberg (1971).

Under \textit{in vivo} conditions, nicotinic acid administration produced an increase in adipose tissue lipoprotein lipase activity in 8-10 hour starved rats (Nikkila (1971), Otway, Robinson, Rogers and Wing (1971)), and the latter group found that nicotinic acid added \textit{in vitro} to
epididymal fat pads reversed the inhibitory effect of epinephrine on lipoprotein lipase. The inhibitory action by epinephrine on lipoprotein lipase is not fully explained by increased cAMP concentration since Khoo, Steinberg, Huang and Vagelos (1976) reported that immunochemically homogenous lipoprotein lipase of chicken adipose tissue was not affected by cAMP-dependent protein kinase added in vitro under conditions which activated hormone-sensitive lipase. However, recent reports of Ashby, Bennett, Spencer and Robinson (1978) postulated that epinephrine acts on adipose tissue lipoprotein lipase at a level prior to its secretion from the cell and after its activation within the cell by a process as yet unknown but which might involve indirectly cAMP.

The adverse effect of nicotinic acid administration goes further than affecting hormone-sensitive lipase, lipoprotein lipase and cAMP. One of the main other effects is the ability of nicotinic acid to reduce plasma cholesterol concentration of human subjects (Froberg, Boberg, Carlson and Eriksson (1971)) and of rats (Gey, Lengsfeld and Lorch (1971)). The mechanism of the hypocholesterimic action of nicotinic acid is not clear, and reports regarding the site of action of nicotinic acid are conflicting (Kritchevsky (1971)) suggesting effects on cholesterol oxidation and cholesterol synthesis (Gey and Carlson (1971)). However, the hydrolysis of esterified cholesterol in adipose tissue and/or other tissue was overlooked. Cholesterol concentration in adipose tissue is quite small when expressed in terms of wet tissue weight, but because of the large mass of adipose tissue, cholesterol amount in the total adipose tissue exceeds that found in total liver (Farkas, Angel and Avigan (1973)). Most of adipose tissue cholesterol is found as free cholesterol in both human and rat adipose tissue being 94% and 90% of the total cholesterol respectively (Schreibman and Dell (1975), Kovanen, Nikkila and Miettinen (1975)). It was also found by these two
groups that the synthesis of cholesterol in adipose tissue is negligible when compared to cholesterol synthesis in liver. Moreover, the latter group found in the adipose tissue of fasted and refed rats, that cholesterol concentrations followed closely the decrease or increase concentrations of adipose tissue triglycerides, and they postulated that the stability of the fat droplet might require a specific concentration of cholesterol.

The preceding discussion gives a brief insight into the multifaceted effect of nicotinic acid in rat and human subjects. In the following experiments, studies were directed towards checking the effect of nicotinic acid administration in vivo and in vitro on rat adipose tissue cytosolic cholesterol ester hydrolase and triacylglycerol lipase.

For the in vivo experiments, twelve female rats (150-170 g) were divided randomly into two groups of six rats. One group received i.p. injections of 0.2 ml saline while the other received i.p. injection of 30 mg nicotinic acid in 0.2 ml saline. Fifteen minutes later, both groups were severely stressed by subjecting the animals to 10 min ether anaesthesia, a condition known to raise the plasma ACTH concentration by about 50 fold (Cook, Greer and Kendall (1972)). Thereafter, adipose tissue of saline and nicotinic acid injected animals was processed as described in Section II for the preparation and assay of cytosolic cholesterol ester hydrolase and triacylglycerol lipase using cholesterol Δ-14C oleate and glycerol tri-Δ-14C oleate respectively.

Table 6.3 shows the results of three identical experiments. In two experiments nicotinic acid decreased the activity of cholesterol ester hydrolase possibly by blocking its stimulation in vivo by endogenous ACTH. Trzeciak and Boyd (1973) showed that on exposure to ether anaesthesia for a 10 min period, rat adrenal cholesterol ester hydrolase was stimulated by approximately two fold and this was accompanied by a rise in cAMP-dependent protein kinase activity. However the reasons why
<table>
<thead>
<tr>
<th>Experiment</th>
<th>Cholesterol Ester Hydrolase (pmole oleic acid/min/mg protein)</th>
<th>Triacylglycerol Lipase (pmole oleic acid/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Saline-Stressed</td>
<td>Nicotinic-Stressed</td>
</tr>
<tr>
<td>1</td>
<td>4900±243</td>
<td>3537±161</td>
</tr>
<tr>
<td>2</td>
<td>4594±16</td>
<td>3838±34</td>
</tr>
<tr>
<td>3</td>
<td>4934±16</td>
<td>4321±184</td>
</tr>
</tbody>
</table>

Table 6.3. The effect of nicotinic acid administration in vivo (30 mg, i.p.) on the stimulation of rat adipose tissue cytosolic cholesterol ester hydrolase and triacylglycerol lipase. Rats were injected with nicotinic acid or saline 15 min prior subjecting the animals to 10 min ether anaesthesia. Results are means value ± S.D.
triacylglycerol lipase was not inhibited by nicotinic acid pre-treatment in two experiments are not clear, but may be best explained by de-activation through phosphoprotein phosphatases of triacylglycerol lipase from adipose tissue of saline injected rats. There is also the possibility of slight activation of triacylglycerol lipase in the nicotinic acid treated animals by catecholamine and ACTH release which could have taken place prior to the agent reaching its target due to the handling of the animals during administration of nicotinic acid. Hypophysectomised rats would have provided a better control for such an experiment but were not available. Although the data presented in Table 6.3 shows, to some extent, the \textit{in vivo} antilipolytic action of nicotinic acid on both cholesterol ester hydrolase and triacylglycerol lipase of rat adipose tissue, the significance of these results are questionable. Accordingly, \textit{in vitro} experiments were carried out in another attempt to provide a more decisive answer to the question of the effect of nicotinic acid on these two enzymes.

The effect of nicotinic acid administration \textit{in vitro} on adipose tissue cholesterol ester hydrolase and triacylglycerol lipase was studied using isolated rat adipocytes prepared as described in Section II. The pooled cell-suspension was stirred gently by swirling the conical flask and aliquots of 10 ml were pipetted into four different polythene tubes. To the first and third tubes was added 100 \(\mu\)l Krebs-Ringer bicarbonate buffer pH 7.4 containing 1% bovine serum albumin. The second and fourth tubes received 100 \(\mu\)l of 0.1M nicotinic acid in Krebs-Ringer buffer pH adjusted to 7.4 with NaOH. The four polythene tubes containing the adipocytes were incubated at 37\(^{\circ}\)C for 20 min. After addition of 10 \(\mu\)l 10 mM epinephrine to tubes 3 and 4, the incubation was continued for a further 10 min. The cells were separated from the medium by centrifugation at 1000 x g for 1 min and washed three times with Krebs-
Ringer bicarbonate buffer pH 7.4 (without bovine serum albumin). After the final wash, the contents of each tube were homogenised in 5 ml ice-cold medium consisting of 0.25M sucrose, 10 mM Tris-HCl buffer pH 7.4, 1 mM EDTA and 1 mM theophylline. Individual homogenates were fractionated by ultracentrifugation and the 105,000 x g supernatants were prepared as described in the methods section. Cholesterol ester hydrolase and triacylglycerol lipase in the control, inactive and active forms were assayed in the cytosol of adipocytes in the presence of buffer only or magnesium or cAMP plus ATP and magnesium respectively as described under the activation procedure in the method section. Fig. 6.1 shows the protocol used for performing this type of experiment.

Table 6.4 and 6.5 show the effect of nicotinic acid addition in vitro on the activities of isolated rat adipocyte cytosolic cholesterol ester hydrolase and triacylglycerol lipase respectively. The results are the mean percentage change from the control of four independent experiments. It seems that nicotinic acid by itself does not affect the basal activity of both enzymes in the cytosol pre-incubated with buffer, magnesium or cyclic AMP. It follows that at least the cAMP-dependent protein kinase and the magnesium-sensitive phosphoprotein phosphatase are as sensitive to in vitro pre-incubation with cAMP and magnesium ions as they are sensitive in the cytosol from control adipocytes.

The stimulation of adipocytes cholesterol ester hydrolase and triacylglycerol lipase by epinephrine showed only a 14% increase over the control which is quite consistent with that observed in the in vivo stimulation of these enzymes by ACTH reported in Table 6.1 of this section. However, triacylglycerol lipase activation in vitro by cAMP was quite high in the cytosol of adipocytes from the four different incubations while cholesterol ester hydrolase was not. The reasons for this high in vitro activation of triacylglycerol lipase are not clear.
Pooled epididymal fat bodies, trimmed from blood vessels and chopped into small pieces of about 50 mg.

Portions of about 2 g were incubated with 5 ml Krebs-Ringer bicarbonate buffer (KRB) pH 7.4 containing 1% bovine serum albumin (KRBB) and 0.5 mg/ml collagenase (Worthington) in a capped polythene tubes.

Cell dispersion was achieved by incubation at 37°C for 40-50 min in a metabolic shaker (120 oscillation/min).

Cells were separated from floating oil droplets, blood vessel cells and unfragmented tissue by centrifugation at 1000 x g for 1 min at room temperature.

Cells were washed twice with KRBB, and finally cells from different tubes were pooled and suspended in the desired volume of KRBB.

Cells suspension was divided as follows:

- 10 ml adipocyte + 0.1 ml KRBB
- 10 ml adipocyte + 0.1 ml 100 mM Nicotinic acid
- 10 ml adipocyte + 0.1 ml KRBB
- 10 ml adipocyte + 0.1 ml 100 mM Nicotinic acid

**INCUBATION OF ALL FOUR MIXTURES FOR 20 MIN AT 37°C IN A SHAKER**

- +0.01 ml KRBB
- +0.01 ml KRBB
- +0.01 ml 10 mM Epinephrine
- +0.01 ml 10 mM Epinephrine

**INCUBATION WAS CONTINUED AT 37°C FOR ANOTHER 10 MIN. THE REMAINING STEPS APPLIES FOR ALL THE FOUR ADIPOCYTE INCUBATION MIXTURE.**

The adipocyte mixtures were centrifuged for 1 min at 1000 x g. The incubation medium was withdrawn from under the adipocyte layer.

The adipocytes layer was washed three times with 10 ml KRB by suspending the cell followed by removal of the washings after centrifugation. The washed adipocytes was homogenised in 5 ml buffer mixture (250 mM sucrose, 10 mM potassium phosphate buffer pH 7.4, 1 mM EDTA and 1 mM theophylline).

The cell homogenate was centrifuged to isolate the cell cytosol as described in Fig. 2.1.

The delipidated cell cytosol was assayed for cholesterol ester hydrolase and triacylglycerol lipase after 10 min preincubation of the cytosol with buffer (control enzyme), Mg**++** ions (inactivated enzyme) and with cAMP/ATP/Mg**++** (activated enzyme). For incubation details and enzyme assay see the methods section.

Fig. 6.1 The protocol of adipocyte preparation and the effect of nicotinic acid on the stimulation by epinephrine of cholesterol ester hydrolase and triacylglycerol lipase of isolated rat adipocyte cytosol.
<table>
<thead>
<tr>
<th>Adipocytes incubation</th>
<th>Cholesterol Ester Hydrolase (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (+ Buffer)</td>
</tr>
<tr>
<td>Control</td>
<td>100±0.9 (2150)</td>
</tr>
<tr>
<td>+ Nicotinic acid (1 mM)</td>
<td>103±4.2</td>
</tr>
<tr>
<td>+ Epinephrine (10 μM)</td>
<td>114.6±5.4*</td>
</tr>
<tr>
<td>+ Nicotinic acid + Epinephrine</td>
<td>79.3±2.5*</td>
</tr>
</tbody>
</table>

Table 6.4. The effect of nicotinic acid (1 mM) addition to isolated rat adipocyte cytosolic cholesterol ester hydrolase. Results are the mean percentage values of four separate experiments ± S.E.M. Figure in parenthesis is the mean of enzymic activity of control expressed as pmoles/min/mg protein. Cytosol of each adipocyte incubation was pre-incubated with buffer, Mg$$^{++}$$ or cAMP to get control, inactivated and activated enzyme preparation prior the enzymic assay. For adipocyte incubations see the text. Asterisk indicates a significant change (P<0.01) from control.
<table>
<thead>
<tr>
<th>Adipocytes incubation</th>
<th>Triacylglycerol Lipase (%)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (+ Buffer)</td>
<td>Inactivated (+ Mg^{++})</td>
</tr>
<tr>
<td>Control</td>
<td>$100\pm1.2$ (351)</td>
<td>$85.5\pm4.9$</td>
</tr>
<tr>
<td>+ Nicotinic acid (1 mM)</td>
<td>$103.2\pm7.4$</td>
<td>$89.4\pm3.2$</td>
</tr>
<tr>
<td>+ Epinephrine (10 mM)</td>
<td>$123.8\pm4.6^*$</td>
<td>$106.7\pm5.4$</td>
</tr>
<tr>
<td>+ Nicotinic acid + Epinephrine</td>
<td>$86.1\pm3.9^*$</td>
<td>$82.4\pm2.7$</td>
</tr>
</tbody>
</table>

Table 6.5. The effect of nicotinic acid addition in vitro to isolated rat adipocytes cytosolic triacylglycerol lipase. For details see legend of Table 6.4.
The basal activity of triacylglycerol lipase from isolated adipocyte cytosol was quite low compared to that of adipose tissue cytosol reported in Table 6.1 of this section, and, although the basal activity of cholesterol ester hydrolase was slightly lower than that reported in Table 6.1, it was not reduced as much as the triacylglycerol lipase. Thus the comparatively lower apparent activity of triacylglycerol lipase would show a higher apparent percentage activation due to the cAMP-dependent protein kinase. It is possible that the two different substrates might not be identical in their interaction with their respective enzyme active site(s) since both are acting on the substrate at an interface and hence different apparent activities might be observed.

When nicotinic acid was added prior to epinephrine addition to an isolated rat adipocyte suspension it blocked the stimulation in vitro by epinephrine of cholesterol ester hydrolase and triacylglycerol lipase. Moreover it lowered the activities of both enzymes to a level significantly below the normal control values. However the inhibition by nicotinic acid of cholesterol ester hydrolase and triacylglycerol lipase from epinephrine-stimulated adipocytes was reversed by more than 50% when the cytosol was pre-incubated with cAMP in vitro. Thus cAMP-dependent protein kinase was not totally suppressed and exogenous cAMP was able to re-activate the enzyme.

The mechanism by which nicotinic acid inhibits the stimulation by epinephrine of cholesterol ester hydrolase and triacylglycerol lipase is difficult to explain. Nicotinic acid appears to be active on adipose tissue when lipolysis is stimulated; that is when a certain concentration of cAMP is attained, nicotinic acid would stimulate certain regulatory site(s)? These control site(s) could be of cAMP accumulation in fat cells, the synthesis of cAMP, accumulation of endogenous feedback regulators of lipolysis, which could include prostaglandins, fatty acids, adenosine, AMP or agents which have not been fully characterised such as that found by Pain and Shepherd (1976) and released into the medium
of adipocytes only in the presence of lipolytic hormones (see reviews by Fain (1977) and Fredholum (1978)). Whatever the mechanism may be, it seems more likely that nicotinic acid does not act directly on adipocyte cholesterol ester hydrolase or triacylglycerol lipase, but rather at stages prior to cAMP-dependent protein kinase activation. It is possible as well, that nicotinic acid has an indirect effect on lipase phosphatase or lipase phosphatase-heat stable inhibitor described by Severson and Sloan (1977). These highly hypothetical postulates will remain so until every step of the regulation of adipose tissue lipolysis is fully characterised.

