STUDIES ON CHOLESTEROL ESTER HYDROLASE IN STEROID

HORMONE PRODUCING TISSUES

by

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This thesis was composed by myself and the results described therein are the product of my own work.

ABBREVIATIONS

The following abbreviations will be used throughout the text:-

ACTH — Adrenocorticotrophic hormone

ATP — Adenosine 5'-triphosphate

BSA — Bovine serum albumin

Cholesterol — 5 cholesten-3β-ol

Cyclic AMP — Cyclic adenosine 3,5'-monophosphate

Dexamethasone — 1,4,Pregnandiene-9-fluoro-16α-methyl-11β, 17α,21-triol-3,20-dione

EDTA — Ethylenediamine tetra-acetic acid

EPR — Electron paramagnetic resonance

FSH — Follicle stimulating hormone

GLC — Gas liquid chromatography

HCG — Human chorionic gonadotrophin

LH — Luteotrophic hormone, Luteinizing hormone

NAD(H) — Nicotinamide adenine dinucleotide (reduced)

NADP(H) — "   "   " phosphate (reduced)

Pregnenolone — 3β-Hydroxypregn-5en-20-one

Pregnenol — Pregn-5-ene-3β-ol

Progesterone — Pregn-4-ene-3,20-dione

S.D.S. — Sodium dodecyl sulphate

Enzymes

Cholesterol ester hydrolase or cholesterol esterase or sterol ester hydrolase (EC. 3.1.1.13.)

Protein kinase or ATP protein phosphotransferase (EC. 2.7.1.37)

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

Glycogen Synthetase (EC. 2.4.1.11)

Phosphorylase kinase phosphatase (EC. 3.1.3)

Alkaline phosphatase (EC 3.1.31)

Pyruvate kinase (EC. 2.7.1.40)

SUMMARY

A brief summary of the work described in this thesis is given below:-

(a) The general properties of bovine adrenal, rat adrenal and rat luteal cholesterol ester hydrolase is are described.

(b) Cofactor addition in vitro of ATP, cyclic AMP and magnesium ions was shown to stimulate cholesterol ester hydrolase activity in rat and bovine adrenal 105,000 x g supernatant, while cyclic AMP did not appear to be required for the in vitro activation of the luteal cholesterol ester hydrolase.

(c) Activation of luteal cholesterol ester hydrolase by LH injection or cofactor addition of cyclic AMP, ATP and magnesium in vitro was not inhibited by prior injection of cycloheximide.

(d) Conditions where plasma LH and ACTH concentrations are elevated were shown to cause an increase in the ratio of cholesterol/cholesterol ester in the luteal and adrenal cortical lipid droplets. Cycloheximide injection enhanced the accumulation of cholesterol.

(e) Two purification procedures are described for the purification of bovine adrenal cholesterol ester hydrolase.

(f) The bovine adrenal cortical cholesterol ester hydrolase was shown to be phosphorylated in vitro in the presence of cyclic AMP and ATP and this resulted in the activation of the enzyme. Activation of purified cholesterol ester hydrolase was shown to require cyclic AMP, ATP and cyclic AMP dependent protein kinase.

Activation of the purified cholesterol ester hydrolase by these protein kinases resulted in the phosphorylation of the cholesterol ester hydrolase.

(g) Dephosphorylation of the bovine adrenal cholesterol ester hydrolase by alkaline phosphatase or phosphorylase kinase phosphatase resulted in a decrease in the activity of cholesterol ester hydrolase. Cholesterol ester hydrolase activity was found to be proportional to the amount of phosphate bound to the enzyme. Evidence is also presented which indicates that a magnesium dependent phosphoprotein phosphatase is responsible for the dephosphorylation of cholesterol ester hydrolase.

(h) A control mechanism is postulated for cholesterol ester hydrolase. Activation of cholesterol ester hydrolase is accomplished by a phosphorylation of the enzyme catalysed by a cyclic AMP dependent protein kinase in the presence of ATP. Deactivation of cholesterol ester hydrolase is achieved by a dephosphorylation of the enzyme by a magnesium dependent phosphoprotein phosphatase. Evidence is also presented to show that a similar process also occurs in the rat adrenal.

(i) Ether anaesthesia stress in vivo which increases the plasma ACTH concentrations or ATP and cyclic ~~ATP~~ AMP addition in vitro was shown to stimulate cholesterol ester hydrolase activity in the rat adrenal 105,000 x g supernatant.

(j) Prior feeding of the rats with rape seed oil for 6 weeks abolished the activation of cholesterol ester hydrolase observed in control animals on ether anaesthesia.

This diet also abolished the _in vitro_ response of cholesterol ester hydrolase to additions of cofactors. Activation of protein kinase on ether stress in the rat adrenal was also abolished by feeding the animals rape seed oil. Corticosteroid production on ether stress in the animals fed rape seed oil was also lower than the levels found in the control animals fed olive oil and subjected to an ether stress.

(k) From the results it is proposed that cholesterol ester hydrolase plays an important role in the control of steroidogenesis by controlling the supply of cholesterol to the mitochondrial side chain cleavage enzyme.

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CHAPTER 1

GENERAL INTRODUCTION

The function of the adrenal cortex and corpus luteum

The enzyme systems involved in steroidogenesis in the adrenal cortical and ovarian tissue have much in common. The adrenal cortex is involved in the formation and secretion of glucocorticoids and mineralocorticoids which in turn play a part in the control of carbohydrate, protein and lipid metabolism and also electrolyte and water balance. The major steroid hormones produced by the adrenal cortex are shown in Figure 1.1.

The corpus luteum of the ovary produces progesterone which is required for the maintenance of pregnancy and other functions. Progesterone is the major product of steroidogenesis in the corpus luteum of most animals but oestrogens and androgens are also produced by the luteal cells (review Savard (1973)).

The precursor of the steroid hormones

The early studies on adrenal steroid biosynthesis established that the hormones could be synthesised from both acetate and cholesterol (see review Simpson and Mason (1975)). The role of cholesterol as an obligatory intermediate, however, has been a matter of controversy.

Stone and Hechter (1954) using bovine adrenal glands perfused with $[^{14}C]$ acetate, $[^{14}C]$-cholesterol and $[^{14}C]$-progesterone in the presence and absence of ACTH demonstrated that ACTH only markedly increased the incorporation of $[^{14}C]$ into corticosteroids when cholesterol
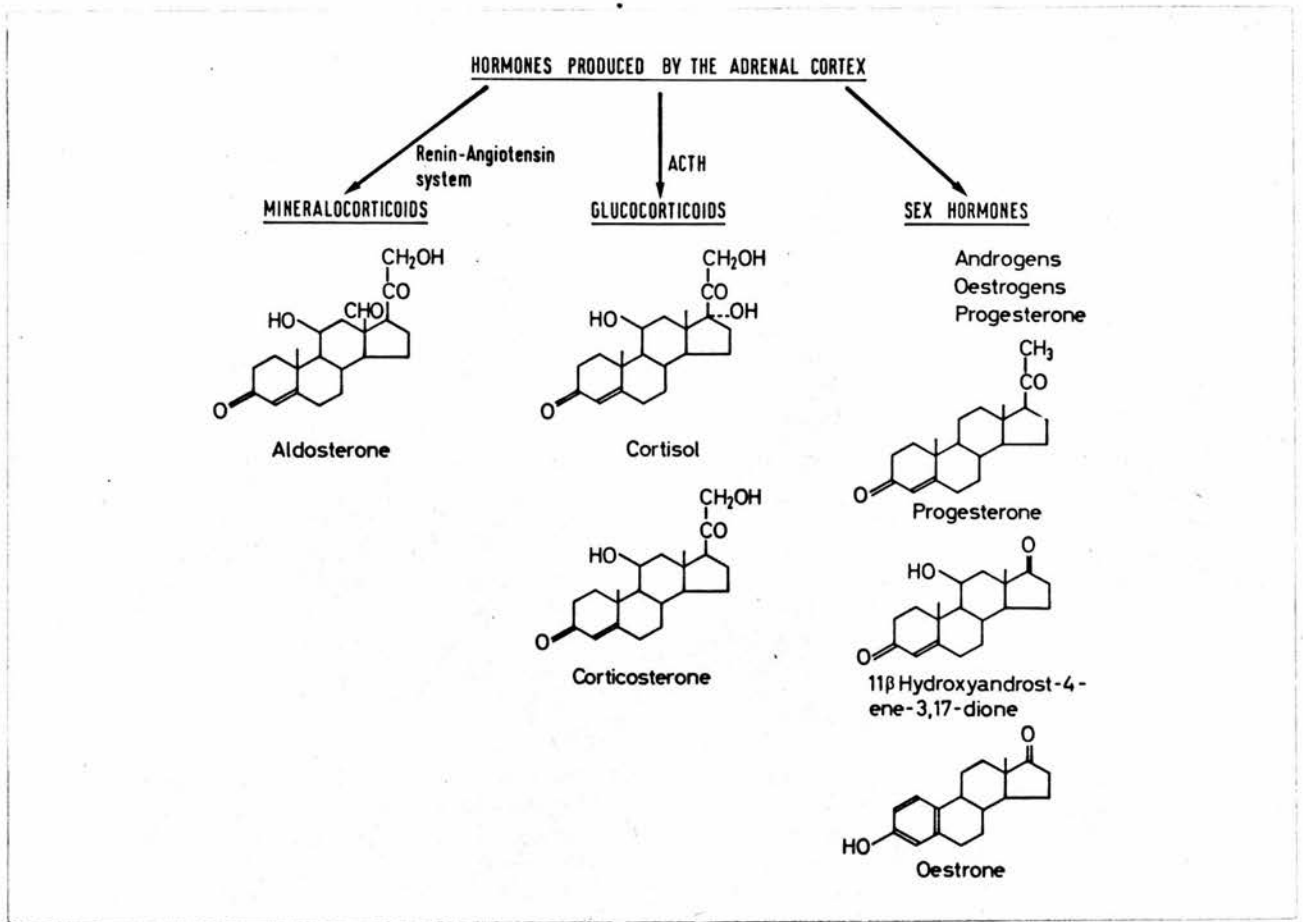
Figure 1.1 Steroid hormones produced by the adrenal cortex.

was used as a precursor. In these experiments ACTH was shown to inhibit the incorporation of $[^{14}C]$ acetate into cholesterol. It was suggested that a pathway existed from acetate to corticosteroids which did not involve the formation of cholesterol as an intermediate. This proposed pathway was allegedly not significantly influenced by ACTH.

In support of the concept that cholesterol is an obligatory intermediate in the formation of the adrenal steroids, it has been observed that the specific radio-activity of urinary cortisol and adrenal cholesterol were equal after $[4-^{14}C]$ cholesterol was fed to guinea pigs for long periods of time (Werbin and Chaikoff (1961)). Differ-ences in the specific radioactivities of the cholesterol and the steroid hormones were observed on short term feeding, but this was probably due to differences in the rate of equilibration of different pools of cholesterol within the adrenal cortical cells. These differences were eliminated by long term feeding of the labelled precursor. It has also been suggested that desmosterol could serve as a substrate for side chain cleavage (Goodman et al. (1962a and 1962b)) as homogenates of rat adrenals could convert $[^{14}C]$ cholest-erol and $[^{14}C]$ desmosterol to $[^{14}C]$ pregnenolone and $[^{14}C]$ progesterone in equal yields from the added substrates.

As discussed previously, ACTH inhibits the total conversion of radioactive acetate into cholesterol (Stone and Hechter (1954); Garren et al. (1971)). This inhibition was prevented by cycloheximide injection. The increased incorporation of $[^{14}C]$ acetate into cholesterol and steroids after ACTH treatment of Sato murine adrenal cortex tumour

monolayer cultures (Kowal (1970)) has been demonstrated.
However the increase in the labelling of the cholesterol
from acetate in response to ACTH was less than the increased
labelling of steroids. Although the initial steroid
secretion represented a relatively small fraction of the
total cholesterol pool, substantial quantities of radio-
activity, one third to one half of that found in cholesterol,
were incorporated into the steroids. This suggested that
either a small pool of cholesterol was being turned over
rapidly in response to the ACTH stimulus, or that cholesterol
synthesis from acetate was by-passed. The use of an
inhibitor of cholesterol biosynthesis, AY-9944, showed that
inhibition of cholesterol synthesis from $[^{14}C]$ acetate was
accompanied by a comparable inhibition of incorporation of
$[^{14}C]$ label into the steroids. Mevalonate was also incorpor-
ated into steroids, but in this case little stimulation
was produced by ACTH. This suggests that the site of
activation of cholesterol biosynthesis was at a point prior
to mevalonate synthesis.

It would appear therefore that ACTH might block the
formation of cholesterol from labelled acetate or else
effect a redirection of cholesterol precursors into an
alternative pathway.

The adrenal cortex contains more cholesterol per gram
wet weight than any other tissue in the body. In fact 4-6%
of the wet weight of the tissue is cholesterol (Carrol (1962);
(Deuel (1955)), which is much more than is found in plasma
and liver (Swell et al. (1961)) or the 1-1.5% found to
occur in the ovary (Behrman et al. (1970)). Most of the

cholesterol in the adrenal gland (Garren et al. (1971))
and the luteinized ovary (Flint and Armstrong (1972)) is
esterified to long chain fatty acids. After [^{3}H] cholesterol
was exchanged with rat adrenal cholesterol by intravenous
injection, the radioactive cholesterol in the adrenal
cortex was found in the intracellular lipid droplets
(Moses et al. (1969)). These droplets are easily observed
under the light or electron microscope (Figure 1.2).