Experiments reported in this section showed the presence of cholesterol ester hydrolase in adipose tissue cytosol, a fraction which is also rich in triacylglycerol lipase activity (Pittman, Khoo and Steinberg (1975)). The sensitivity of these enzymes to lipolytic hormones both in vivo and in vitro was quite similar. Moreover, nicotinic acid, an antilipolytic agent, was shown in this study to modulate the acute response of cholesterol ester hydrolase and triacylglycerol lipase to lipolytic hormones. The similarity of response of these two enzymic activities to hormonal signals, which is like the situation in the rat adrenal (Section III) poses again the question of their identities. With the limited experimental evidence obtained and reported in this section it is very difficult to speculate about the relationship between cholesterol ester hydrolase and triacylglycerol lipase in rat adipose tissue cytosol. However, evidence from the literature (Pittman, Khoo and Steinberg (1975)) lends support to the view which is reported in the previous sections on rat and bovine adrenal cytosolic enzymes, that either a complex of two or more enzymes is present or a single-low specificity enzyme is responsible for the hydrolysis of both cholesteryl esters and triacylglycerol.
The role of cholesterol ester hydrolase in adipose tissue is not known yet. Although the amount of esterified cholesterol in adipose tissue is very small (10-20% of total cholesterol) its contribution through enzymic hydrolysis, to adipocyte lipid droplet integrity has been postulated but never confirmed (Kovanen, Nikkila and Miettinen (1975)). It is also reported that the uptake of very low density lipoprotein cholesterol esters by rat adipose tissue, though very small, involves a very rapid hydrolysis of these cholesterol esters (Brot, Lossow and Chaikoff (1964)). Finally, nicotinic acid treatment of hypercholesterolaemic subjects induced a rapid fall of serum cholesterol which was associated with a rise in fecal neutral steroids of endogenous origin, and it was suggested that nicotinic acid caused an increased mobilisation of tissue cholesterol (Miettinen (1968)). It will remain to be determined which tissue was depleted of its cholesterol content described in that report, and hence which enzyme(s) system was more sensitive to nicotinic acid treatment, whether it was the synthesis of cholesterol or the hydrolysis of cholesterol esters. If it is found that the hydrolysis of cholesterol esters is a sensitive system, then the characteristics of this cholesterol ester hydrolase might be different from the hydrolase reported in this study.

6.4 SUMMARY

1. Rat adipose tissue cytosol contains an activatable cholesterol ester hydrolase as well as triacylglycerol lipase.

2. Administration of ACTH in vivo stimulated both enzymes from adipose tissue cytosol.

3. The in vivo stimulation of these enzymes was enhanced when the adipose tissue cytosol was pre-incubated in vitro with cAMP.

4. Nicotinic acid administration in vivo blocked the stimulation in vivo of cholesterol ester hydrolase and, to a lesser extent, triacylglycerol lipase of rat adipose tissue cytosol.
5. Isolated rat adipocytes incubated with nicotinic acid showed a consistent response in that both hydrolytic enzymes, cholesterol ester hydrolase and triacylglycerol lipase failed to be stimulated with epinephrine.

6. The similarity of the response of adipose tissue cytosolic cholesterol ester hydrolase and triacylglycerol lipase to both in vivo and in vitro manipulation suggested that these enzymes may be associated in a manner similar to that found in bovine and rat adrenal cytosol.
SECTION VII

STUDIES ON RAT OVARIAN CHOLESTEROL ESTER HYDROLASE AND OVARIAN STEROIDOGENESIS

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SECTION VII: STUDIES ON RAT OVARIAN CHOLESTEROL ESTER HYDROLASE AND OVARIAN STEROIDOGENESIS

7.1 INTRODUCTION

The early studies of Haynes and Berthet (1957) on the role of cAMP in the steroidogenic response of the adrenal to ACTH initiated several studies on the possible role of cAMP in other steroidogenic tissues such as the testis and the ovaries. The mammalian ovary has been the subject of intensive research (for reviews see Marsh (1975), Channing, Thanki, Lindsey and Ledwitz-Rigby (1978), Dufau and Catt (1978)). The corpus luteum of the mammalian ovary has as its primary function the synthesis and secretion of progestins. This tissue has been shown to be under the influence of luteinising hormone (LH) at least in the human, cow and rat (Greep (1971)). LH was found to increase cAMP accumulation in cow and rat corpora lutea, and this accumulation was due to stimulation of adenylate cyclase (Marsh (1970), Lamprecht, Zor, Tsafriri and Linder (1973)). The in vitro addition of cAMP to bovine corpora lutea slices was shown to stimulate progesterone production (Marsh and Savard (1964)) and it was concluded that cAMP enhances steroidogenesis in corpora lutea at a step(s) between cholesterol and pregnenolone (Hall and Koritz (1965)).

Using corpora lutea preparation isolated from pseudopregnant immature female rats, Flint, Grinwicht and Armstrong (1973) showed that LH administration in vivo caused a marked decrease in the corpus luteum esterified cholesterol. Following that, Beckett (1975) showed that LH administration in vivo to pseudopregnant rats was accompanied by an activation of a cytosolic cholesterol ester hydrolase, and it was postulated that the activation might be through the phosphorylation of the enzyme by a cAMP-dependent protein kinase similar to what was found in the rat adrenal (Boyd, Arthur, Beckett, Mason and Trzeciak (1975)).
Studies on ovarian tissue with regard to cAMP and ovarian steroidogenesis are few. The main reason for this is the complications posed by the fact that there are three components in the mammalian ovary namely the Graafian follicle, the corpus luteum and the interstitial tissue (Marsh (1975)). Therefore the results are very difficult to interpret. However, cAMP accumulation in response to LH was studied in the ovarian tissue of the rat, and the stimulation of cytosolic cAMP-dependent protein kinase in response to LH was also observed (Mason, Schaffer and Toomey (1973), Lamprecht, Tsafriri and Linder (1973)).

It was of interest to study the effect of LH on the ovarian cytosolic cholesterol ester hydrolase at the ovulation stage and shortly after that event, linking the hydrolase activity with mitochondrial steroidogenesis and ovarian steroid content. The experimental animals chosen for this study were immature female rats (21-24 days old) pre-treated with Pregnant Mare's Serum Gonadotrophin (PMSG) which induces follicular development in these rats. The rats were killed two days after such treatment. The full experimental conditions of the assay of progesterone, pregnenolone, free cholesterol, esterified cholesterol and cholesterol ester hydrolase are described in the methods section. The experimental animal model chosen was shown to respond to low doses of PMSG by induced ovulation, and this ovulation was similar to spontaneous ovulation (Guillet and Rennels (1964), Herlitz, Khan and Ahren (1976)). Moreover, under these conditions the ovaries from these animals respond to LH by increased production of intracellular cAMP (Herlitz, Khan and Ahren (1976)). Under these specific experimental conditions an attempt was made to study rat ovarian cytosolic cholesterol ester hydrolase and correlate this activity with ovarian free cholesterol, esterified cholesterol and ovarian steroidogenesis.
7.2 THE EFFECT OF LH ADMINISTRATION IN VIVO ON OVARIAN CHOLESTEROL ESTERS, FREE CHOLESTEROL, PROGESTERONE AND SERUM PROGESTERONE IN THE PMSG-PRIMED RAT

Using the experimental animal model described above, Goff and Henderson (1979) showed that a single dose of LH (10 μg) which induced ovulation in these PMSG-primed rats, produced a dramatic change in follicular and serum steroid concentrations. These changes were similar to those found on the day of ovulation in the mature female rat (Meijs-Roelofs, Uilenbrocek, DeGreef, DeJong and Kramer (1975)). Moreover, Goff and Henderson (1979) substantiated the evidence of Guillet and Rennells (1964) that 4 iu of PMSG administered previously to immature female rats coupled with a single ovulatory dose of LH does provide a useful experimental model from immature female rats, which simulates the mature cyclical female rats with regard to ovulation, follicular steroid content, luteinisation and serum progesterone concentration. Optimum steroidogenic activity in the ovaries from these PMSG-primed rats after receiving LH was established so that a proper study of cholesterol ester hydrolase could be carried out. Cholesterol and cholesterol esters were measured in the ovaries of these rats together with serum and ovarian progesterone. Fig. 7.1 shows the effect of LH administration in vivo on serum and ovarian steroid concentrations of PMSG-primed immature female rats. Animals were killed by decapitation at the indicated time after LH injection, and trunk blood was collected for serum progesterone measurement. The ovaries were also removed, trimmed of adhering fat and oviduct, dried on filter paper and weighed individually. They were homogenised in chloroform/methanol mixture (2:1, v/v) and measurements of free and esterified cholesterol as well as serum and ovarian progesterone were performed as described in the methods section.
Fig. 7.1 The effect of 10 μg LH administration in vivo on ovarian cholesterol, cholesterol ester, progesterone and serum progesterone concentrations in PMSG-primed immature female rats. Results are the mean values ± SEM (4 animals). The estimation of the steroids was carried out as described in the methods section.
Serum progesterone increased 10 fold, 6 h after LH administration to about 60 ng/ml serum. At the same time ovarian progesterone concentration rapidly increased after LH injected reaching a maximum of 11 ng/mg tissue 6 h after the animal received LH. These data are consistent with the finding of Goff and Henderson (1979) that LH administration to PMSG-primed immature female rats caused a change in ovarian and serum progesterone at the time of ovulation. Moreover, measurement of steroid content in this study was extended to include the luteal phase of these ovaries. After LH administration the serum and ovarian progesterone concentration rose rapidly to a peak at 6 h and then declined to a low point at 24 h. Thereafter, there was a secondary rise which reached a new peak at 48 h after the initial LH administration, the progesterone concentration of both serum and ovary continued to decline. It is thought that the fall in progesterone production 12 h after LH injection may be caused by receptor desensitisation of luteal cells, while the second rise of progesterone production could indicate functional corpora lutea before the start of luteal regression.

Free and esterified cholesterol were measured in the ovaries of these PMSG-primed rats after they received the LH injection. In Fig. 7.1 it can be seen that the amount of free cholesterol in the ovaries of these rats did not change significantly from the value observed at 0 h (time of LH administration). On the other hand, esterified cholesterol did not change significantly during the first 12 h after LH injection, it increased in the ovaries of these rats in 24 h reaching a 2 fold increase by 48 h after LH administration and appeared to stabilise at that level up to 72 h from the hormone injection time. Although in Fig. 7.1 there is a small fall in the amount of esterified cholesterol in the ovaries of the PMSG-LH treated immature female rats, this fall was not significant in four separate experiments. These results show that LH-induced ovulation while accompanied by a surge of progesterone
synthesis and secretion, does not deplete the ovarian free or esterified cholesterol, and accumulation of esterified cholesterol was observed at 24 h after LH administration when the luteinisation phase had started. These results are similar to those obtained recently by Schuler, Scavo, Kirsh, Flickinger and Strauss (1979) who found that 50 iu PMSG-primed immature female rats (the Parlow (1958) preparation on which most corpora lutea studies are based) started from the first day of 50 iu hCG (human chorionic gonadotrophin) administration to accumulate esterified cholesterol reaching a peak value 4 days later when corpora lutea are quite mature under such conditions. It is of importance to stress that after the Parlow (1958) treatment on the immature female rats, the luteinised ovaries of these rats secrete progesterone at the peak of their steroidogenic activity to establish progesterone concentration of about 400-500 ng/ml plasma (Schuler, Scavo, Kirsh, Flickinger and Strauss (1979)). This level of progesterone production is 10 times the amount recorded in this study. It is also 10 times the highest concentration of progesterone in the serum of mature pregnant rats (Henderson and Tsang (1980)). This note will be discussed later in this section.

7.3 THE EFFECT OF LH ADMINISTRATION IN VIVO ON OVARIAN CYTOSOLIC CHOLESTEROL ESTER HYDROLASE IN PMSG-PRIMED IMMATURE RATS

Although many studies have provided evidence for luteal cholesterol ester depletion upon LH administration in vivo to rat (Behrman and Armstrong (1969)) and rabbit (Flint and Armstrong (1973)), the effect of LH addition to bovine corpora lutea slices did not produce any evidence for the activation of bovine corpora lutea cholesterol ester hydrolase (Goldstein and Marsh (1973)). Using rat corpora lutea Beckett (1975) found that LH administration in vivo caused a depletion of esterified cholesterol of the lipid droplets isolated from these ovaries, and
associated with this depletion a two fold stimulation of cytosolic cholesterol ester hydrolase. The discrepancy between these reports could be attributed to one main reason, that the bovine corpora lutea studied by Marsh group is a system isolated from animals in a more physiological condition, and different from rat corpora lutea studied by others who obtain the tissue after presenting the animal with a massive hormonal stimulus. It seems unlikely that under normal conditions the rat would ever suffer such a large gonadotrophin surge, even at the maximum peak of progesterone production during pregnancy. With such non-physiological condition the results may be difficult to generalise and extend to other physiological situations. The lack of studies on rat ovarian cholesterol ester hydrolase activity under physiological conditions prompted this investigation on the role of LH on cytosolic cholesterol ester hydrolase.

From the results presented in Fig. 7.1 it was decided to study the effect of LH administration in vivo on the cytosolic cholesterol ester hydrolase from ovaries of PMSG-primed immature female rats. These animals were killed 6 h after receiving either 0.2 ml saline solution (Saline group) or 10 µg LH dissolved in 0.2 ml saline which was given in the tail vein. The time after the animals were injected was decided from the results shown in Fig. 7.1. Progesterone concentrations in serum and ovary are maximal and cholesterol ester hydrolase, if it is stimulated, should be at its highest level 6 h after LH administration. Table 7.1 summarises the results of three different experiments with 12 rats per group per experiment.

Contrary to what was expected, LH administration in vivo, 6 h before the animals were sacrificed, did not cause any stimulation of cholesterol ester hydrolase but significantly in two experiments an inhibition was observed in the cytosol incubated with buffer, Mg++ ions or cAMP.
Table 7.1. The effect of LH administration (10 μg) in vivo on cytosolic cholesterol ester hydrolase of PMSG-primed immature female rats killed 6 h after LH injection. Data are obtained from 12 rats/group in each experiment. Results are mean values ± S.D. of five replicates. The cytosol of each saline and LH injected rat was preincubated for 10 min before the enzymic assay was started with the addition of emulsified substrate as described in the methods section.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Addition in vitro to enzyme incubation</th>
<th>Ovarian Cytosolic Cholesterol Ester Hydrolase (pmole oleic acid/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control saline injected rat</td>
</tr>
<tr>
<td>1</td>
<td>None</td>
<td>116±12</td>
</tr>
<tr>
<td></td>
<td>+2 mM Mg++</td>
<td>88±14</td>
</tr>
<tr>
<td></td>
<td>+10 μM cAMP/ATP/Mg++</td>
<td>170±9</td>
</tr>
<tr>
<td>2</td>
<td>None</td>
<td>193±29</td>
</tr>
<tr>
<td></td>
<td>+2 mM Mg++</td>
<td>158±19</td>
</tr>
<tr>
<td></td>
<td>+10 μM cAMP/ATP/Mg++</td>
<td>154±4</td>
</tr>
<tr>
<td>3</td>
<td>None</td>
<td>209±15</td>
</tr>
<tr>
<td></td>
<td>+2 mM Mg++</td>
<td>314±20</td>
</tr>
<tr>
<td></td>
<td>+10 μM cAMP/ATP/Mg++</td>
<td>573±11</td>
</tr>
</tbody>
</table>
Moreover, incubating ovarian cytosol from both saline and LH injected rats with magnesium ions did not consistently lower the basal activity (pre-incubation with buffer as discussed in the activation procedure (Section II)) on two occasions produced a stimulation. The reasons for this activation by Mg$^{++}$ ions are unknown and Beckett (1975) speculated that it could be activation through cAMP-dependent protein kinase which lacks only Mg$^{++}$ and has sufficient ATP and cAMP. However, the results in Table 7.1 show that addition of exogenous cAMP and ATP to ovarian cytosol produced a marked stimulation of both saline and LH injected rats ovarian cholesterol ester hydrolase reaching up to 150% increase in one experiment (exp. 3).