If the adrenal glands or luteinized ovaries are homogen-
ised and subjected to subcellular fractionation, the intra-
cellular lipid droplets can be isolated as a floating layer
(Claesson (1954)). These lipid droplets have been isolated
and characterised in the rat adrenal cortex (Garren et al (1971);
Boyd and Trzeciak (1973)) and the luteinized ovary (Armstrong
and Flint (1973)). The principal lipids in these droplets
are cholesterol ester, and phospholipid with only small
amounts of triglyceride and free cholesterol.

The cholesterol in the adrenal appears to be selectively
esterified to unsaturated fatty acids such as oleate,
linoleate and arachidonate (Boyd and Trzeciak (1973);
Goodman (1965) Table 9.3).

The administration of ACTH to rats is known to cause
a decrease in total adrenal cholesterol and ascorbic acid
(Sayers et al.(1946)) and this is associated with an
increase in the adrenal steroid hormone production. This
fall in total adrenal cholesterol has been accounted for
by a fall in the cholesterol esters of the lipid droplets;
the cholesterol content of the other cell fractions were
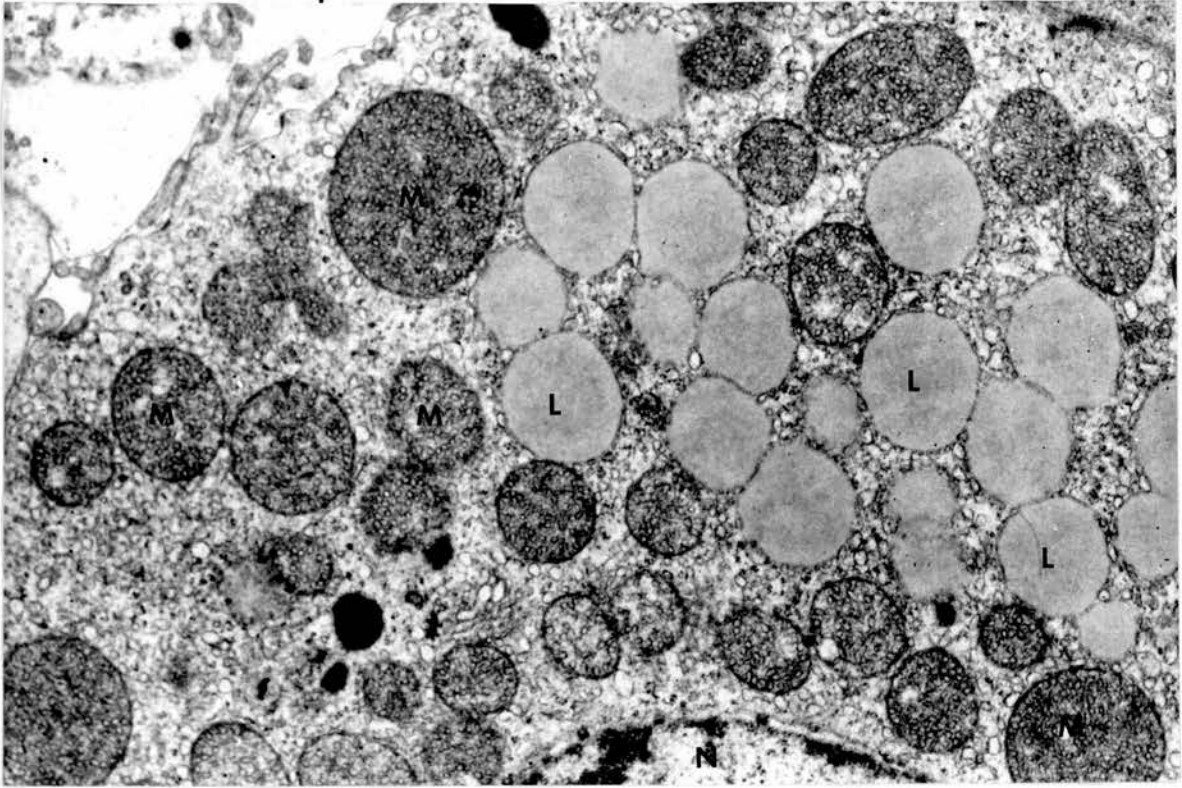not altered. A similar effect has been observed when

Figure 1.2 Electronmicrograph of a section of rat
adrenal cortex. The white lipid droplets (L),
mitochondria (M) and Nuclei (N) can clearly be
seen.

animals are subjected to ether anaesthesia (Trzeciak and Boyd (1973)) a condition which elevates the plasma ACTH levels (Matsayuma et al. (1971)).

Injection of cycloheximide prior to ACTH injection, which blocks the ACTH stimulated fall in adrenal cholesterol (Davies and Garren (1968)) by inhibiting the conversion of cholesterol to pregnenolone has been shown to cause a marked increase in free cholesterol in the adrenal lipid droplets (Davies and Garren (1966);(1968) Garren et al. (1969)). Davies (1969) has reported that perfusion of the adrenal gland with ACTH or dibutyryl cyclic AMP similarly activated the hydrolysis of cholesterol esters and it has been reported (Garren et al. (1971)) that free cholesterol seemed to leak into the cytosol from the lipid droplets of the adrenal cells of ACTH treated rats. Brecher et al. (1973) have also shown that in adrenal cell suspensions, ACTH decreased the amount of cholesterol esters.

These studies provide evidence that the cholesterol esters stored in the lipid droplets are an important source of precursor cholesterol for steroidogenesis. The amount of cholesterol which accumulates in the presence of ACTH plus cycloheximide, was less than half the amount of cholesterol ester lost. This suggests that a fraction of the free cholesterol being produced as a result of the hydrolysis of the cholesterol esters is equilibrating with other subcellular membranes or is secreted by the gland rather than being utilised for steroid hormone production. Another possibility is that cholesterol esters are secreted from the adrenal cell under the influence of ACTH.

Rhodin (1971) has shown that endoplasmic reticulum profiles form an almost complete membranous casing around the lipid droplet. As the lipid droplet reached the cell surface the endoplasmic reticulum merged with the cell membrane and the lipid droplets were ejected. The extrusion produced membranous ghosts in the cell cytoplasm. Rhodin (1971) also showed that dexamethasone treatment, which inhibits ACTH release from the pituitary, greatly increased the number of lipid droplets in the zona fasiculata while ACTH treatment for ten minutes greatly reduced the number of lipid droplets. Rhodin postulated that the final steps in the formation of steroid hormones may not occur until the moment of discharge of the lipid droplet and this would explain why progesterone and pregnenolone are secreted by the adrenal. If the lipid droplets extrude their contents before complete conversion of the cholesterol ester to cholesterol and other steroid products this would explain why depletion of the cholesterol ester was larger than the free cholesterol accumulating in the rats treated with cycloheximide and ACTH.

A rapid loss of cholesterol ester from the corpus luteum associated with a decrease in the size and the number of the luteal lipid droplets on the treatment of rats with a number of hormones has been observed (Deane et al. (1948); Barker (1951); Claesson (1954); Ashworth et al. (1959); Giacomelli et al. (1965)). These luteal lipid droplets have also been suggested as being the precursors for the luteal steroid hormones (Everett (1945).