It seems from the results presented in Table 7.1 that LH slightly inhibited rather than stimulated ovarian cytosolic cholesterol ester hydrolase. This pattern of LH action was consistent throughout three different experiments. The time after LH injection, and the dose of LH were chosen for maximum steroidogenic response as judged by progesterone synthesis and secretion (Fig. 7.1). The data in Fig. 7.1 suggests that LH administration to PMSG-primed rats under the conditions used, results in a large stimulation of ovarian steroidogenesis and this stimulation could not be linked with any significant fall in ovarian cholesterol esters or change in free cholesterol.

How can the results in this study be interpreted in relation to the role of LH in steroidogenesis, cAMP-dependent protein kinase activation and LH inhibition of cholesterol ester hydrolase? There are several lines of evidence in the literature which could explain the results of this study. First, using mature bovine ovaries Caffrey, Fletcher, Dickman, O'Callaghan and Niswender (1979) found that cholesterol ester hydrolase activity was not increased during the oestrous cycle until the sixth day of the cycle, and reached its maximum activity by day ten.
The activity was mostly associated with the cytosol fraction and moreover it was stimulated with cAMP, ATP, Mg++ in the presence of a phosphodiesterase inhibitor. Secondly, even with the Parlow (1958) treated rats (50 iu PMSG followed by 25 iu hCG) which causes ovulation and eventually luteinisation of immature female rat ovaries, cholesterol ester hydrolase activity was not stimulated with hCG treatment until the fourth day after hCG injection and reached a peak on the 8th day. At this time the esterified cholesterol in these ovaries is increased 14-fold (Strauss, Schuler, Kirsh and Flickinger (1978)). Thirdly, it was shown recently that in the first day of hCG injection to the 50 iu PMSG-primed immature female rats, a sharp and several fold increase of ovarian microsomal HMG-CoA reductase activity was observed. This was followed two days later by a gradual stimulation of microsomal acyl-CoA cholesterol acyltransferase (ACAT) activity reaching its peak by the 8th day after hCG injection (Schuler, Scava, Kirsch, Flickinger and Strauss (1979)). Finally, bovine corpora lutea slices responded to LH incubation in vitro by enhancement of progesterone production while cholesterol ester hydrolase was not affected by LH at all (Goldstein and Marsh (1973)).

LH administration to female rats with fully luteinised ovaries produces a stimulation of cytosolic ovarian cholesterol ester hydrolase, a stimulation which may be mediated by cyclic AMP-dependent protein kinase (Beckett (1975), Behrman and Armstrong (1969)). However under the conditions used in this study such a stimulation was not observed. No one reason could be found for this discrepancy, except that it is possible that female rat ovaries accumulate esterified cholesterol in large quantities, as in these PMSG-hCG treated rats, while LH may stimulate ovarian cytosolic cholesterol ester hydrolase, possibly through cAMP-dependent protein kinase. However, in Table 7.1 the results show
that in vitro pre-incubation of ovarian cytosol with cAMP, caused a significant stimulation of cholesterol ester hydrolase in both saline and LH injected rats. This means that the mechanism of cholesterol ester hydrolase activation, at least in vitro, was not impaired, and earlier studies of Lamprecht, Zor, Tsafriri and Linder (1973) showed that rat ovaries responded to LH with increased cAMP production in the second week of postnatal development. For such explanations, one question remains to be answered and that is whether LH has a dual action on cholesterol ester hydrolase. It is very unlikely, but further investigation regarding the role of LH on ovarian steroidogenesis is required with more consideration being given to recent findings on the activation of ovarian HMG-CoA reductase and ACAT after hCG administration (Schuler, Scavo, Kirsch, Flickinger and Strauss (1979)).

To validate that LH did not activate ovarian cholesterol ester hydrolase at a time not linked with maximum progesterone production, the following experiments were carried out. In the first experiment, animals received LH or saline by injection and were sacrificed 3 or 6 hours later. Serum progesterone concentration as well as ovarian cytosolic cholesterol ester hydrolase were measured as described in the method sections. Table 7.2 illustrates the result of that experiment and it shows clearly that LH administration in vivo 3 or 6 hours before sacrifice did not produce any stimulation of ovarian cytosolic cholesterol ester hydrolase in the 4 iu PMSG-primed immature female rat. The Table also shows that progesterone secretion into serum was increased in animals sacrificed 6 hours after LH administration.

The results in Table 7.2 were further assessed by the following experiment. Instead of using saline-injected rats, 36 immature female rats (21-24 days) which as before, had been treated 2 days earlier with 4 iu PMSG, were injected in the tail vein with 10 μg LH in 0.2 ml saline.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Saline injected rats</th>
<th>10 μg LH injected rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time after injection</td>
<td>3 h</td>
<td>6 h</td>
</tr>
<tr>
<td>Incubated with buffer only</td>
<td>139±3</td>
<td>209±15</td>
</tr>
<tr>
<td>Incubated with cAMP/ATP/Mg++</td>
<td>163±13</td>
<td>573±11</td>
</tr>
</tbody>
</table>

Ovarian progesterone (ng/mg ovarian tissue) | 0.11±0.04 | 0.12±0.05 | 1.3±0.6 | 6.3±1.8

Table 7.2. The effect of LH administration in vivo on ovarian cytosolic cholesterol ester hydrolase and ovarian progesterone concentration in PMSG-primed immature female rats sacrificed 3 and 6 hours after receiving saline or 10 μg LH injection. Results are mean values ± S.D. of five determinations.
Table 7.3. The changes in cytosolic cholesterol esterase hydrolase activity in cytosol of ovaries from rat sacrificed at 0 h after LH was considered 10% (116 pmoles oleic acid released/min/mg protein) to which had previously been exposed. All other figures referred to as in the Methods section.

<table>
<thead>
<tr>
<th>Time after LH administration (Hours)</th>
<th>Cholesterol Ester Hydrolase (per cent)</th>
<th>+Mg</th>
<th>+cAMP/ATP/Mg</th>
<th>+GppNHp/Mg</th>
<th>No addition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(Mean - S.D.) (n=5)</td>
<td>(Mg)</td>
<td>(Mg)</td>
<td>(Mg)</td>
<td>(Mg)</td>
</tr>
<tr>
<td>0</td>
<td>58 ± 8</td>
<td>26 ± 7</td>
<td>161 ± 34</td>
<td>4 ± 0.7</td>
<td>74 ± 2.2</td>
</tr>
<tr>
<td>6</td>
<td>76 ± 5.4</td>
<td>63 ± 4</td>
<td>268 ± 49</td>
<td>3 ± 0.9</td>
<td>72 ± 5.8</td>
</tr>
<tr>
<td>48</td>
<td>71 ± 12</td>
<td>74 ± 4</td>
<td>517 ± 3</td>
<td>0 ± 0.3</td>
<td>74 ± 2.2</td>
</tr>
</tbody>
</table>

Ovarian progesterone, pregnenolone, and cholesterol content of ovaries of PMSG-primed rats sacrificed at 0 h after LH was considered 10% (Mean - S.D. n=5). Ovarian progesterone (Mean - S.D. n=5) and mitochondrial progesterone, pregnenolone and cholesterol (Mean - S.D. n=5) were measured as described in the Methods section.
Twelve of these rats were killed immediately after the LH injection (0 hour), and the rest were killed 6 and 48 hours after administration of LH, i.e., twelve rats per group.

Serum progesterone was measured as before from trunk blood. Cholesterol ester hydrolase was measured in the ovarian cytosol as before, using cholesteryl\(_{14}^{14}\)oleate. In addition, ovarian mitochondria were prepared for pregnenolone, progesterone and free cholesterol estimations using the following procedure. Ovaries were homogenised in 0.25M sucrose pH 7.0, and the homogenate was centrifuged in an MSE centrifuge at 650 x g for 10 min to sediment cell debris and nuclei. The supernatant was further centrifuged at 8,500 x g for 15 min to sediment mitochondria. The mitochondrial pellet was washed once with sucrose (0.25M, pH 7.0) and centrifuged at 8,500 x g for a further 15 min. The pellet was suspended in 0.25M sucrose, pH 7.0 and used for protein and steroid measurements. Pregnenolone, progesterone and free cholesterol were extracted from the mitochondrial suspension by a chloroform:methanol mixture (2:1 v/v). The efficiency of pregnenolone and progesterone extraction was 95% as checked by addition of radioactive tracers. Pregnenolone and progesterone were measured in the chloroform:methanol extract using the respective radioimmunoassays which were described in Section II, while cholesterol was also measured in the extract using the fluorometric assay described in the same section. Table 7.3 summarises the results of such experiments in which it can be seen that although ovarian progesterone concentration was increased 10 fold in six hours after LH administration, a similar increase could not be detected in ovarian cytosol cholesterol ester hydrolase and a significant inhibition was observed which persisted for up to 48 hours after the injection of LH. Moreover, data obtained from ovarian mitochondria showed that upon LH injection there was a five fold increase in mitochondrial pregnenolone and
progesterone production. Ovarian mitochondrial free cholesterol decreased 3-6 hrs after LH administration which is about 40% of its concentration at 0 hours. However 42 hrs later the concentration of mitochondrial free cholesterol was restored to normal values. The decline of mitochondrial progesterone at 48 hour from the high level observed at 6 hour, is consistent with the result described in Fig. 7.1 where serum and total ovarian progesterone were lower 48 hour after LH injection although higher than the progesterone concentration at zero time.

The data in Table 7.2 and 7.3 show that a single dose of LH to 4 iu-PMSG-primed immature female rats initiated ovarian mitochondrial steroidogenesis with a maximum response observed about 6 hours after LH. The stimulation of ovarian steroidogenesis was associated with a decrease in the mitochondrial free cholesterol. However, measurements of cholesterol ester hydrolase at 6 and 48 hour after LH injection showed a significant inhibition which increased with time after the LH injection. These results, together with the increase in esterified cholesterol reported in Fig. 7.1 indicated that a single dose of LH, could stimulate ovulation in these 4 iu PMSG-primed immature female rats although such a treatment did not stimulate ovarian cytosolic cholesterol ester hydrolase. However, the depletion of free cholesterol in ovarian mitochondria reported in Table 7.3, and the later increase in free cholesterol to within the normal range observed at zero hours may be explained as follows. The indirect action of LH on the sterol side-chain cleavage reaction could be followed by stimulation of cholesterol synthesis through HMG-CoA reductase as recently observed (Schuler, Scavo, Kirsh, Flickinger and Strauss (1979)). It is not known why LH stimulates cholesterol ester hydrolase in luteinised rat ovaries of the Parlow (1958) type while in a semi-physiological induced ovulation it does not produce such a stimulation despite the presence of a responsive cAMP-dependent protein kinase.
7.4 THE EFFECT OF LH ADMINISTRATION IN VIVO ON OVARIAN MITOCHONDRIAL SIDE-CHAIN CLEAVAGE AND 3β-HYDROXysteroid DEHYDROGENASE ACTIVITIES IN VITRO

One of the possible sites of action of LH is the mitochondrial sterol side-chain cleavage reaction. Most studies indicate that LH action on the complex reactions of the mitochondrial sterol side-chain cleavage lies at a site between cholesterol and pregnenolone formation (Hall and Koritz (1964) and Armstrong, Lee and Miller (1970)). The tropic action could be brought about by an increase in the substrate available to the active site of the sterol side-chain cleavage enzyme or the stimulation of one component of the sterol side-chain cleavage complex. Arthur and Boyd (1974) found in isolated ovarian mitochondria that the rate of pregnenolone formation from endogenous substrate decreased in 4-5 minutes for the addition of exogenous cholesterol to these mitochondria and maintained the rate of pregnenolone formation for up to 30 minutes. They suggested that the supply of cholesterol to the luteal sterol side-chain cleavage complex is a critical factor in the regulation of ovarian steroidogenesis. On the other hand, cAMP was reported to stimulate pregnenolone synthesis and inhibit the Δ⁵-3β-hydroxysteroid dehydrogenase and Δ⁵-3-ketosteroid isomerase which are enzymes involved in the conversion of pregnenolone to progesterone (Sulimovici and Boyd (1968)). Since most studies of ovarian steroidogenesis were carried out in the presence of inhibitors which block the further metabolism of pregnenolone (cyanoketone), it was of interest to assess the effect of LH administration in vivo on the sterol side-chain cleavage reaction in ovarian mitochondria of PMSG-primed immature female rats. In these experiments, 4 iu PMSG-primed immature female rats were given LH (10 µg) in the tail vein, while a control group received saline solution. The animals from both groups were killed six hours later and their ovaries were collected separately. Mitochondria were prepared from the homogenate of these
ovaries as described in the previous sub-section. Mitochondrial sterol side-chain cleavage activity was assessed by the amounts of pregnenolone and progesterone, measured by their respective radio-immunoassay, as described in the method section. The mitochondria from ovaries of both saline and LH treated rats were pre-incubated for 15 min with either 100 μl ethanol or with added exogenous cholesterol or 24-hydroxycholesterol (100 μM) delivered in 100 μl ethanol. Sterol side-chain cleavage activity was initiated by the addition of the electron donor DL-isocitrate (10 mM), the incubation was continued for 20 min at 37°C. The reaction was stopped at the indicated times by extraction with chloroform:methanol (2:1 v/v), and pregnenolone and progesterone were measured in the extract. Fig. 7.2 summarises the results of such experiment.

LH administration in vivo increased pregnenolone and progesterone formation in ovarian mitochondria when the mitochondria were not supplied with exogenous substrate. Consistent with the findings of Arthur and Boyd (1974), the ovarian mitochondrial pregnenolone synthesis rate decreased (in both saline and LH injected rats) in less than 5 min. However, addition of exogenous cholesterol or 24-hydroxycholesterol in vitro to these mitochondria not only stimulated pregnenolone synthesis but also maintained the elevated rate for up to 20 min. Exogenous substrate addition in vitro to ovarian mitochondria, prepared from both saline and LH-injected rats abolished the difference between the two groups in terms of pregnenolone synthesis.

Luteinising hormone (LH) administered in vivo produced a marked stimulation of progesterone from ovarian mitochondria incubated without exogenous sterol substrate. The rate of progesterone synthesis did not decrease as rapidly as pregnenolone synthesis. Moreover, in contrast to pregnenolone synthesis in the presence of exogenous substrate,
Fig. 7.2. The production of pregnenolone and progesterone in vitro in ovarian mitochondria of PMSG-primed immature female rats. Mitochondria were prepared from these rats after receiving either i.v. saline injection or 10 µg LH injection 6 h before sacrifice. Results are mean values ± SEM (six replicate determination). The preparation of ovarian mitochondria and estimation of ovarian sterol side chain cleavage activity were carried out as described in the methods section.
progesterone synthesis was not enhanced by the addition of cholesterol or 24-hydroxycholesterol to mitochondria from saline-injected rat ovaries. However, in vitro addition of exogenous sterol substrate stimulated progesterone synthesis in ovarian mitochondria from LH-treated rats, but stimulation was observed in such mitochondria pre-incubated with acetone. These results indicate that although pregnenolone synthesis was enhanced in ovarian mitochondria of saline injected rats, when these mitochondria were pre-incubated in vitro with exogenous cholesterol, the increase of pregnenolone formation was not followed by an increased progesterone production. Thus, the availability of sterol substrate to these mitochondria is not enough to promote progesterone synthesis. It seems that LH may act directly on the \( \Delta^5-3\beta \)-steroid dehydrogenase and \( \Delta^5,\Delta^4 \)-ketosteroid isomerase enzyme complex, as well as its well documented action on the translocation of cholesterol to the active site of the mitochondrial side-chain cleavage enzyme complex (Marsh (1975)). It is not known whether the apparent stimulation of the pregnenolone to progesterone transformation by LH is mediated by cAMP or not. It must be noted that the 3\( \beta \)-hydroxysterol dehydrogenase is located in mitochondria and in the endoplasmic reticulum, in these studies only the mitochondrial enzyme was studied. Recently, using procine corpora lutea slices, Downing and Dimino (1979) showed that 10 \( \mu \)g LH added to these slices produced a 3-fold stimulation of progesterone synthesis. This stimulation coincided with the activation of mitochondrial cAMP-dependent protein kinase. However, these authors could not detect any increase in the mitochondria sterol side-chain cleavage enzyme activity, measured as the percentage conversion of added \( \Delta^4-\text{\textsuperscript{14}C} \)-cholesterol in mitochondria prepared from such LH-treated corpora lutea.
7.5 **SUMMARY**

1. Administration of LH to PMSG-primed immature female rats produced a several fold increase in both serum and ovarian progesterone concentration which reached its peak value 6 h after LH injection.

2. The increased progesterone synthesis and secretion was not linked with any significant change in ovarian free cholesterol. However the esterified cholesterol concentration was increased 24 h after the LH injection.

3. The changes in progesterone and esterified cholesterol concentrations in the ovaries of these rats was not correlated with any stimulation of ovarian cytosolic cholesterol ester hydrolase.

4. Although *in vitro* incubation of ovarian cytosol with cAMP resulted in an activation of cholesterol ester hydrolase, it seemed that LH did not affect the enzyme through cAMP-dependent protein kinase.

5. Studies on ovarian mitochondrial sterol side-chain cleavage activity indicated that LH affected the 3β-steroid dehydrogenase apart from its effect on the facilitation of cholesterol accessibility to the sterol side-chain cleavage enzyme complex.

6. It was concluded that *in vivo* administration of LH to PMSG-primed immature female rats produced a significant change in progesterone synthesis and secretion, a build-up of esterified cholesterol in the ovaries without stimulation of cholesterol ester hydrolase. Therefore, earlier results of studies on Parlow (1958) treated immature female rats were not supported by the results obtained in this study.
SECTION VIII

STUDIES ON RAT LIVER CYTOSOLIC CHOLESTEROL ESTER HYDROLASE

8.1 Introduction

8.2 The assay of rat liver cholesterol ester hydrolase

8.3 The effect of protein concentration, incubation time and pH of the incubation medium on the estimation of rat liver cytosolic cholesterol ester hydrolase activity

8.4 The effect of Mg++, ATP and cAMP addition in vitro on the rat liver cytosolic cholesterol ester hydrolase activity

8.4.1 The effect of Mg++ concentration

8.4.2 The effect of the addition of equimolar concentrations of ATP and Mg++ on rat liver cholesterol ester hydrolase

8.4.3 Comparison of the in vitro activation by cAMP of cholesterol ester hydrolase in rat liver, adrenal and adipose tissue

8.5 The effect of glucagon administration in vivo on rat liver cytosolic cholesterol ester hydrolase

8.6 The effect of dietary cholesterol on rat liver cytosolic ester hydrolase

8.7 Summary
CHAPTER VIII STUDIES ON RAT LIVER CYTOSOLIC CHOLESTEROL ESTER HYDROLASE

8.1 INTRODUCTION

In the search for the distribution of cholesterol ester hydrolase activated by the phosphorylation-dephosphorylation mechanism similar to that found in rat adrenal and adipose tissue, attention focussed on rat liver because of its exclusive role in bile salt synthesis and it is known that free cholesterol serves as the primary precursor of the bile salts (Boyd, Hattersley, Mason, Arthur and Beckett (1977)). It is also known that diets high in cholesterol fed to the rat results in a massive accumulation of esterified cholesterol in the liver (Gould (1977)). It is firmly established that the liver plays an important part in the rapid uptake of chylomicron remnant under various physiological states (Sherrill (1978)). This rapid uptake of chylomicron esterified cholesterol and its storage in rat liver prompted Deykin and Goodman (1962) to study the hydrolysis of cholesterol esters by rat sub-cellular fractions. These authors showed that rat liver cytosol contained most of the total liver cholesterol ester hydrolase when they assayed the enzyme by the use of exogenous labelled substrate delivered in an acetone solution. However, the regulation of the enzymic activity was not studied in that report nor in subsequent studies which dealt with liver cholesterol ester hydrolase in general (Lundberg, Klemets and Lovgren (1979), Stokke (1972), Nilsson, Norden and Wilhelmsson (1973)). The lack of information regarding how cholesterol ester hydrolase in the liver is regulated, and the significance of the possible modulation of this enzyme on the metabolism of cholesterol in the liver prompted this study. An attempt was made to compare this liver enzyme with a similar enzyme in rat adrenal.
8.2 THE ASSAY OF RAT LIVER CHOLESTEROL ESTER HYDROLASE

These studies on rat liver cytosolic cholesterol ester hydrolase were carried out before the adaptation of the emulsified substrate assay described in the methods section. The assay which was used in the liver study was essentially that described by Beckett (1975) and based on that devised by Chen and Morrin (1971). In this assay labelled $^{14}$C cholesteryl oleate was added in a small volume of acetone to the delipidate liver cytosol in potassium phosphate buffer with the subsequent isolation of radioactive cholesterol by precipitation with ethanolic solution of digitonin (Sperry and Webb (1950)). In a final volume of 0.5 ml the following concentrations were used as follows: $^{14}$C cholesteryl oleate, 60 μM (0.025-0.05 μCi/assay), potassium phosphate buffer pH 7.4, 20 mM, enzyme protein, 0.1-1 mg, and acetone, 2% (v/v). Any other additions were added without altering the final volume of 0.5 ml incubation mixture. Incubations were carried out for 30 min at 37°C. Fig. 8.1 illustrates the procedure up to the final step of $^{14}$C cholesterol estimation.

8.3 THE EFFECT OF PROTEIN CONCENTRATION, INCUBATION TIME AND pH OF THE INCUBATION MEDIUM ON THE ESTIMATION OF RAT LIVER CYTOSOLIC CHOLESTEROL ESTER HYDROLASE ACTIVITY

Rat liver cytosol prepared as described in the methods section was used in these experiments. The effect of varying the protein concentration on the assay of cholesterol ester hydrolase was carried out in order to optimise the condition of the hydrolysis of $^{14}$C cholesteryl oleate. Fig. 8.2a shows that the hydrolysis of $^{14}$C cholesteryl oleate was linear up to about one mg protein of rat liver cytosol, thereafter the relationship was not linear. Although in the assay of Deykin and Goodman (1962) a similar amount of substrate was incubated with protein concentration of up to 4 mg protein, the liver cytosol in this study
Liver cytosol (0.1-1.0 mg protein) in 20 mM potassium phosphate buffer pH 7.4, 60 \mu M \text{[^14]}C/cholesteryl oleate was added in 10 \mu l acetone. The final volume of the assay was 0.5 ml with or without any further addition.

Incubation at 37°C for 30 min, thereafter the reaction was stopped with 2 ml acetone-ethanol mixture (1:1, v/v).

Carrier cholesterol was added (100 \mu g in 100 \mu l acetone) followed by the addition of 1 ml of 1% digitonin (10 g in a 9:1 ethanol/water mixture, one litre). The tubes were left overnight for the precipitation of cholesterol (Sperry and Webb (1950)). The digitonide[^14]C cholesterol was separated by centrifugation.

The digitonide precipitate was washed twice with 2 ml acetone-ether mixture and finally with 2 ml ether. The precipitate was dissolved in 0.5 ml redistilled methanol and aliquots were taken for the determination of \[^{14}C\text{cholesterol in 5 ml scintillation liquid (4 g 2',5'-diphenyloxazole (PPO)) and 0.03 g 1',4'-bis-5-phenyloxazoly-2-benzene (POPOP) in a litre of dry toluene.}

Counting of \[^{14}C\text{cholesterol was performed in a Packard Tri Carb liquid scintillation spectrometer. Blank assay containing boiled enzyme was run in parallel, and the recovery of \[^{14}C\text{cholesterol was checked using separate tube containing known amount of tracer cholesterol and was found routinely to be more than 80%.}

Fig. 8.1. The flow diagram of the assay of rat liver cholesterol ester hydrolase using \[^{14}C\text{cholesterol oleate delivered in acetone.}
Fig. 8.2. The effect of liver cytosolic protein concentration (a) and of the incubation time (b) on the determination of rat liver cytosolic cholesterol ester hydrolase. Cholesterol ester hydrolase assay was carried out as described in Fig. 8.1 except varying protein concentrations (a) or various time intervals (b) were used. Results are the mean values of triplicate determinations.
Fig. 8.3. The effect of hydrogen ion concentration on the determination of rat liver cytosolic cholesterol ester hydrolase by the radioassay described in Fig. 8.1. Results are mean values of duplicate determination.
was delipidated of any visible droplets, the dilution of the substrate could not be envisaged to be the cause of the non-linearity of the hydrolysis of $\overset{\alpha}{\Delta}^{14}$cholesteryl oleate. From the results obtained with this experiment, subsequent assays of cholesterol ester hydrolase were performed using a protein concentration well within the linear limits shown in Fig. 8.2a.

The effect of incubation time on the linearity of the assay of cholesterol ester hydrolase was studied over a 30 min period to give the most reasonable count rate of $\overset{\alpha}{\Delta}^{14}$cholesterol, and the linearity of hydrolysis of $\overset{\alpha}{\Delta}^{14}$cholesteryl oleate with respect to time is shown in Fig. 8.2b.

Using a protein concentration of less than 1 mg and an incubation time of 30 min, rat liver cytosolic cholesterol ester hydrolase activity was found to be much less variable than adrenal cholesterol ester hydrolase with a mean value ranging between 40-60 pmole cholesterol ester hydrolase/min/mg cytosolic protein. This activity was well below the activity found in rat adrenal and adipose tissue, but considering the total protein of the liver cytosol, the total activity of cholesterol ester hydrolase in liver cytosol exceeds that in the adrenals.

Fig. 8.3 shows the effect of pH of the incubation medium on the activity of rat liver cytosolic cholesterol ester hydrolase. The liver enzyme, like that of adrenal cytosol (Beckett (1975)), has a broad range of pH optimum between pH 6.8 and 7.8. Deykin and Goodman (1962) reported similar findings with regard to the pH optimum of liver cholesterol ester hydrolase. The enzymic activity was greater in potassium phosphate buffer than in tris-HCl buffer at the same hydrogen ion concentration.

These experiments showed that, using the molar concentration of 60 $\mu$M $\overset{\alpha}{\Delta}^{14}$cholesteryl oleate presented in acetone, liver cytosolic
cholesterol ester hydrolase assay was linear within the 30 min incubation period using liver cytosolic protein concentrations not exceeding 1 mg/assay at a pH of 7.4 and in potassium phosphate buffer. These parameters were standardised for the subsequent studies on rat liver cytosol.

8.4 THE EFFECT OF Mg²⁺, ATP AND CAMP ADDITION IN VITRO ON THE RAT LIVER CYTOSOLIC CHOLESTEROL ESTER HYDROLASE

In the previous sections, evidence was presented that upon pre-incubation of rat adrenal and adipose tissue cytosol with cAMP in vitro plus ATP and magnesium ions, a substantial activation of cholesterol ester hydrolase occurred. In several pilot experiments, rat liver cytosolic cholesterol ester hydrolase was not consistently activated when cAMP, ATP and magnesium were added to the assay medium at concentrations previously shown by Beckett (1975) to activate rat adrenal cholesterol ester hydrolase. It was decided therefore to study the activation of rat liver cholesterol ester hydrolase in a much more systematic manner.

8.4.1 The effect of Mg²⁺ concentration

When rat liver cytosolic cholesterol ester hydrolase was assayed in the presence of different millimolar concentrations of Mg²⁺ an unexpected result was obtained. Magnesium at concentrations of 1-5 mM stimulated cholesterol ester hydrolase by up to 100% of the basal activity. Unlike rat and bovine adrenal cytosolic cholesterol ester hydrolase, magnesium ions did not deactivate but activated rat liver cholesterol ester hydrolase. Addition of magnesium ions at a concentration higher than 5 mM did not seem to affect the enzyme significantly. Fig. 8.4a illustrates such experiments on the effect of magnesium ions on rat liver cytosolic cholesterol ester hydrolase using as substrate [14C]cholesteryl oleate added to the incubation mixture in acetone as described in Fig. 8.1.
Fig. 8.4. The effect of varying Mg$^{++}$ concentration (a) and of varying the concentration of equimolar mixture of Mg$^{++}$ and ATP (b), in the presence and absence of 10 μM cAMP, on the activity of rat liver cytosolic cholesterol ester hydrolase. The enzymic assay was carried out as described in Fig. 8.1 except Mg$^{++}$ or Mg$^{++}$/ATP mixture were added without alteration of the final assay volume. Results are mean values of triplicate incubations (a) and means values ± SEM of triplicate incubations (b).
8.4.2 The effect of the addition of equimolar concentrations of ATP and Mg\(^{++}\) on rat liver cholesterol ester hydrolase

When ATP was added to the assay medium of rat liver cholesterol ester hydrolase in the presence of equimolar concentrations of magnesium ions, no additive effect was observed. The enzyme was stimulated with Mg\(^{++}\)/ATP complex concentration as it was by magnesium alone as shown in Fig. 8.4a. The combined effect of Mg\(^{++}\) and ATP is illustrated in Fig. 8.4b and from these results it was decided in later experiments to use a concentration of 2-5 mM Mg\(^{++}\) and ATP at an equimolar concentration.

In the same experiment, when 10 \(\mu\)M cAMP was added to the incubation medium of rat liver cytosolic cholesterol ester hydrolase in the presence of 2, 5 or 10 mM magnesium and ATP, the same pattern was observed as with magnesium alone, i.e. greater stimulation at 2 and 5 mM and without effect at 10 mM concentration of magnesium or magnesium and ATP together. Thus, Fig. 8.4b shows clearly that the addition of cAMP did not result in the stimulation of cholesterol ester hydrolase and all the apparent activation above the control value was due to magnesium alone.

The effect of cAMP, ATP and Mg\(^{++}\) on rat liver cytosol was further assessed by pre-incubated desalted rat liver cytosol with and without the addition of 10 \(\mu\)M cAMP, 0.5 mM Mg\(^{++}\) and 0.5 mM ATP for 15 min at 37\(^\circ\)C. Both incubation mixtures were then chilled in an ice-bath and desalted by running them through a Sephadex G-25 column (2.5 x 20 cm) equilibrated with 20 mM potassium phosphate buffer pH 7.4 at 4\(^\circ\)C, and the incubation mixtures were eluted individually with the starting buffer. The protein fractions were pooled and the assay of cholesterol ester hydrolase was performed as described in Fig. 8.1 using \(^{14}\)C-cholesteryl oleate, with or without further addition of 10 \(\mu\)M cAMP, 0.5 mM ATP and 0.5 mM Mg\(^{++}\).

It has been shown by Beckett and Boyd (1977) that the addition of \(^{32}\)P-ATP with 10 \(\mu\)M cAMP to bovine adrenal cytosol resulted in the activation
and phosphorylation of cholesterol ester hydrolase and simple removal of excess $\gamma^{-32}_P$ATP and cAMP by desalting the adrenal cytosol did not affect the amount of bound radioactive phosphate nor the state of activation of adrenal cholesterol ester hydrolase. However, as it is illustrated in Fig. 8.5, pre-incubation with cAMP and Mg$^{++}$/ATP clearly indicated that when liver cytosol was pre-incubated with cAMP and ATP no activation of cholesterol ester hydrolase was observed. Control liver cytosol did not respond to further additions of ATP and cAMP, but the cytosol which was pre-incubated with cAMP showed a slight increase of cholesterol ester hydrolase when a further addition of ATP and cAMP was made to the final assay medium for cholesterol ester hydrolase.