The role of ACTH and LH in steroidogenesis

The initial action of the trophic hormones is thought to be a binding of the hormone to a receptor on the cell surface. It has been shown that ACTH bound to cellulose, a molecule which cannot cross the plasma membrane, can still stimulate steroidogenesis in the adrenal cell (Selinger and Civen (1971); Schimmer et al. (1968)). Haynes and Berthet (1957) have shown that ACTH induced the formation of cyclic AMP in the adrenal gland and that cyclic AMP could stimulate steroidogenesis in the absence of ACTH (Haynes et al. (1959)). The increase in cyclic AMP levels on ACTH treatment has been associated with an activation of adenyl cyclase (Lefkowitz et al. (1970); Schimmer et al. (1968)). Protein synthesis has been implicated as an essential step in the steroidogenic response to ACTH and LH (Ferguson (1962); Farese (1964); Garren et al. (1965); Garren et al. (1971)). From these studies it was postulated that a labile protein was involved, whose synthesis was stimulated by LH or ACTH. This protein factor was considered to take part in the steroidogenic response and the production of this protein is thought to be stimulated by cyclic AMP. However the mechanism of the action of this protein on steroidogenesis has not yet been determined.

Calcium ions have been shown to be important in mediating the effects of LH and ACTH in the adrenal and corpus luteum. Calcium ions are required for the formation of cyclic AMP under the influence of ACTH (Sayers et al. (1972)) and it is proposed that calcium ions are involved in the transmission of the signal arising from the ACTH - receptor interaction

to the adenyl cyclase. Calcium has also been shown to increase in vitro the amount of cholesterol bound to the adrenal mitochondrial cholesterol side chain cleavage cytochrome P 450 (Simpson et al. (1974)) a situation which could increase pregnenolone production.

Prostaglandins also seem to play an important role in the steroidogenic response to LH in the corpus luteum and ACTH in the adrenal cortex. Indomethacin, as inhibitor of prostaglandin synthetase has been shown to reduce elevated corticosterone levels achieved by ACTH injection to hypophysectomised rats. Indomethacin did not however reduce corticosterone levels in hypophysectomised rats injected with dibutyryl cyclic AMP. Injection of prostaglandin E_2 (PGE$_2$) restored the normal response (Gallant and Brownie (1973)) to ACTH, suggesting that prostaglandins may be involved in the action of ACTH.

Behrman et al. (1971) have shown that prostaglandin $F_{2\alpha}$ (PGF$_{2\alpha}$) depressed the level of ovarian cholesterol esters by 75%. Hypophysectomy produced a similar effect but the administration of prolactin restored cholesterol ester levels in these hypophysectomised animals. The trophic effect of the prolactin however was abolished if the PGF$_{2\alpha}$ was administered together with prolactin. Progesterone output was also reduced to 50% of the control in ovarian slices by prior in vivo treatment with PGF$_{2\alpha}$. In vivo treatment with PGF$_{2\alpha}$ and prolactin could not restore the levels of progesterone production. Prolactin itself has little effect on steroidogenesis when administered to control rats (Huang and Pearlman (1962)) but it is required

to maintain the function and the structure of the corpus luteum in the hypophysectomised rats (Macdonald et al. (1970)).

"Cholesterol ester synthetase" activity in the corpus. luteum is decreased by the administration of $PGF_{2\alpha}$ and cholesterol ester hydrolase activity is also decreased. Injection of LH was found to reverse these effects (Behrman et al. (1971)). Recently it has been shown that prostaglandin $F_{2\alpha}$ stimulates progesterone synthesis in human corpus luteum in vitro (Santos et al. (1973)). Prostaglandins of the E series have also been reported to stimulate progesterone synthesis when added in vitro to the rat luteal slices (Behrman et al. (1971); Marsh (1970)). Despite all these studies the exact role of prostaglandins in steroidogenesis is still uncertain.

It has been suggested that mitochondrial cholesterol side chain cleavage is the rate limiting step in steroidogenesis and is under the control of the trophic hormones (Stone and Hechter (1954); Hall and Eik-Nes (1964); Hall and Koritz (1965); Hall and Young (1968)). Only recently however has prior ACTH treatment been shown to influence cholesterol side chain cleavage in the isolated rat adrenal mitochondria (Simpson et al. (1973)). There are several ways in which trophic hormone stimulation could affect the metabolism of cholesterol in the mitochondria. The hormone could increase the availability of NADPH, cholesterol and oxygen for the cholesterol side chain cleavage reaction. The hormone could also have a direct effect on the cholesterol metabolising enzymes through some activator or inhibitor.

Bell and Harding (1974) and Bell et al. (1973) have concluded that the supply of cholesterol to the cholesterol side chain cleavage enzyme is the rate limiting step in adrenal steroidogenesis. They do not deduce however the origin of the cholesterol supply. It seems therefore that the supply of cholesterol to the mitochondria is an important factor controlling steroidogenesis.

If there is a decrease in the quantity of esterified cholesterol in the adrenal and corpus luteum under conditions which elevate plasma ACTH and LH levels it would be reasonable to suggest that a hydrolytic enzyme might be activated under these conditions.

The injection of LH has been shown to increase luteal cholesterol ester hydrolase activity (Behrman and Armstrong, (1969)) and LH antiserum has been shown to decrease luteal cholesterol ester hydrolase activity (Behrman et al. (1972)).

Shima et al. (1972) have shown that adrenal cholesterol ester hydrolase is stimulated in rats injected with ACTH whilst cholesterol ester synthetase is inhibited. Trzeciak and Boyd (1973) have shown that ether anaesthesia stress, which increases plasma ACTH levels (Matsayuma et al. (1971)) also stimulates cholesterol ester hydrolase activity in the rat adrenal and this stimulation was not prevented by prior injection with cycloheximide. This would tend to support the view of Garren et al. (1971) that the depletion of adrenal cholesterol esters is due to an activation of a preformed enzyme rather than de novo synthesis. From these results it has been postulated that ACTH activated adrenal cortical cholesterol ester hydrolase could act on the lipid

droplets rich in cholesterol ester to produce cholesterol for utilisation in the adrenal mitochondria (Figure 1.3). The free fatty acid released could be utilised by the mitochondria for energy purposes as suggested by (Boyd and Trzeciak (1973)) or as a precursor for prostaglandin production (Boyd and Trzeciak (1973)).

Pregnenolone formation from cholesterol is thought to involve hydroxylation at the C20 and C22 but the exact nature and sequence of formation of the intermediates still has to be established. Pregnenolone production from isolated rat adrenal mitochondria follows a biphasic course with an initial rapid production lasting 2 to 5 minutes Simpson et al. (1972)). Mitochondria isolated from rats subjected to ether anaesthesia had an increased rate of pregnenolone production from endogenous cholesterol. Prior injection of cyclo-heximide blocked the effect of ether stress. From these results it was suggested that one action of ACTH was to affect the distribution of cholesterol in the mitochondria to allow more of it to bind to the cholesterol side chain cleavage cytochrome P 450. As prior injection of the cycloheximide blocked the effect of ACTH it was postulated that some labile protein factor synthesised in response to ACTH may be involved in transporting cholesterol to the sterol side chain cleavage cytochrome P450 enzyme system.

Boyd and Trzeciak (1973) have shown that the rat adrenal mitochondria undergo a sizable depletion of total mitochondrial cholesterol during a 30 minute incubation. If mitochondrial cholesterol is being consumed for pregnen-olone synthesis it is quite possible that the major adrenal

METABOLISM OF CHOLESTEROL ESTER TO PREGNENOLONE

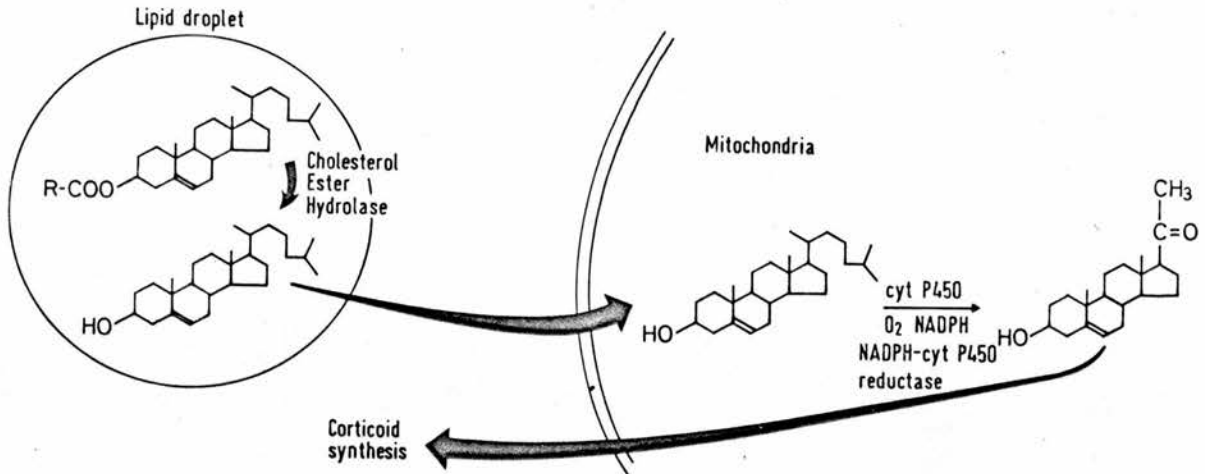Figure 1.3 The metabolism of lipid droplet cholesterol
ester to pregnenolone. Cytoplasmic cholesterol ester
hydrolase acts on the cholesterol esters in the lipid
droplet to produce free cholesterol. This   cholesterol
is then available to the mitochondrial side chain
cleavage enzymes for the production of pregnenolone.
The pregnenolone produced can then undergo further
enzymatic reaction to produce corticosteroids.

reservoir of cholesterol esters, the lipid droplets, in
conjunction with a hormone sensitive cholesterol ester
hydrolase play an important role in supplying the substrate,
cholesterol, for pregnenolone production induced by ACTH, and
for maintaining the integrity of the mitochondrial structure.

Cyclic AMP and cyclic AMP dependent protein kinase in the adrenal cortex and luteal tissue

Trophic hormones have been shown to produce changes
in cyclic AMP concentration and protein kinase activity in
the luteal and adrenal tissues as well as changes in
steroidogenesis.

Before the work of Garren, despite evidence for the
involvement of cyclic AMP in the adrenal cortical function,
the mechanism of action of the nucleotide remained unknown.
Gill and Garren (1969) demonstrated that cyclic AMP receptor
proteins existed in the bovine adrenal cortex cell in the
cytosol and microsomal fractions. When the microsomal
fractions were further fractionated into rough and smooth
endoplasmic reticulum and free ribosomes it was found that
binding resided predominantly in the endoplasmic reticulum.

The cyclic AMP bound by the cytosol protein did not
appear to be covalently linked or metabolised (Gill and
Garren (1969)). Using other cyclic nucleotides it was
demonstrated that the receptor protein specifically and
tightly bound cyclic AMP and this led these workers to
conclude that the initial action of cyclic AMP in the
adrenal cell is the binding of the nucleotide to this
receptor protein. A similar binding has been observed in
several other tissues of the rat (Walton and Garren (1970))
and in the bovine corpus luteum (Menon (1973)).

Walsh et al. (1968) have suggested that in the rabbit skeletal muscle the initial action of cyclic AMP is the binding of the nucleotide to phosphorylase kinase to activate the kinase.

Gill and Garren (1970) demonstrated the existence of a cyclic AMP dependent protein kinase in the cytosol of the bovine adrenal cortical tissue. The protein kinase exhibited the same nucleotide specificity as did the cyclic AMP binding protein and it also showed the same subcellular location.

When both the cyclic AMP dependent protein kinase and the receptor protein were purified it was found that the receptors sedimented at 4S while the fraction containing both the protein kinase and cyclic AMP binding properties sedimented at 7S. Addition of cyclic AMP to a protein fraction containing protein kinase and receptor protein complex (7S) resulted in the separation of the complex into a 4S receptor fraction and a 7S protein kinase fraction. In some preparations addition of cyclic AMP to the protein kinase-receptor complex (7S) resulted in a protein kinase fraction (3.8S) and a receptor fraction (4S).