8.4.3 Comparison of the in vitro activation by cAMP of cholesterol ester hydrolase in rat liver, adrenal and adipose tissue

To strengthen the evidence that rat liver cytosolic cholesterol ester hydrolase did not respond to in vitro activation by cAMP, experiments were performed using the assay of emulsified cholesteryl $\Delta^{-14}_\text{oleate}$ described in the methods section. In these experiments, rats were killed and the liver, adrenals and adipose tissue were homogenised and the cytosol of each tissue was prepared as described in the methods section. The assay of cholesterol ester hydrolase in all tissues was carried out simultaneously after 10 min pre-incubation with cAMP (see the activation procedure in Section II). The results of such experiments are illustrated in Table 8.1. The results show that no matter how cholesteryl oleate is presented to the liver cytosolic cholesterol ester hydrolase this enzyme is not activated by cAMP. In the same experiment as previously shown in the preceding sections, rat adrenal and adipose tissue cytosolic cholesterol ester hydrolase were activated significantly by the in vitro pre-incubation of the cytosol
Fig. 8.5. The effect of desalting of rat liver cytosol, pre-incubated with either buffer only or with 0.5 mM Mg$^{++}$, 0.5 mM ATP and 10 µM cAMP, on the activity of rat liver cytosolic cholesterol ester hydrolase. The assay of cholesterol ester hydrolase was carried out as described in Fig. 8.1 except that Mg$^{++}$/ATP and cAMP were added (dashed column) to the final assay medium.
<table>
<thead>
<tr>
<th>Addition</th>
<th>Cholesterol Ester Hydrolase (pmole oleic acid produced/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>4651 1506 18</td>
</tr>
<tr>
<td>+ cAMP/ATP/Mg^{++}</td>
<td>6945 2641 20</td>
</tr>
</tbody>
</table>

Table 8.1. The effect of in vitro addition of cAMP on cytosolic cholesterol ester hydrolase of rat adipose tissue, adrenal and liver. The enzyme was prepared from the cytosol of the indicated tissue, and the assay of cholesterol ester hydrolase was carried out using emulsified cholesteryl[^14]C7 oleate as described in the methods section after 10 min preincubation with or without 10 μM cAMP, 2 mM magnesium ions and 2 mM ATP.
with cAMP. Table 8.1 also gives a comparison of the activity of cholesterol ester hydrolase in the cytosol of rat liver, adrenal and adipose tissue. It seemed that the adipose tissue had the highest enzyme activity, while the liver had the lowest and the adrenal enzyme fell between these extremes.

Under the experimental conditions used, rat liver cytosolic cholesterol ester hydrolase showed no activation when liver cytosol was incubated with cAMP. Early experiments (Fig. 8.4a) demonstrated that magnesium ions activated the enzyme, and the mechanism of this activation is not known. The addition of ATP and cAMP in the presence of magnesium did not further affect cholesterol ester hydrolase. In the case of simultaneous addition of Mg++, ATP and cAMP, an activation was observed, but the halting of this apparent activation and the removal of excess ions by simple desalting procedure suggested that no covalent modification of cholesterol ester hydrolase had occurred in contrast to the rat adrenal enzyme (Beckett (1975)). The Mg++ stimulation of cholesterol ester hydrolase was challenged by the desalting procedure (Fig. 8.5) and by interfering with the activation by the removal of Mg++ using EDTA and by employing emulsified substrate (Table 8.1). In a simple comparative study, rat liver cytosolic cholesterol ester hydrolase was not activated by cAMP as under conditions which could activate the enzyme from rat adrenal and adipose tissue cytosol. It was not necessary to add exogenous cAMP-dependent protein kinase since rat liver cytosol has a cAMP-dependent protein kinase (Yamamura, Takeda, Kumon and Nishizuka (1970)). From these experiments described in Fig. 8.4a,b, Fig. 8.5 and Table 8.1, it was concluded that cAMP did not seem to have a direct role on rat liver cytosolic cholesterol ester hydrolase.
8.5 THE EFFECT OF GLUCAGON ADMINISTRATION IN VIVO ON RAT LIVER CYTOSOLIC CHOLESTEROL ESTER HYDROLASE

When all experiments on the activation in vitro of rat liver cytosolic cholesterol ester hydrolase gave negative results, a trial was carried out in order to determine whether an in vivo stimulation of rat liver cytosolic hydrolase could be achieved. In this experiment, rats were given i.v. injections of either saline or 100 μg glucagon in saline. Rats of both groups were killed 10 and 30 min after receiving the injection and their livers were excised quickly and homogenised for the preparation of the liver cytosol as described in the methods section.

The assay of cholesterol ester hydrolase was performed as described in Fig. 8.1 with and without the addition of 2 mM Mg++, 2 mM ATP and 10 μM cAMP. Table 8.2 illustrates the results of such an experiment, in which glucagon did not seem to affect liver cholesterol ester hydrolase either from animals killed 10 min after receiving glucagon or in those sacrificed 30 min after injection. It was shown that glucagon caused a marked increase in cAMP-dependent protein kinase of rat liver (Byus, Haddox and Russel (1978)). Moreover in perfused rat liver, glucagon restored the progressive decline of phosphorylase a (Saitch and Ui (1975)). It seemed that conditions which altered the activity of cAMP-dependent protein kinase did not activate liver cytosolic cholesterol ester hydrolase. Thus the negative results of the in vivo stimulation of liver cholesterol ester hydrolase are consistent with the negative findings of the in vitro attempted stimulation by cAMP of this enzyme. These experiments showed the different nature of liver cholesterol ester hydrolase from rat adrenal and adipose tissue cholesterol ester hydrolase. The liver enzyme did not seem to be regulated by cAMP-dependent protein kinase and phosphoprotein phosphatase as indicated by the result shown in Fig. 8.4a in which it is clear that magnesium ions did not inhibit
Table 8.2. The effect of glucagon administration in vivo on rat liver cytosolic cholesterol ester hydrolase. Three groups of rat were injected i.v. with 0.2 ml saline, and 100 μg glucagon in 0.2 ml saline. Those rats received the glucagon were killed at 10 and 30 min after the hormone injection. Cholesterol ester hydrolase was measured in liver cytosol as described in Fig. 8.1. The results are the mean values ± S.D.
the enzyme. Thus no strong evidence was obtained regarding the role of cAMP in these studies together with the failure to phosphorylate the enzyme with $\gamma-^{32}P$ followed by a simple purification step similar to these used with bovine adrenal cholesterol ester hydrolase described in Section IV (experiment not shown) indicated that liver cytosolic cholesterol ester hydrolase was not regulated by a phosphorylation-dephosphorylation mechanism.

Since the liver cytosol was shown to have both cAMP-dependent and independent protein kinase (Yamamura, Takeda, Kumon and Nishiuka (1970)), all the experiments of in vitro activation of rat liver cytosolic cholesterol ester hydrolase by cAMP were carried out without the addition of exogenous protein kinase. However it was thought that the addition of protein kinase might enhance or alter the activity of liver cholesterol ester hydrolase. This experiment showed that upon the addition of 50 μg cAMP-dependent protein kinase cholesterol ester hydrolase was not affected even in the presence of 2 mM Mg$^{++}$, 2 mM ATP and 10 μM cAMP. These results, illustrated in Table 8.3, also showed that exogenous protein kinase prevented the rise of cholesterol ester hydrolase observed with Mg$^{++}$. The lack of any stimulation due to ATP alone indicated that at least cAMP-independent protein kinase, which uses ATP as a phosphate donor, was not involved in the regulation of liver cholesterol ester hydrolase. The stimulation observed with addition of Mg$^{++}$ and ATP was not due to ATP since the degree of stimulation obtained was not more than that observed with Mg$^{++}$ alone. This was reinforced by the observed effect of the combination of Mg$^{++}$ with ADP and AMP (Table 8.3).

The series of experiments described in this section indicated the lack of the regulation of liver cholesterol ester hydrolase by cAMP-dependent protein kinase and the experiment described in Table 8.3 gave
<table>
<thead>
<tr>
<th>Incubation</th>
<th>Cholesterol Ester Hydrolase (pmole/min/mg protein)</th>
</tr>
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<tbody>
<tr>
<td>No addition</td>
<td>14±1</td>
</tr>
<tr>
<td>2 mM Mg⁺⁺</td>
<td>34±2</td>
</tr>
<tr>
<td>2 mM ATP</td>
<td>12±3</td>
</tr>
<tr>
<td>2 mM ADP</td>
<td>17±4</td>
</tr>
<tr>
<td>2 mM AMP</td>
<td>9±1</td>
</tr>
<tr>
<td>10 μM cAMP</td>
<td>19±2</td>
</tr>
<tr>
<td>Mg⁺⁺ + ATP</td>
<td>36±4</td>
</tr>
<tr>
<td>Mg⁺⁺ + ADP</td>
<td>28±2</td>
</tr>
<tr>
<td>Mg⁺⁺ + AMP</td>
<td>28±5</td>
</tr>
<tr>
<td>cAMP + Mg⁺⁺ + ATP</td>
<td>44±1</td>
</tr>
<tr>
<td>cAMP + Mg⁺⁺ + ADP</td>
<td>42±3</td>
</tr>
<tr>
<td>cAMP + Mg⁺⁺ + AMP</td>
<td>42±5</td>
</tr>
<tr>
<td>+ 50 μg Protein kinase</td>
<td>12±2</td>
</tr>
<tr>
<td>+ 50 μg Protein kinase + Mg⁺⁺ + ATP + cAMP</td>
<td>23±2</td>
</tr>
</tbody>
</table>

Table 8.3. The effect of in vitro addition of magnesium, adenine nucleotides and rabbit skeletal muscle cAMP-dependent protein kinase on rat liver cytosolic cholesterol ester hydrolase. Results are mean values ± S.D. of triplicate incubations. The enzyme was assayed as described in Fig. 8.1.
the more positive evidence that supports these findings. It would remain to be determined whether the hydrolysis of liver cholesterol esters is regulated and whether cytosolic cholesterol ester hydrolase plays any role in such a regulation.

8.6 THE EFFECT OF DIETARY CHOLESTEROL ON RAT LIVER CYTOSOLIC CHOLESTEROL ESTER HYDROLASE

Although rat liver cholesterol is mostly in the free form (about 85% free and 15% esterified) dietary cholesterol caused a large increase of up to 50 fold in the esterified fraction only (Gould (1977)). This accumulated esterified cholesterol in rat liver is associated with a rapid decline of microsomal HMG-CoA reductase (Gould); stimulation of microsomal cholesterol-7α-hydroxylase (Boyd, Scholan and Mitton (1969)) and stimulation of the microsomal acyl-CoA:cholesterol acyltransferase (ACAT) (Balasubramaniam, Mitropoulos and Venkatesan (1978)). Although cytosolic cholesterol did not change under these conditions of feeding (Gould (1977)), no attention was paid to the fate of liver cholesterol esters which were accumulated. It was thought that the hydrolysis of these esters could provide a means by which free cholesterol could be utilised for further metabolic events. Accordingly cholesterol ester hydrolase in liver cytosol might be affected under such conditions. It was decided to investigate the effect of dietary cholesterol on rat liver cytosolic cholesterol ester hydrolase.

In this type of experiment a group of female rats were given standard soft diet supplemented with 1% (w/w) cholesterol dissolved in 10% (v/w) olive oil. Control animals were given soft diet and the effect of olive oil was also checked by feeding a third group of animals a 10% (v/w) olive oil in soft diet. Feeding such diets was continued for seven days, thereafter all three groups were fed standard diet and animals from each group were sacrificed as indicated considering the day of diet replacement
as day 0. It was shown that under these experimental conditions and using the same strain of rats, liver esterified cholesterol was increased several fold and both microsomal cholesterol-7α-hydroxylase and acyl-CoA; cholesterol acyltransferase (ACAT) were stimulated after one week of feeding 1% cholesterol (P. Rae, personal communication). It was for this reason and the several fold increase in esterified cholesterol in rat liver, that the assay of cytosolic cholesterol ester hydrolase was carried out after a full week of feeding cholesterol. If the enzyme was to show any effect it might be predicted to change in activity when esterified cholesterol is abundant and further dietary intake of cholesterol is halted. Such a modulation of enzyme activity might arise from the need to remove the extra esterified cholesterol and the possible need for free cholesterol upon the removal of dietary cholesterol.

Table 8.4 summarises the results of such experiments in which cholesterol ester hydrolase was assayed in the liver cytosol of rats fed the soft diet, cholesterol diet and olive oil diet. The assay of the enzyme was carried out as described in Fig. 8.1 using the acetone-
solubilised [3-14C]cholesteryl oleate.

It seemed that the replacement of the cholesterol diet by the standard diet did not produce any effect on rat liver cytosolic cholesterol ester hydrolase in the three consecutive days immediately following the cessation of the high cholesterol dietary intake. It was shown by Harry, Dini and McIntyre (1973) that upon the replacement of the high cholesterol diet by standard diet, rat liver cholesterol esters concentration fell to normal by the third day of diet replacement in a roughly linear fashion. The lack of response of cytosolic cholesterol ester hydrolase in rat liver under these conditions could not be explained except by the assumption that the enzyme did not play any significant role in the degradation of the accumulated esterified cholesterol. This kind of
<table>
<thead>
<tr>
<th>Days after diet replacement</th>
<th>Cholesterol Ester Hydrolase (pmole cholesterol/min/mg protein)</th>
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<tbody>
<tr>
<td></td>
<td>Soft Diet</td>
</tr>
<tr>
<td>1</td>
<td>31±6</td>
</tr>
<tr>
<td>2</td>
<td>62±16</td>
</tr>
<tr>
<td>3</td>
<td>57±18</td>
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Table 8.4. The effect of dietary cholesterol feeding on rat liver cytosolic cholesterol ester hydrolase. Rats were fed soft diet, 1% (w/w) cholesterol in 10% (v/w) olive oil diet and 10% (w/v) olive oil diet for one week before diets were replaced with soft diet (day 0). Liver cholesterol ester hydrolase was estimated in the cytosol of livers of rats killed 1, 2 and 3 days after diet replacement. Results are the mean values ± S.D. of five replicate.
negative response of cholesterol ester hydrolase was also found when the enzymic assay was carried out by using the standard emulsified cholesterol $^{\Delta-14}$C-oleate procedure which is described in the methods section (data not shown, experiment carried out in collaboration with P. Rae). This finding ruled out any doubt about the technicalities of the enzyme assay which might be attributed to the substrate presentation and substantiated the results of Table 8.4.

The lack of any stimulation of rat liver cytosolic cholesterol ester hydrolase in the presence of a high liver cholesterol ester content and the total suppression of cholesterol synthesis (Harry, Dino and McIntyre (1973)) posed a question about its role in liver cholesterol metabolism. The sensitivity to the fluctuation in liver cholesterol concentration of microsomal cholesterol-7α-hydroxylase (Boyd, Hattersley, Mason, Arthur and Beckett (1977)), microsomal acyl-CoA:cholesterol acyl transferase (Balasubramaniam, Mitropoulos and Venkatesan (1978)) and 3-hydroxy-3-methylglutaryl-CoA reductase (Edwards (1975)), and the insensitivity of liver cytosolic cholesterol ester hydrolase under these conditions may exclude the latter enzyme from the regulation of hepatic cholesterol metabolism. This view is supported by the following observations. First, the subcellular localisation of liver cholesterol ester hydrolase was investigated by Nilsson, Norden and Wilhelmsson (1973). These authors found by making two substrate presentations to liver cholesterol ester hydrolase that the enzyme was more active at pH 4.0 than at neutrality. The use of rat serum lipoproteins, which were labelled with cholesteryl oleate, and phospholipid-cholesteryl oleate emulsion assays agreed very well with each other and suggested that liver cholesterol ester hydrolase is more active at an acidic pH. The subcellular localisation showed that this activity resided mainly in the lysosomes, which also possessed an esterifying activity. Secondly, feeding rats with a cholesterol diet for two weeks followed by replacement
to standard diet, it was found that liver microsomes accumulated large quantities of esterified cholesterol which decreased to normal value within three days of diet replacement (Harry, Dino and McIntyre (1973)). From such observations and from the stimulatory effect of dietary cholesterol on microsomal ACAT, it was suggested that ACAT in liver microsomes is associated with the synthesis of lipoprotein and the transport of cholesterol from the liver to the plasma (Balasubramaniam, Venkatesan, Mitropoulos and Peters (1978)). It may be possible that through lipoprotein synthesis in the endoplasmic reticulum and the export of these lipoproteins, the accumulated cholesterol ester could be removed from the liver as lipoproteins. Therefore, if any stimulation of cytosolic cholesterol ester hydrolase occurs it would result in an increased concentration of free cholesterol which might be taken up by the endoplasmic reticulum where ACAT exerts its action. The result of the consequent re-esterification of the free cholesterol would be a futile cycle of cholesterol ester hydrolysis.