When the 4S fraction was added to the 7S fraction which contained protein kinase activity, the kinase activity was supressed. The addition of cyclic AMP after the addition of the 4S fraction was found to reverse the inhibition of the protein kinase activity. These results led Garren et al. (1971) to suggest that cyclic AMP binds to the receptor protein of a receptor protein-protein kinase complex and causes the dissociation of the regulatory subunit from the catalytic subunit thus activating the protein kinase (Figure 1.4).
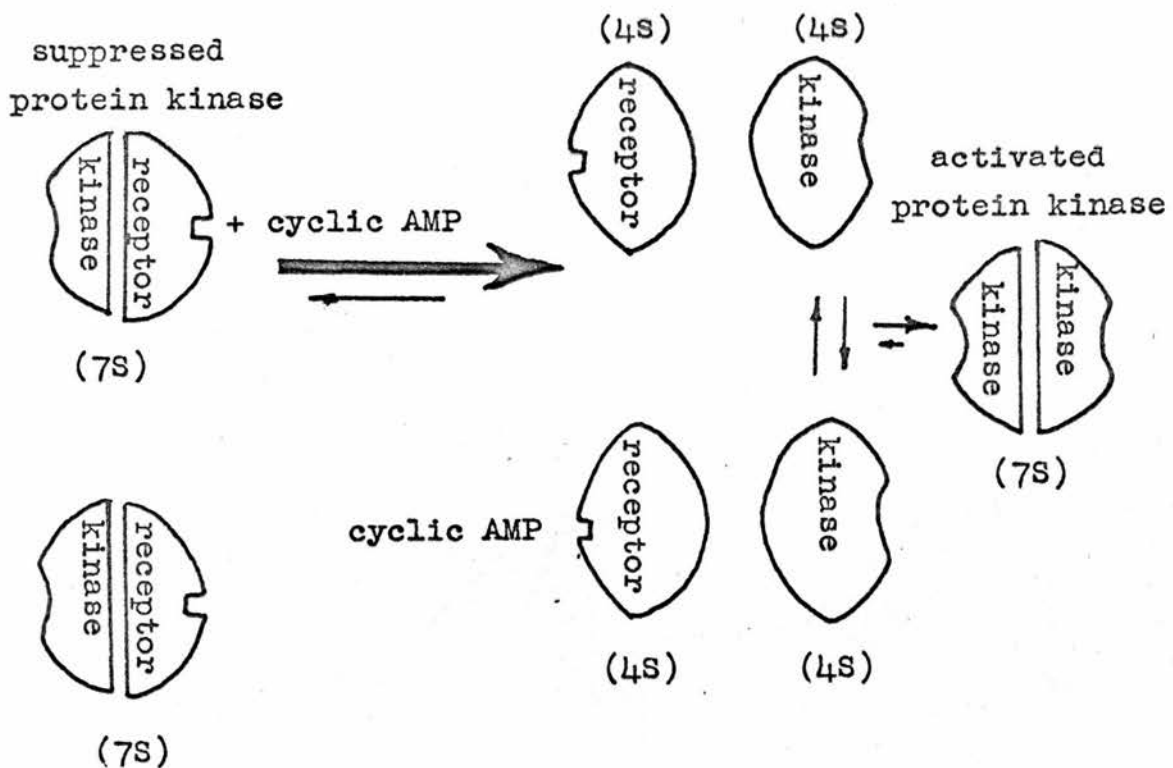
Figure 1.4 Activation of protein kinase by cyclic AMP

Cyclic AMP in binding to the receptor causes an
allosteric change in the protein that results in the
dissociation of the receptor from the protein kinase,
thereby activating the enzyme. The protein kinase-
receptor complex sediments at 7S; the binding of
cyclic AMP to the receptor results in the sedimentation
of the receptor at 4S. When protein kinase is not
observed at 4S, even after the addition of cyclic AMP
it is proposed that the protein kinase molecules, in
the active state , associate in a 7S complex.
(After Garren et al (1971)).

Menon (1973) has isolated a cyclic AMP dependent protein kinase from bovine corpus luteum KI which appears to be activated by the same mechanism as the protein kinase . isolated from the bovine adrenal. Menon has also isolated a second protein kinase KII which could be activated by cyclic AMP or in this case directly by the in vitro addition of LH.

From these studies it was inferred that LH may have a direct control on the activity of KII. It has been shown however (Azhar and Menon (1975a)(1975b)) that while KII is activated by cyclic AMP via dissociation of the catalytic-regulatory subunit complex, LH activation does not cause such a dissociation. Again, while cyclic AMP activated KII will phosphorylate luteal ribosomes, LH activated KII will not.

There are no reports of a kinase activated directly by ACTH in the adrenal but Shima et al. (1973) demonstrated cyclic AMP dependent protein kinase activity in the rat adrenal and Ichii (1972) has found both cyclic AMP dependent and independent protein kinase activity in the rat adrenal.

Walsh et al. (1971) have isolated a heat stable cyclic AMP dependent protein kinase inhibitor. This protein is not the receptor protein for the protein kinase catalytic - regulatory subunit structure but it appears to exert its effect by promoting a five fold increase in the binding constant of the protein kinase for cyclic AMP. This protein kinase inhibitor has been shown to inhibit the activation of cyclic AMP dependent protein kinases from the brain, heart, skeletal muscle and adipose tissue of the rabbit.

Mechanism of activation of cholesterol ester hydrolase

As discussed previously cholesterol ester hydrolase activity in the adrenal and luteal tissues is increased in conditions where plasma concentrations of ACTH and LH are elevated. Garren et al. (1971) has suggested without evidence that a protein kinase may be involved and that this protein kinase, activated by cyclic AMP would activate cholesterol ester hydrolase by a mechanism similar to that of hormone sensitive lipase.

Glucagon, adrenalin, noradrenalin, and ACTH are known to stimulate free fatty acid release from adipose tissue (Vaughan (1960)). Rizack (1964) showed that ATP, magnesium and cyclic AMP additions in vitro to cell free extracts of adipose tissue activated lipolytic activity. Caffeine which inhibits phosphodiesterase also increased the degree of activation.

Using a purified preparation of hormone sensitive lipase it was shown that the activation of hormone sensitive lipase required ATP, cyclic AMP, magnesium ions and rabbit muscle protein kinase (Huttunen et al. (1970a); Huttunen and Steinberg (1971)). If $[\gamma-^{32}P]$ ATP was used $[^{32}P]$ radioactivity was transferred to the protein fraction containing lipase activity.

Huttunen and Steinberg (1971) showed that the transfer of the $[^{32}P]$ radioactivity from the $[\gamma-^{32}P]$ ATP to the protein on preincubation showed the properties of a serine -O- phosphate linkage. This led to the postulation (Tsai et al. (1970); Huttenen et al. (1970a) that hormone sensitive lipase is activated by a phosphorylation of the

enzyme catalysed by a protein kinase. A similar mechanism
of the activation of human adipose tissue hormone sensitive
lipase has been postulated by Khoo et al. (1972).

It has been suggested (Tsai and Vaughan (1972); Tsai et al.
(1973)) that inactivation of hormone sensitive lipase is
accomplished by dephosphorylation by a phosphoprotein
phosphatase.

Boyd and Trzeciak (1973) have shown that in the rat
adrenal protein kinase activity increases and cyclic AMP
levels rise immediately after rats are subjected to ether
anaesthesia stress. Prior injection of cycloheximide did
not block this increase. Activation of cholesterol ester
hydrolase in the rat and bovine adrenal can be reproduced
in vitro by the addition of ATP and cyclic AMP. Theophylline
potentiated this effect (Trzeciak and Boyd (1973)(1974)).
Trzeciak and Boyd (1974) have also shown that preincubation
of a crude adrenal cortical supernatant protein fraction
with cyclic AMP, magnesium ions and $[\gamma-^{32}P]$ ATP followed
by a partial purification of the cholesterol ester hydrolase,
resulted in the transfer of the $[^{32}P]$ radioactivity to the
protein fraction containing cholesterol esterol ester
hydrolase activity. Activation of cholesterol ester
hydrolase in the purified preparation also required an
adrenal cortical supernatant fraction which did not contain
cholesterol ester hydrolase activity but did contain
protein kinase activity.

From this evidence Trzeciak and Boyd (1974) postulated
that activation of cholesterol ester hydrolase in the rat
and bovine adrenal occurred by a phosphorylation of an

inactive enzyme. This phosphorylation was postulated to be catalysed by a cyclic AMP dependent protein kinase system similar to that of Garren et al. (1971).

Aims of the present study

The ACTH induced cholesterol ester depletion of the adrenal gland is well established. The work of Trzeciak and Boyd (1973) and (1974) has shown that under conditions where ACTH is elevated a cholesterol ester hydrolase is activated and from their investigations it has been proposed that a phosphorylation of cholesterol ester hydrolase takes place to activate it. This phosphorylation is catalysed by a protein kinase.

The purified phosphorylated protein fraction of Trzeciak and Boyd (1974) containing cholesterol ester hydrolase activity was only purified seven fold. As pointed out by them it cannot be excluded that the phosphorylated protein in their preparation is another protein which is phosphorylated and not cholesterol ester hydrolase. The protein may stimulate or participate in the hydrolysis of cholesterol esters or it may have nothing to do with cholesterol ester hydrolysis.

The object of this study was to investigate and characterise the properties of cholesterol ester hydrolase in the adrenal and ovarian tissue.

Using both crude and purified preparations of bovine cholesterol ester hydrolase the mechanism of activation and deactivation of cholesterol ester hydrolase was investigated. The role of cyclic AMP, ATP and cyclic AMP dependent protein kinase in the activation of cholesterol

ester hydrolase was also investigated. From these invest-
igations it was hoped to obtain information about the role
of ACTH in the control of steroidogenesis.

From the proposed investigations it was hoped to
determine if cholesterol ester hydrolase was under hormonal
control and show that it plays an important role in supply-
ing cholesterol to the mitochondria and hence plays a part
in the control of steroidogenesis.

CONTENTS

CHAPTER 2

MATERIALS AND METHODS

Animals and pretreatment

The experiments described in chapters 7 and 9 were performed on male Wistar rats (Edinburgh University Small Animal Breeding Centre) weighing 200 to 400 gms and were maintained on a stock diet of 25% skimmed milk powder, 5% dried yeast and 70% wholemeal flour. The animals were given water _ad_ _libitum_.

The experiments described in chapter 8 were performed on female Wistar rats obtained from Edinburgh University Small Animal Breeding Centre. Luteinized ovaries were induced into these animals by the pretreatment method of Parlow (1958). Rats 21 to 24 days old were given subcutaneous injections of 50 international units (iu) of Pregnant Mare Serum Gonadotrophin (Gestyl-Organon) dissolved in 0.5 mls of 0.9% saline. This solution induces heavy follicular growth in the ovaries of the rat as it contains FSH like activity. After a period of three days rats were given a second injection of 50 (iu) of Pregnant Mare Serum Gonadotrophin (Sulimovici and Boyd (1968)) or 25 (iu) Human Chorionic Gonadotrophin (Pregnyl-Organon). These preparations contain luteotrophic activity and cause ovulation in the rat ovaries (Schmidt-Elmendorf _et_ _al_. (1962)). The granulosa and thecal cells of the ruptured ovarian follicles then develop into corpura lutea. After the second injection of Human Chorionic Gonadotrophin the animals were left for 5-7 days. The ovaries obtained from these animals varied from 120-150 mgs compared with less than 10 mgs in the untreated animal (Arthur (1975)).

Treatment of animals prior to killing

a) Ether anaesthesia stress

Animals requiring ether anaesthesia stress were subjected to an ether anaesthesia stress for ten minutes prior to killing by decapitation (Boyd et al. (1972)).

b) Injections of cycloheximide and LH

Cycloheximide, 5mgs per rat, was dissolved in 0.2mls of 0.9% saline and administered by intra-peritoneal injection. LH 10μg per rat (N.I.H. LH-S-18) was administered in 0.2mls of 0.9% saline by sub-cutaneous injection behind the neck of each rat. The rats were killed after the injections at the times indicated in the text.

Preparation of tissue homogenates

a) Rat corpus luteum

After pretreatment the rats were killed by cervical dislocation, their ovaries were quickly removed and trimmed free of adherent fat and connective tissue. The ovaries were then kept in 0.25 M sucrose prior to homogenisation within 3 minutes of killing and all subsequent operations were performed at 4°C. The trimmed ovaries were cut into small pieces with scissors and homogenised in 5 volumes of 0.25 M sucrose per gram of tissue using a loose fitting teflon pestle Potter Elvehjem homogeniser. Only three passes were made with the homogeniser to avoid disrupting the luteal mitochondria.

b) Rat adrenal

After pretreatment the animals were killed by decapitation. The adrenals of the animals were quickly removed after killing, trimmed free of adherent fat, pooled and homogenised

in 5 volumes of ice cold 0.25 M sucrose using a loose
fitting teflon pestle, Potter-Elvehjem homogeniser as
described in the previous section.