8.7 SUMMARY

1. The activity of rat liver cytosolic cholesterol ester hydrolase was found to be low when compared to the activity of the enzymes in the cytosol of rat adrenal, rat adipose tissue and bovine adrenal.

2. In vitro incubation of the enzyme with cAMP did not produce any activation as compared to the activation of the enzyme of rat adrenal and adipose tissue.

3. The in vivo administration of glucagon which is known to increase hepatic cAMP concentrations did not result in the stimulation of liver cytosolic cholesterol ester hydrolase.

4. Addition of exogenous cAMP-dependent protein kinase of rabbit skeletal muscle did not alter the activity of cholesterol ester hydrolase. The enzyme does not seem to be regulated by a phosphorylation-dephosphorylation mechanism involving cAMP-dependent protein kinase.
5. Feeding rats a high cholesterol diet, a condition which alters the activities of the enzymes involved in liver cholesterol metabolism, produced no effect on the activity of cytosolic cholesterol ester hydrolase. The possibility of excluding any role of the enzyme in liver cholesterol metabolism has been posed.
SECTION IX
GENERAL DISCUSSION

REFERENCES
SECTION IX: GENERAL DISCUSSION

The studies in this thesis had two main aims, (1) to investigate the specificity of adrenal cytosolic cholesterol ester hydrolase towards other fatty acid esters and (2) to study the mechanism by which this enzyme is regulated in other tissues. These objectives stemmed from the facts that purified cholesterol ester hydrolase of bovine adrenal cortical cytosol as reported by Beckett and Boyd (1977) had not been studied with respect to substrate specificity. It was not known whether the purified enzyme preparation had any activity towards long chain fatty acid esters especially those of glycerol. This point was important because in rat adipose tissue, when triacylglycerol lipase was purified one hundred fold from the tissue cytosol cholesterol ester hydrolase activity was detected and was enriched as the triacylglycerol lipase activity increased. Furthermore, adrenal cytosolic cholesterol ester hydrolase was found to be regulated in vitro by a cAMP-dependent protein kinase and phosphoprotein phosphatase involving the phosphorylation and dephosphorylation of the enzyme (Beckett and Boyd (1977)). This type of enzymic regulation has been the subject of intensive research, and several enzyme have been shown to share this unique kind of regulation (for review see Krebs and Beavo (1979)). The regulation of cholesterol ester hydrolase in the adrenal cortex by cAMP furnishes the tissue with a rapid process for the activation of the enzyme to hydrolyse the stored cholesterol esters to provide free cholesterol for steroid hormones synthesis and secretion. The known rapid response of the adrenal to the tropic hormone ACTH to synthesise and secrete steroid hormones necessitates the rapid and controlled supply of free cholesterol from esterified cholesterol. However, many other tissues contain substantial amounts of esterified cholesterol as well as cytosolic cholesterol ester hydrolases. It could be deduced that cholesterol ester hydrolase in
tissues such as liver, ovary and adipose tissue does not require to be activated as rapidly as the adrenal cortex because there is no need for these tissues to mobilise and utilise free cholesterol rapidly. It follows that not all mammalian tissues may possess cytosolic cholesterol ester hydrolases capable of activation and deactivation through the phosphorylation-dephosphorylation mechanism. Therefore cAMP may not be involved in the regulation of this enzyme in all tissues.

The studies presented in Sections III, IV and V deal with the characterisation of triacylglycerol lipase from rat and bovine adrenal cytosol. Triacylglycerol lipase activity was stimulated in vivo by ACTH administration to about the same extent as cholesterol ester hydrolase (Table 3.2). The degree of stimulation of both enzymes was remarkably similar. Moreover, both enzymes were activated in vitro when the adrenal cytosol was pre-incubated with cAMP (Table 3.2). These results presented two possibilities, either, adrenal cytosolic cholesterol ester hydrolase has a very low specificity in that it hydrolyses both cholesterol and glycerol esters of long chain fatty acids or there may be two different enzymes one for esterified cholesterol and the other for triglyceride hydrolysis. These possibilities were investigated using physical and chemical procedures to test the validity of either hypothesis.

In terms of a physical approach to the problem, triacylglycerol lipase was purified from bovine adrenal cytosol. It had been found that bovine adrenal cortical cytosol possesses enzymic activity against triolein as well as the well documented enzyme activity against cholesterol ester hydrolysis (Table 4.1). Both enzyme activities were activated in vitro by cAMP preincubation of the adrenal cytosol, indicating that triacylglycerol lipase may be regulated by the same mechanism that affects cholesterol ester hydrolase, namely, enzyme phosphorylation. Experiments with a purified preparation containing both enzyme activities
strongly support the proposal, of a common mechanism of enzyme regulation by cAMP in both cases.

The purification scheme of triacylglycerol lipase and cholesterol ester hydrolase from bovine adrenal cortical cytosol (Fig. 4.1) was adapted from published methods (Beckett and Boyd (1977), Wallat and Kanau (1976), Huttunen and Steinberg (1971), and it was found to give a reasonably purified fraction in less than three days with a modest recovery of both enzymes (Table 4.1). Although other methods were used such as adsorption and ion exchange chromatography, these techniques either did not work with this enzyme preparation or such steps did not improve the apparent purification or the enzymic recoveries.

Bovine adrenocortical cytosol was prepared by differential centrifugation (Fig. 2.1) and subjected to acid titration in order to concentrate triacylglycerol lipase and cholesterol ester hydrolase into a smaller and manageable volume. Performing these steps, every fraction was checked for the two enzymic activities cholesterol ester hydrolase, and triacyl glycerol lipase activity and their ratio to each other as well as the response of these enzymic activities to cAMP and magnesium ion pre-incubations. It was found that cytosolic fractions precipitated between pH 7.4 and 5.0 exhibited the enzymic activation – inactivation of both cholesterol ester hydrolase and triacylglycerol lipase when these fractions were incubated with cAMP or magnesium ion respectively (Table 4.2). Although the percentage recovery of cholesterol ester hydrolase and triacylglycerol lipase in the acid titration stage was poor (Table 4.4), this loss of about 30-50% of the total enzymic activity could not be prevented. It is shown in Section IV that the pre-incubation of the adrenal cytosol with cAMP or Mg\(^{++}\) did not enhance or decrease the enzymic recovery. However this percentage recovery was not inferior to that obtained by ammonium sulphate precipitation reported by Wallat and
Kanau (1976) and Beckett and Boyd (1977). It is also possible that the apparent specific activities of those enzymes differs dramatically from fraction to fraction due to the change in the phospholipid content (Wallat and Kanau (1976)). There could be the loss of some factor(s) that enhance or diminish the accessibility of the substrate to the active centre of the enzyme at the lipid-water interface (Scallen, Seetharam, Srikantaiah, Hansbury and Lewis (1975)).

The elution of cholesterol ester hydrolase and triacylglycerol lipase from agarose gel gave two peaks containing both enzymes. One peak eluted in the void volume of the agarose gel column while the other peak runs at twice the void volume of the column (Fig. 4.2). The perfect coincidence of the two peaks of both enzymes was similar to that found with rat adrenal enzymes (Pittman and Steinberg (1977)), and suggested a very strong relationship between these two enzymes. As for the elution of both cholesterol ester hydrolase and triacylglycerol lipase in two peaks each, it is likely that each peak represent one form of the enzyme and each form differs from the other in their lipid content especially phospholipid, resulting in two sizes of enzyme protein-phospholipid aggregate (Wallat and Kanau (1976)). These two peaks of enzymic activities were not changed even after a mild delipidation procedure was performed on the pH 5.0 precipitate fraction. Thus, the removal of free cholesterol by butanol-acetone homogenisation (R. Hume, personal communication) followed by lyophilisation did not affect significantly the enzyme activity (Table 4.3). Furthermore, delipidating produced the same elution pattern of cholesterol ester hydrolase and triacylglycerol lipase as was observed in the control (Figs. 4.2 and 4.3). However, when the delipidation process was carried out with an ethanol-ether mixture, which removes phospholipids, the enzymic activities were lost and hence assessment of the two peaks which emerged from the agarose gel chromatography step remained unknown.
When the chromatographic fractions containing cholesterol ester hydrolase and triacylglycerol lipase activities which had been eluted in the void volume of the agarose column were pooled and subjected to a linear density gradient centrifugation procedure, both enzyme activities floated in a fraction of a density less than 1.21. Again, both enzymes were enriched in that fraction to a similar extent and their distribution throughout the density gradient was identical (Fig. 4.5). The identical flotation of these hydrolytic enzymes from bovine adrenal is similar to the flotation of their counterparts from rat adipose tissue (Pittman, Khoo and Steinberg (1975)). Thus, conventional methods of enzyme separation failed to differentiate the two activities of cholesterol ester hydrolase and triacylglycerol lipase of bovine adrenocortical cytosol, and as shown in Table 4.4, the ratio of the two enzymic activities throughout the different fractions remained fairly constant. Although it was not intended that the figures in Table 4.4 should be translated into absolute specific enzyme activity, the Table 4.4 summarised the apparent specific activities of both enzymes in the different purified fraction when the assay of all fractions were run simultaneously using one batch of substrate. It seemed from the apparent specific activities that both cholesterol ester hydrolase and triacylglycerol lipase activities were increased many folds. Pilot experiments with disc gel electrophoresis in the presence of sodium dodecylsulphate showed that the final purified preparation was still heterogeneous. Consequently it was not possible to estimate the size and subunit structure of the enzyme complexes. Although Beckett and Boyd (1977) estimated the molecular weight of cholesterol ester hydrolase from the phosphorylated subunit of the enzyme, that approach could not be used in the present study because it is impossible to ascertain the phosphorylated subunit(s) of either enzyme, and the possibility that
these subunit(s) might be shared by both enzymes could not be ruled out. The situation with respect to both enzymes in rat adipose tissue is similar to the bovine adrenal, and no characterisation of the adipose tissue enzymes has been achieved (Pittman, Khoo and Steinberg (1975)). Even after an elegant and laborious purification scheme for triacylglycerol lipase, though purified more than 2000-folds, it was still not convincingly homogeneous (Belfrage, Jergil, Stralfors and Tornqvist (1977)). Moreover, these authors did not test the final partially purified enzyme preparation against cholesteryl esters, which possibly might have been hydrolysed at a rate similar to the hydrolysis of the substrate triglyceride.

The separate identity of cholesterol ester hydrolase and triacylglycerol lipase of bovine adrenocortical cytosol was examined using two inhibitors of cholesterol hydrolase. It was shown that chloropyrifos oxone inhibited adrenal steroidogenesis at a point beyond cAMP synthesis and before pregnenolone production, and that locus was shown to be cholesterol ester hydrolase (Civen, Lifrak and Brown (1977)). This toxic organophosphate insecticide was shown to differentiate, in rat adrenal, between the activity of cholesterol ester hydrolase from that of triacylglycerol lipase (Pittman and Steinberg (1977)). Using a purified fraction of bovine adrenocortical cholesterol ester hydrolase and triacylglycerol lipase, chloropyrifos oxone failed to give a clear cut differentiation between the two enzymic activities (Fig. 5.5). The use of the partially purified fraction of both enzymes permitted the detection of any changes in activity such that these alterations would have been obscured if a crude preparation of the enzymes had been employed. Moreover, chloropyrifos oxone failed to give any separation between the two enzymic activities in chicken adipose tissue (R. Pittman, personal communication). Thus, the results presented in Fig. 5.5 did not
support the concept of a separate identity of these enzymes as suggested in rat adrenal. This difference could be due to the use of a crude fraction in the latter study. The small changes in triacylglycerol lipase which was detected in the purified fraction from bovine adrenal enzymes might have been obscured in the crude fraction.

It was hoped that cholesteryl oleyl ether might be used as a specific inhibitor for cholesterol ester hydrolase being similar to cholesteryl olate it might bind selectively to cholesterol ester hydrolase without being hydrolysed and thus reduce the binding of the substrate ester and consequently its hydrolysis. The competition for the active centre of cholesterol ester hydrolase might be different from the competition for the active centre of triacylglycerol lipase. However, as the results showed (Table 5.2) both enzymes were inhibited to an identical degree by cholesteryl oleyl ether. These results indicated that both enzymic activities either had similar binding properties or they had a single binding unit. In a mixed substrate emulsion study it was shown that further addition of either substrate inhibited both activities of cholesterol ester hydrolase and triacylglycerol lipase of rat adrenal cytosol and to the same degree (Pittman and Steinberg (1977)). Thus in both rat and bovine adrenal, these two enzymic activities showed such a strong physical association that it has proved very difficult to disengage one activity from the other activity. These enzyme activities, at least in bovine adrenal, exhibited a remarkable similarity in their response to a selective organophosphate inhibitor as well as to substrate analogues. Moreover, both enzymic activities were stimulated in vivo by ACTH administration and the degree of their stimulation in rat adrenal was identical (Table 3.3). Activation of both enzymic activities in rat and bovine adrenal was achieved when the cytosol was pre-incubated with cAMP, and this in vitro activation was shown with a purified bovine
adrenal fraction to be correlated with the phosphorylation of protein fraction (Fig. 5.4). Although the precise judgement whether both enzymes (if they are separate proteins) are phosphorylated or only one specific subunit is phosphorylated can not be drawn from such evidence. The experiments on the peptide mapping of a phosphorylated purified preparation of cholesterol ester hydrolase and triacylglycerol lipase showed that digestion did not produce identifiable phosphopeptides which could relate to any enzyme activity (Figs. 5.10, 5.11 and 5.12). Therefore the location and the number of the phosphoryl groups per enzyme(s) could not be determined. Unlike rabbit skeletal muscle phosphorylase kinase (Cohen (1978)) where the role of phosphorylation in the regulation of the enzyme has been firmly established through the precise identification of the molecular mechanism involved in the process, the heterogeneity of cholesterol ester hydrolase in the bovine adrenal has hampered such progress.

The evidence presented in Section III, IV and V showed that in both rat and bovine adrenal, there was a triacylglycerol lipase activity which matched the activity of cholesterol ester hydrolase in the response to in vivo modulation such as ACTH stimulation and nicotinic acid inhibition and in vitro manipulation such as cAMP, chloropyrifos oxone and cholesteryl oleyl ether. These two hydrolytic activities were inseparable by conventional enzyme purification procedures. The possibilities are that either these two activities represent a single-low specificity enzyme, or these two activities reside in two distinct but closely related proteins, are both valid. With regard to the specificity, both rat and bovine adrenal enzyme preparations have been shown to hydrolyse cholesteryl linoleate at a faster rate than cholesteryl oleate or glycerol trioleate (Tables 3.6 and 5.1). The similar response of both activities to
in vitro and in vivo treatment support the concept that there may be a single enzyme. However, a failure to separate these activities, if they are two independent enzymes, contrasts with the failure to show the homogeneity of either enzyme. None of the experimental evidence presented in this thesis disproves either hypothesis.

Thus far the role of triacylglycerol lipase in the adrenal has not been discussed because the experimental evidence for such a study is outside the scope of this investigation. However, the stimulation of the adrenal triacylglycerol lipase in vivo by ACTH administration to the rat (Table 3.3) and the adrenal triacylglyceride depletion by ACTH in hypophysectomised rats (Rudman and Garcia (1966)) are reminiscent of the stimulation of cholesterol ester hydrolase by ACTH (Table 3.3) and cholesterol ester depletion by ACTH (Sayers, Sayers, Fry, White and Long (1944)). Thus under conditions that stimulate adrenal steroidogenesis, both adrenal triglycerides and cholesteryl esters were depleted. The hydrolysis of adrenal triglycerides upon the hormonal signal of ACTH may be in some way either obligatory or supportive for adrenal steroidogenesis. The fate of liberated free fatty acids could be one of at least three routes and they are, for example, oxidation for energy purposes, elongation for prostaglandin biosynthesis, export from the tissue as free fatty acids and other functions that support steroidogenesis. There are several lines of evidence which lend support for the functional necessity of free fatty acids in adrenal steroidogenesis. Recently it was shown that ACTH stimulates prostaglandin PGF and PGE biosynthesis and release in cat adrenals (Laychock and Rubin (1977)) and rat adrenocortical cells (Chanderbhan, Hodges, Treadwell and Vahouny (1979)). In both studies it was shown that the precursor of these adrenal prostaglandins was arachidonic acid, which in the rat was furnished by the hydrolysis of cholesteryl esters of the adrenal.
However, triglyceride hydrolysis was overlooked in the latter study where it was shown that the major fatty acid in adrenal triglycerides was linoleic acid. It is possible that linoleic acid in the adrenal undergoes enzymic elongation to produce arachidonic acid similar to the events occurring in rat liver (Sprecher (1977)). Although both prostaglandin PGE₁ and PGE₂ were shown to bind to human and ovine adrenal preparations, at sites distinct from that of ACTH, with the consequent stimulation of adenylate cyclase (Dazord, Morera, Bertrand and Saez (1974)), prostaglandin biosynthesis and release may not be obligatory events in ACTH-induced adrenal steroidogenesis but they may modulate steroid production and/or release (Laychock and Rubin (1977)). Other aspects of free fatty acids produced by adrenal triglyceride may include augmentation of free fatty acid concentration to a level of feed back inhibition such as that shown cholesterol ester hydrolase inhibition by oleic acid (Beckett and Boyd (1975)), and exchange with the fatty acids of membrane phospholipids which are required for the integrity of the linkage between the two units of adenylate cyclase (Gill (1979)).

The finding of triacylglycerol lipase activity in the adrenal cytosol, may provide adrenal steroidogenesis with another fine tune control locus. This activity was shown to be under ACTH regulation (Table 3.3), adding a locus to the many sites of action of ACTH in adrenal steroidogenesis. Some of the early sites affected by ACTH directly or indirectly are depicted in Fig. 9.1. Although many of these steps have not been shown or confirmed to be regulated by ACTH, the scheme in Fig. 9.1 integrates current models of adrenal steroidogenesis (Boyd, Arthur, Beckett, Mason and Trzeciak (1975), Gill (1979)) and has reviewed some of the recent information available on the effect of ACTH on other enzymic process in the adrenal. In this scheme, the primary action of ACTH on adrenal
Fig. 9.1. A scheme for the various loci affected by ACTH in the adrenal cell. For details see the text. (HSIP) indicate protein inhibitors of cAMP-dependent and cAMP-independent protein kinases.
steroidogenesis lies in the well documented rate limiting step of pregnenolone production from cholesterol. This mitochondrial-occurring reaction which is catalysed by the side-chain cleavage mixed function oxidase system requires the supply of the precursor free cholesterol, which is limited in adrenal mitochondria (Boyd, Arthur, Beckett, Mason and Trzeciak (1975)). Thus, the modulation of cholesterol conversion into pregnenolone requires the supply of free cholesterol from outside the mitochondria, the transport of this cholesterol to the mitochondria and finally its translocation within the mitochondrial cristae.

The latter process may involve protein synthesis (Boyd, Arthur, Beckett, Mason and Trzeciak (1975), Gill (1979)). The initial event in ACTH action on adrenal steroidogenesis is the stereospecific binding of ACTH to the adrenal cell membrane (Lefkowitz, Roth, Pricer and Pastan (1970)). This reversible binding, which is specific for ACTH, results in the stimulation of adenylate cyclase (Lefkowitz, Roth, Pricer and Pastan (1970)). The stimulation of corticosteroid production does not involve the entry of ACTH into the adrenal cell (Selinger and Civen (1971), Richardson and Schulster (1972)). The stimulation of adenylate cyclase increases the conversion of ATP into cAMP (Haynes (1958)). The effect of cAMP on steroidogenesis was first shown in adrenal slices where corticosteroidogenesis was stimulated upon the addition of cAMP to the incubation medium (Haynes, Koritz and Peron (1959)). This was consequently confirmed in numerous studies (for review see Halkerston (1975)). The rise in intracellular concentration of cAMP upon ACTH interaction with the adrenal cell precedes the rise of mitochondrial steroidogenesis (Podesta, Milani, Steffen and Neher (1979)), and this relationship supports the concept of the role of cAMP as the intracellular mediator of ACTH action on adrenal steroidogenesis (Grahame-Smith, Butcher, Ney and Sutherland (1967)).
The elevation of cAMP concentration in the adrenal results in the activation of cAMP-dependent protein kinase (Boyd and Trzeciak (1973), Saez, Evain and Gallet (1978)) which was shown to be composed of two subunits, a cAMP binding protein and a catalytic kinase unit (Gill and Garren (1971)). Thus the binding of cAMP to the regulatory unit of the holo-protein kinase produces the cAMP-binding protein complex which dissociates from the catalytic unit of the protein kinase. The free catalytic unit of the kinase functions to transfer the γ-phosphate of ATP to various protein substrates. The action of cAMP thus involves protein phosphorylation, and for such a process there are several criteria to be met before any cAMP action is said to be through protein phosphorylation. Of these criteria, such as those laid down by Krebs (1972), it is suggested that there must be a protein acceptor that undergoes functional modification in vitro and in vivo when it is phosphorylated in the presence of cAMP and cAMP-dependent protein kinase. Although very few proteins rigorously meet these criteria (Nimmo and Cohen (1977)), three proteins (or two?) in Fig. 9.1 seem to meet most of these phosphorylation process's criteria. These proteins are cholesterol ester hydrolase, triacylglycerol lipase and phosphoprotein phosphatase inhibitor. Discussion will be limited to highlight their importance, upon their phosphorylation, and their possible effect on adrenal steroidogenesis.

The depletion of rat adrenal lipid droplet cholesterol esters was shown to be associated with an increased cAMP concentration in the adrenal of ether stressed rats associated with stimulation of cAMP-dependent protein kinase and stimulation of cytosolic cholesterol ester hydrolase (Boyd and Trzeciak (1973)). The phosphorylation of cholesterol ester hydrolase was proposed, but not confirmed until a partially purified enzyme preparation was shown to be activated rapidly by
exogenous cAMP-dependent protein kinase and the activation apparently paralleled the transfer of the terminal phosphate of ATP to acid-precipitatable protein (Beckett and Boyd (1977)). Moreover, a later study showed that upon incubation of the phosphorylated and purified cholesterol ester hydrolase preparation with magnesium-dependent phosphoprotein phosphatase, the hydrolase enzyme was deactivated in parallel with the loss of radioactive phosphate. The phosphorylation of cholesterol ester hydrolase was subsequently confirmed in other studies (Wallat and Kanau (1976), Naghshineh, Treadwell, Gallo and Vahouny (1978), and also in this study Section V). Thus in the adrenal cortex, upon the interaction of ACTH with the cell membrane, the adrenal cAMP concentration increases resulting in a binding to the regulatory unit of the inactive protein kinase with the resultant dissociation of the active catalytic unit of protein kinase. The latter in turn mediates the transfer of phosphate from ATP to a less active non-phosphorylated cholesterol ester hydrolase in the adrenal cytosol. Consequently, the phosphorylated and active hydrolase attacks the esterified cholesterol stored in the lipid droplets which are dispersed in the cytosol. The release of free cholesterol from the hydrolysed esters is then transported by, as yet, unknown mechanisms to the adrenal mitochondria. ACTH may exert its action on the mitochondria possibly through facilitation of cholesterol translocation to the mixed function oxidase centre, and this process may involve the synthesis of a labile protein (Boyd, Arthur, Beckett, Mason and Trzeciak (1975), Gill (1979)).

As discussed earlier in this section, the association of triacylglycerol lipase with cholesterol ester hydrolase in the adrenal cytosol seems to be very strong so that their separate identities remained unknown. However, the triacylglycerol lipase activity was shown, assuming it to be a separate protein, to be modified in vivo and in vitro
in a process mediated by cAMP. Moreover, in a purified preparation of this activity it was shown that the activation of this enzymic activity was paralleled by a transfer of the terminal phosphate from labelled ATP onto acid-precipitatable protein (Fig. 5.4). Thus the evidence presented in Section III-V suggested that triacylglycerol lipase activity in adrenal cytosol was under ACTH regulation, and cAMP in the cell mediated the hormonal action by protein phosphorylation. The identity of the phosphorylated protein, however, remained ambiguous. This postulate is supported by the following observations. First, ACTH is known to deplete adrenal triglycerides (Rudman and Garcia (1966)) and this depletion was associated with the stimulation of triacylglycerol lipase activity in the adrenal cytosol (Section III). Second, ACTH increased the intracellular concentration of cAMP in bovine and rat adrenocortical cell (Haynes, Koritz and Peron (1959), Podesta, Milani, Steffen and Neher (1979)). Third, cAMP activated in vitro a cytoplasmic inactive cAMP-dependent protein kinase (Gill and Garren (1970), Saez, Evain and Gullet (1978)). Fourth, in adrenal cytosol, cAMP activated in vitro triacylglycerol lipase and upon the purification of this activity, and cAMP-dependent protein kinase is required for this activation (Sections III, V, Pittman and Steinberg (1977)). Fifth, the activation of triacylglycerol lipase associated with phosphate transfer (Section V), is a very rapid process. Sixth, incubation of adrenal cytosol with Mg++ ions resulted in a reversible deactivation of triacylglycerol lipase activity (Section IV and V). This reversible deactivation is similar to the reversible deactivation of cholesterol ester hydrolase catalysed by phosphoprotein phosphatase (Beckett and Boyd (1977)) which suggests the possibility of the action of a phosphoprotein phosphatase such as that described for the deactivation of phosphorylase (Merlevede and Riley (1966)). Thus the hormonal depletion of triglycerides in the
adrenal was associated with cAMP, the reversible functional alteration of triacylglycerol lipase which could be a phosphate acceptor in the cAMP-dependent protein kinase catalysed transfer of the γ-phosphate from ATP, and finally the Mg++ ion inactivation which could be catalysed by a phosphatase; all these observations are in line with the proposal of Krebs (1972) for protein phosphorylation criteria.

In recent years, there has been much interest in the regulation of phosphoprotein phosphatases (Nimmo and Cohen (1977), Tao, Huang, Lynch and Glinsmann(1978)). It was shown that there is in the beef adrenal cortex a relatively heat-stable protein which upon phosphorylation in vitro by a cAMP-dependent protein kinase becomes active and inhibits phosphorylase phosphatase (Huang, Tao, Glinsmann (1977)). This inhibitor was also found in rabbit skeletal muscle and, more interesting, it was found to be dephosphorylated and inactivated by one of its substrates namely the multifunctional phosphatase-1 (Cohen (1978)). Moreover, in a perfused rat hindlimb, epinephrine infusion rapidly increased muscle cAMP concentration and increased the phosphatase inhibitor activity while there was a decreased phosphorylase activity (Tao, Huang, Lynch and Glinsmann (1978)). What has this to do with the adrenal lipolytic enzymes studied in this thesis? The answer lies in the fact that the phosphatase activity in the tissues examined showed the low specificity of the enzyme (Burchell, Foulkes, Cohen, Condon and Cohen (1978)) and in the adrenal cortex it was shown that phosphorylase phosphatase and protamine phosphatase co-purified together which led the authors to suggest that one enzyme possesses both activities (Ullman and Perlman (1975)). It is possible then that the same phosphatase dephosphorylates and deactivates cholesterol ester hydrolase and triacylglycerol lipase of the adrenal cytosol. It is also possible that a phosphatase inhibitor plays a role in the regulation of these two enzymes through protein phosphatase
modulation. The action of ACTH in adrenal steroidogenesis would be by phosphorylation of cholesterol ester hydrolase and triacylglycerol lipase with their consequent activation. At the same time the phosphorylation of the protein phosphatase inhibitor-1 could lead to the suppression of the dephosphorylation of cholesterol ester hydrolase by protein phosphatase, thus providing a favourable directional reaction towards cholesterol ester hydrolysis by stabilising the phosphorylated and active form of cholesterol ester hydrolase. However, although this is an interesting scheme, the final answer for its validity will require every step in the scheme in Fig. 9.1 to be fully characterised.

The possible role of the cAMP concentration as a regulator of cholesterol ester hydrolase in other tissues was the object of the studies presented in Sections VI, VII and VIII. In these studies rat adipose tissue, ovary and liver were examined to see whether cytosolic cholesterol ester hydrolase in these tissues is regulated in a manner similar to the adrenal enzyme, by a phosphorylation-dephosphorylation mechanism. The study on rat adipose tissue was carried out to compare the response of cholesterol ester hydrolase with triacylglycerol lipase hormone-sensitive lipase since these two activities co-purified and were both stimulated by cAMP (Pittman, Khoo and Steinberg (1975)).

The response of adipose tissue cytosolic cholesterol ester hydrolase to in vivo administration was consistent with the finding of Pittman, Khoo and Steinberg (1975) in that epinephrine stimulated in vitro cholesterol ester hydrolase of isolated adipocytes. Moreover the incubation of adipose tissue cytosol with cAMP enhanced further the in vivo stimulation due to ACTH administration to the rat (Table 6.1). The response of adipose tissue triacylglycerol lipase to ACTH was similar to that found with the adrenal enzymes. However, in vivo stimulation of both enzymes from adipose tissue was not consistent as in the adrenal,
and the reason for the disparity was attributed to several factors such as losses during tissue processing, active phosphoprotein phosphatase and finally stimulation of these hydrolytic enzymes in control animals due to the stress of handling them for the short period before sacrifice (see Section VI). This inconsistency was also observed when the effect of nicotinic acid was studied under in vivo conditions. The antilipolytic action of nicotinic acid is well documented, however its action on enzymes of lipid metabolism is not fully understood (for review see Gey and Carlson (1971), Fain (1973)). Nicotinic acid was shown to block, in rat epididymal fat pads incubated with epinephrine, the stimulation of a cytosolic-microsomal lipase (Shafrir (1971)). It was shown subsequently that hormone-sensitive lipase is located mainly in adipose tissue cytosol (Khoo, Jarett, Mayer and Steinberg (1972)). It was interesting to re-evaluate the effect of nicotinic acid on the cytosolic lipase and compare this effect with the effect on cholesterol ester hydrolase. Although nicotinic acid blocked the stimulation of both enzymes when the rat was subjected to acute ether anaesthesia, a condition that elevates plasma ACTH, this nicotinic acid blockade was also not consistent. Moreover nicotinic acid in vivo had no effect on the basal activities of both enzymes. This in part supports the concept that nicotinic acid exerts its antilipolytic action through an alteration of steps prior to the lipolytic enzymes themselves (Fain (1973)). This concept was further assessed when isolated adipocytes were incubated with nicotinic acid in the presence and absence of epinephrine. Under these in vitro conditions more control could be exerted on the level of enzyme stimulation because the known lipolytic hormones could be controlled and confined to the desired additions. Using isolated adipocytes, evidence was presented that epinephrine stimulated both cholesterol ester hydrolase and triacylglycerol lipase of the adipocyte cytosol. This stimulation was consistent
in all experiments, and thus supports the in vivo (less controllable) stimulation by ACTH (Table 6.4). Moreover, the addition of nicotinic acid to the adipocyte medium did not affect the basal activities of cholesterol ester hydrolase and triacylglycerol lipase. However, when nicotinic acid was added to the cell medium prior to epinephrine addition no stimulation of either hydrolytic enzymes occurred. This inhibition was also consistent in all experiments performed, thus removing any doubt about the observed in vivo inhibition of both enzymes when they were stimulated under ether anaesthesia (Table 6.3). These experiments showed that nicotinic acid inhibited triacylglycerol lipase and cholesterol ester hydrolase to the same degree (Table 6.4) thus matching the responses of both enzymes from rat adrenal cytosol (Table 3.5). This similarity of the responses of these enzyme in both adrenal and adipose tissue cytosol adds to the unanswered question of the role of cholesterol ester hydrolase in adipose tissue lipolysis comparable to the role of triacylglycerol lipase in adrenal steroidogenesis. These enzymes may be identical in both tissue or the enzyme may be of a low specificity in both tissue. The physiological effect of the enzyme could be modified according to the existing abundant substrate in each tissue. Certainly neither this study nor the evidence from other laboratories provide an answer to this problem which remains a high priority in lipolytic enzyme research.

As outlined in Section VI the evidence for the involvement of cAMP in mediating the action of lipolytic hormones is well established and this cAMP action appears to be through the phosphorylation of protein. However, two important advances in this field are worth noting. The finding of a phosphoprotein phosphatase that catalysed the deactivation of chicken triacylglycerol lipase and this deactivation could be catalysed by phosphatases from other tissue is interesting (Severson,
Khoo and Steinberg (1977)). The deactivation of the chicken lipase by lipase phosphatase was prevented by the addition of a heat stable protein, a phosphatase inhibitor. It is not known whether this inhibitor is a cAMP-dependent protein kinase substrate or not (Severson and Sloan (1977)). If this protein inhibitor of lipase phosphatase turns out to be regulated by cAMP, this would be consistent with the anticipated scheme depicted in Fig. 9.1 whereby cAMP concentrations regulate both lipolysis in adipose tissue and adrenal steroidogenesis. The second advance regarding adipose tissue is that cGMP-dependent protein kinase of bovine lung activated chicken adipose tissue cholesterol ester hydrolase and triacylglycerol lipase as well as adrenal cholesterol ester hydrolase. However cGMP by itself, unlike cAMP was not able to stimulate both enzymes from both tissue which is consistent with the finding that bovine adrenal cholesterol ester hydrolase did not respond to cGMP (Fig. 5.3, this study) and only cAMP produced the activation (Khoo, Sperry, Gill and Steinberg (1977)). It remains, however, to assess the possible physiological role of this activation by cGMP-dependent protein kinase with regard to adipose tissue lipolytic regulation.

The corpus luteum of the mammalian ovary functions primarily to synthesise and secrete progesterone. This tissue has been shown to be influenced by luteinising hormone (LH) at least in the human, cow and rat (Greep (1971)). Fluctuation in corpus luteum cholesterol ester concentration was reported to occur in bovine and rat corpora lutea during increased progesterone production in these tissues (Hafs and Armstrong (1968), Flint, Grinwich and Armstrong (1973)). Cholesterol ester hydrolase in rat corpus luteum was found to be stimulated by LH administration in vivo (Behrman and Armstrong (1969), Beckett (1975)). These studies were carried out on either isolated bovine corpus luteum or in the superovulated rat ovaries which are composed of mainly corpora
lutea due the massive hormonal stimulus given to immature female rats (Parlow preparation, Parlow (1958)). However an attempt was made in this study to assess the response of cytosolic cholesterol ester hydrolase in rat ovary to a single ovulatory dose of LH administered in vivo and correlating this response to ovarian steroid content and changes of serum progesterone concentration. The experimental animal model used in this study was the immature female rat injected with 4 iu PMSG, and two days later the animals were sacrificed. The ovaries of these immature rats had achieved the properties of mature ovaries of cyclical female rats at ovulation (see Section VII). Under these specified conditions, LH stimulated an increase in serum and ovarian progesterone concentration with a maximum steroid output occurring six hours after LH administration. However neither free nor esterified cholesterol concentrations in the ovaries of these LH injected rats had changed significantly during the 6 h period after LH injection. Moreover, upon the extension of steroid measurement to 72 hr after the rats received the LH injection, the free cholesterol had not changed at all while the esterified cholesterol concentration started to increase 12 h after the LH injection reaching a plateau 36 h later, and stabilised at that value about twice the original concentration for three days from the start of the experiment (Fig. 7.1). The implication of the result of this experiment is two fold. First it provided information on the time of maximum steroidogenic activity of the ovaries of these specific rats. This time value was used later as a guide for the optimum time to assess cholesterol ester hydrolase activity. The other fact obtained from the experiment was that in these rats, LH did not deplete ovarian esterified cholesterol at a time when serum and ovarian progesterone concentration was raised several fold. Thus the link between progesterone and esterified cholesterol concentration was not found to be reciprocally related as was expected.
In assessing ovarian cytosolic cholesterol ester hydrolase of rats treated with LH three and six hours prior to their sacrifice, no stimulation of the enzyme was observed. This was consistent with the observed unchanged esterified cholesterol ester concentration of these rat ovaries. In fact a significant inhibition of cholesterol ester hydrolase was detected in the LH treated animals, in both control, Mg\(^{++}\) or cAMP incubated ovarian cytosol (Table 7.1). Although the \textit{in vitro} activation of the enzyme by cAMP was not impaired, the failure of LH to stimulate cholesterol ester hydrolase could not be attributed to the lack of stimulation of intracellular cAMP concentration. This is because LH stimulated an increase in cAMP concentration in the ovaries of two week old immature female rats (Lamprechet, Zor, Tsafiriri and Linder (1973)). LH-stimulated steroidogenesis in the ovaries of these immature rats was further established by simultaneous measurements of ovarian mitochondrial progesterone, pregnenolone and free cholesterol together with the estimation of cytosolic cholesterol ester hydrolase activity (Tables 7.2, 7.3 and Fig. 7.2). The results of these experiments showed that in these animal models, LH stimulated an increase in both pregnenolone and progesterone synthesis in the mitochondria of these rat ovaries. Consistent with previous experiments, cytosolic cholesterol ester hydrolase was not stimulated. Thus contrary to the observed stimulation of the enzyme in the superovulated rat ovaries (Behrman and Armstrong (1969)) the enzyme was not stimulated in the immature female rat ovaries, previously treated with PMSG to induce ovulation similar to the spontaneous ovulation of mature female rat ovaries (Guillet and Rennels (1964)). The experimental animal model used in the present study was shown to respond to LH with changes in serum and follicular steroid concentrations (Goff and Henderson (1979)) and these changes were similar to those that found on
the day of ovulation of mature female rats (Meijs-Roelof, Ullenbroeck, DeGreef, DeJont and Kramer (1975)). Thus under conditions that simulate physiological conditions, LH did not seem to stimulate rat ovarian cytosolic cholesterol ester hydrolase. It is difficult to reconcile this result with that obtained with the superovulated rat ovarian enzyme. However, a recent report using the superovulated rat ovaries showed that hCG injection stimulated both microsomal ACAT activity on the second day after the hormonal injection, and this ACAT stimulation was coupled with an increased accumulation of esterified cholesterol in the ovaries of these rats (Schuler, Scava, Kirsch, Flickinger and Strauss (1979)). This report is consistent with the finding of the increased esterified cholesterol accumulation observed in this study. It seems unlikely that LH, which is similar to hCG in terms of its action to induce ovulation, would stimulate both cholesterol ester hydrolase and microsomal ACAT because such action would yield a futile cycle of cholesterol ester hydrolysis and esterification. The reported result on cholesterol ester hydrolase inhibition would be more logical in the light of the observed ester accumulation in this study. It follows that the absence of the response of ovarian cholesterol ester hydrolase to an LH signal, and the absence of cellular action, i.e., cholesterol ester hydrolysis, due to LH, constitute the absence of a major criterion of protein phosphorylation mediated by cAMP. Thus, cholesterol ester hydrolase in corpora lutea does not seem to be regulated by cAMP in vivo, at a time when this nucleotide concentration increases in the ovarian tissue in association with the LH signal. This in turn indicates a different mechanism for the regulation of cholesterol ester hydrolase in the ovarian cytosol from that of adrenal cytosol. In this respect, so far no report has shown the phosphorylation of ovarian, or corpus luteum cholesterol ester hydrolase (see Marsh (1975)). Furthermore, it
is worth noting that the in vivo depletion of esterified cholesterol by LH in the superovulated rat ovary was blocked by prior injection of the protein synthesis inhibitor cycloheximide (Flint, Grinwich and Armstrong (1973)). The inhibitory effect of cycloheximide was not observed with adrenal cholesterol ester depletion by ACTH (Davis and Garren (1966)). This is because in the adrenal, cholesterol ester hydrolase, cAMP and cAMP-dependent protein kinase were shown to be insensitive to cycloheximide treatment (Boyd and Trzeciak (1973), Grahame-Smith, Butcher, Ney and Sutherland (1967)).

The accumulation of esterified cholesterol in the liver in response to feeding the rat a high cholesterol diet (Gould (1977)) or in vivo administration of chylomicron enriched with labelled cholesterol ester (Goodman (1962)) is a very rapid process regardless of the physiological condition of the liver (Sherrill (1978)). The fast accumulation of esterified cholesterol initiated the search for the means by which the rat liver disposes of these excessive loads of cholesterol esters.

The study of Deykin and Goodman (1962) showed that there is in the liver a neutral cytosolic cholesterol ester hydrolase which hydrolysed preferentially long chain unsaturated fatty acid cholesterol esters. The regulation of this cytosolic cholesterol ester hydrolase was not reported. It was interesting to study the effect of cAMP on this enzyme and compare the liver enzyme with its counterparts in the adrenal and adipose tissue.

The studies of rat liver cytosolic cholesterol ester hydrolase presented in the previous section were carried out using exogenous labelled cholesteryl oleate substrate delivered in acetone. This assay system and the assay described in the methods section gave comparable results with regard to the specific activity of the enzyme (Table 8.1 and 8.2). The activity of rat liver cholesterol ester hydrolase...
obtained in this study is almost identical to that reported (Deykin and Goodman (1952)) but it is much lower than that found in the adrenal and adipose tissue (Table 8.1). Upon the incubation of rat liver cytosol with varying concentrations of Mg\textsuperscript{++} ions, cholesterol ester hydrolase was activated by Mg\textsuperscript{++} ion addition up to a concentration of 5 mM. The addition of ATP at equimolar concentration to that of Mg\textsuperscript{++} did not affect the enzyme activity any further than that of Mg\textsuperscript{++} alone. Cyclic AMP addition, in the presence of optimal concentrations of Mg\textsuperscript{++} and ATP, did not stimulate cholesterol ester hydrolase significantly (Fig. 8.4 and 8.5). The addition of these cofactors to the assay medium, did not produce a stimulation of the enzyme as observed in the adrenal and adipose tissue. This negative effect was checked using two procedures. The first was to affect stimulation of the cholesterol ester hydrolase, if there was any, by the addition of cAMP, ATP and Mg\textsuperscript{++} ions to the enzyme cytosol followed by their removal through desalting of the incubation mixture. The second procedure was to compare the activation of this enzyme in rat liver with that of rat adrenal and adipose tissue using the activation procedure described in the methods section, and then assay all three enzyme sources simultaneously for cholesterol ester hydrolase using the emulsified substrate assay described in Section II. The reason for using the first procedure was that if any covalent modification of the enzyme by cAMP occurred, the removal of these effectors after their action will render the enzyme active even after the desalting step (Beckett (1975), Severson, Khoo and Steinberg (1977)). However, as the result indicated neither procedure yielded any activation of liver cholesterol ester hydrolase by cAMP preincubation with the liver cytosol in contrast to the marked activation of the enzyme from the adrenal and adipose tissue cytosol (Fig. 8.4b and Table 8.1). These in vitro
experiments were further supplemented by in vivo experiments. The administration of glucagon in vivo to the rat followed by the isolation of liver cytosol did not alter the activity of cholesterol ester hydrolase (Table 8.2). Thus in vivo conditions that alter cAMP concentrations and stimulate liver cAMP-dependent protein kinases (Byus, Haddox and Russell (1978)) did not seem to affect cytosolic cholesterol ester hydrolase. Even the addition of exogenous cAMP-dependent protein kinase of rabbit skeletal muscle together with cAMP, ATP and Mg++ also failed to stimulate the liver cholesterol ester hydrolase (Table 8.3). These in vivo and in vitro experiments positively excluded the activation of the enzyme through a cAMP mediation and no single criterion of protein phosphorylation (Krebs (1972)) involving cAMP could be found. It is proposed that, unlike rat adrenal and adipose tissue, rat liver seem to lack a cAMP-dependent protein kinase activatable cholesterol ester hydrolase and thus enzyme activation by protein phosphorylation catalysed by protein kinase seems very unlikely. The absence of hormonal sensitivity in vivo, and cAMP insensitivity in vitro are indicative of the different mechanisms that regulate cholesterol ester hydrolase in the liver and in adrenal or adipose tissue.

The exclusive role of the liver in bile salts production, coupled with the stimulation of microsomal cholesterol-7α-hydroxylase upon a high cholesterol diet feeding (Boyd, Scholan and Mitton (1969)) focussed the attention on the possible role of dietary cholesterol on the activity of rat liver cytosolic cholesterol ester hydrolase. It was shown that feeding the rat a high cholesterol diet resulted in a massive accumulation of esterified cholesterol in the liver (Gould (1977)). The disposal of these excess esters upon the withdrawal of the high cholesterol diet was predicted to induce an alteration of the
activity of the cytosolic cholesterol ester hydrolase. Therefore, experiments were carried out in which rats were fed 1% cholesterol diet for a week then they were fed the standard low cholesterol diet. Cholesterol ester hydrolase was assayed in the livers of these rats one day after diet replacement and also on the next consecutive two days. As the results show (Table 8.4), it seemed that cholesterol ester hydrolase did not respond to this dietary manipulation. However, the return of hepatic esterified cholesterol ester concentrations to normal values is a slow process and took about three days (Harry, Dino and McIntyre (1973)). Because of the mass of protein in the cytosol of the average liver, even with the low activity of rat cytosolic cholesterol ester hydrolase observed in this study, it may be possible that the liver, through this hydrolase, disposes very slowly of the excess of esterified cholesterol. It was shown that when labelled cholesterol esters were injected in vivo in the rat in a chylomicron preparation, the esterified cholesterol was rapidly taken up by the liver. However, about 80% of the radio-labelled ester in the liver disappeared very slowly in about three hours. These observations support the finding of the lack of a rapid cAMP-activatable cholesterol ester hydrolase. It is well established that the free cholesterol content of the liver which is largely if not solely associated with the membrane components, remain remarkably constant even in the face of a dietary cholesterol load. Since there is no sudden requirement for the supply of free cholesterol for bile salt production, the liver cholesterol ester hydrolase activity will suffice to meet these demands. It is possible that, a rapid activation of the enzyme would be counteracted by activation of the microsomal ACAT which is known to be sensitive to cholesterol concentration (Balasubramaniam, Mitropoulos and Venkatesan (1978)). Therefore, protein phosphorylation mediated by cAMP does not seem to influence liver cytosolic cholesterol ester hydrolase.
The studies presented in this thesis show the presence of a cAMP activatable triacylglycerol lipase in both adrenal and adipose tissue cytosol. This activity was intimately associated with cholesterol ester hydrolase activity from bovine adrenal. Both hydrolytic enzymic activities were activated by cAMP in vitro, and this activation paralleled the rapid transfer of phosphate onto acid-precipitable protein. The response of these enzymes in vivo to ACTH does meet the criteria of protein phosphorylation. The role of triglyceride hydrolysis in the adrenal cortex is not known, it could be obligatory or supportive to adrenal steroidogenesis. The mechanism of cholesterol ester hydrolase regulation in rat ovarian tissue, under the experimental conditions that simulate physiological conditions, does not seem to involve protein phosphorylation. Likewise the liver cytosolic cholesterol ester hydrolase enzyme was not hormonally sensitive neither was it activatable by cAMP in vitro. It seems that the rapid response of the adrenal and adipose tissue to acute hormonal stress make them unique in possessing a sensitive set of enzymes that undergoes functional modification. Such sensitive enzymes are not required in organs not obliged to respond rapidly to an acute and transient action, such as adrenal steroid hormones synthesis and secretion.
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