c) Bovine adrenal cortex

Bovine adrenals were obtained from the local abattoir,
transported to the laboratory in crushed ice and processed
within about 60 minutes of killing the animals. The glands
were trimmed free of adherent fat and cut longitudinally
and the medulla was carefully removed and discarded. The
cortex was then scraped off the capsule with a scalpel and
transferred into a beaker containing ice cold 0.25 M sucrose
solution. A twenty per cent homogenate was then prepared
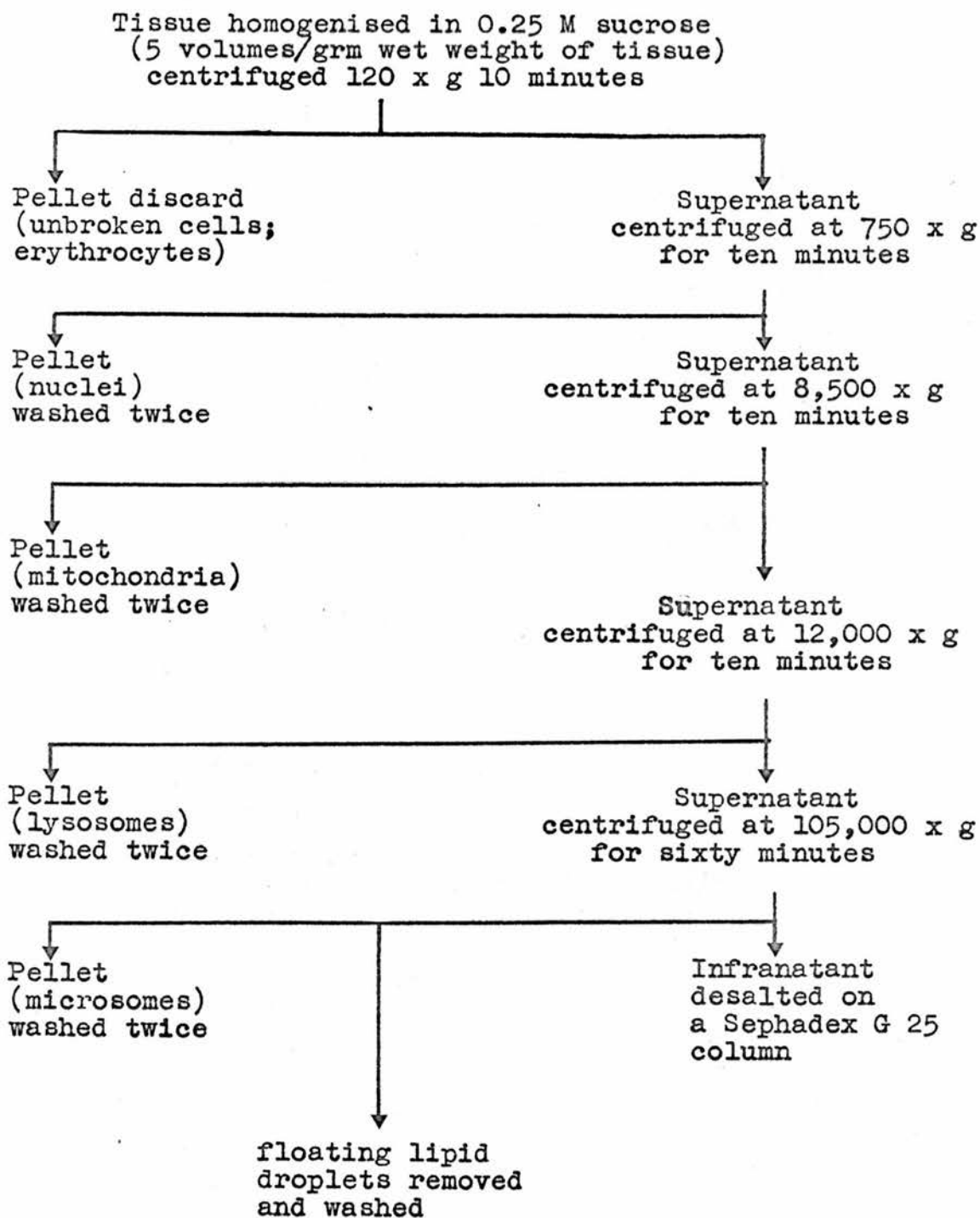by the use of a teflon glass Potter-Elvehjem homogeniser.

Preparation of subcellular fractions

The method for preparing subcellular fractions from
the rat corpus luteum, rat adrenal and bovine adrenal cortex
was the same for each tissue and is shown in Figure 2.1.

The homogenate was centrifuged for ten minutes at
120 x g and the pellet which contained tissue fragments,
unbroken cells and erythrocytes was discarded. The superna-
tant was centrifuged for ten minutes at 750 x g to sediment
the nuclear fraction and the mitochondrial and lysosomal
pellets were isolated by centrifugation of the 750 x g
supernatant for ten minutes each at 8,500 x g and 12,000 x g
respectively. The microsomal fraction was obtained by
centrifugation of the 12,000 x g supernatant for 60 minutes
at 105,000 x g. Each pellet was resuspended in 0.25 M
sucrose and recentrifuged at the required speed. This
washing procedure was carried out twice on each fraction.

Figure 2.1

Procedure for the preparation of subcellular fractions

from adrenal and ovarian homogenates

Tissue homogenised in 0.25 M sucrose
(5 volumes/grm wet weight of tissue)
centrifuged 120 x g 10 minutes

Pellet discard
(unbroken cells;
erythrocytes)

Supernatant
centrifuged at 750 x g
for ten minutes

Pellet
(nuclei)
washed twice

Supernatant
centrifuged at 8,500 x g
for ten minutes

Pellet
(mitochondria)
washed twice

Supernatant
centrifuged at 12,000 x g
for ten minutes

Pellet
(lysosomes)
washed twice

Supernatant
centrifuged at 105,000 x g
for sixty minutes

Pellet
(microsomes)
washed twice

Infranatant
desalted on
a Sephadex G 25
column

floating lipid
droplets removed
and washed

(All procedures carried out at 0 to 4°C)

The 105,000 x g supernatant, which was used for the majority
of the experiments described in the subsequent chapters,
had a floating lipid layer (Claesson (1954)). This lipid
layer which contained the intra-cellular lipid droplets
was carefully removed with a syringe, resuspended in 0.25 M
sucrose and recentrifuged at 105,000 x g for 20 minutes.
The optically clear infranatant obtained from the first
105,000 x g centrifugation was then carefully decanted from
the microsomal pellet and desalted on a Sephadex G-25 column
with the required buffer.

In experiments where only the infranatant was required
a simplified centrifugation procedure was employed and this
is shown in Figure 4.1.

Determination of cholesterol ester hydrolase activity

The assay based on the method of Chen and Morin (1971)
and adapted by Trzeciak and Boyd (1973) was conducted in
a final volume of 1ml with 0.1 to 4.0 mgs of protein per
assay. The reaction mixture contained 30mM potassium chlor-
ide, 20mM potassium phosphate or Tris-HCl buffer pH 7.4
and $30\mu M$ [$4-^{14}C$] cholesterol oleate (75,000-100,000 counts
per minute) added to 10 μl of acetone. Other additions
were made as indicated in the text. The reactions were
initiated by the addition of the substrate and the incubat-
ions were carried out at $37^{o}C$ in air with shaking in a
water bath for 30 minutes. A control incubation containing
1ml of the boiled enzyme was also carried out at the same time.
The incubations were terminated by the addition of 4mls of
a 1:1 (v/v) acetone-ethanol mixture. The precipitated
protein was removed by centrifugation and 2.5 mls of the
supernatant was taken for the determination of [$4-^{14}C$]

cholesterol by the method of Sperry and Webb (1950). Carrier
cholesterol (0.2mgs in 0.5mls of acetone) was added to the
2.5mls of supernatant and 2.0mls of a 0.5% digitonin solut-
ion (5gms in 1 litre of 9:10 (v/v) ethanol-water) was added
to precipitate the free cholesterol. The cholesterol digit-
onide precipitate was centrifuged and the supernatant
discarded by suction. The precipitate was then washed once
with 5mls acetone:ether 1:1 (v/v) and once with 5mls of
ether. The washed precipitate was then dissolved in 1ml
of methanol and 0.5mls was taken for counting on a Packard
Tri Carb liquid scintillation spectrometer. A scintillation
liquid containing 4gms 2',5'-diphenyloxazole (PPO), and
0.03gms 1',4'-bis 5 phenyloxazoly-2-benzene (POPOP) and
5% (v/v) methanol per litre of dry toluene was employed
and under these conditions a counting efficiency of 72%
for $[^{14}C]$ was obtained.

Standards containing $[4-^{14}C]$ cholesterol were processed
as above by precipitation, washing and counting. Using
this method it was found that 80% to 90% of the free
cholesterol was recovered (Table 2.1). Cholesterol ester
hydrolase activity was expressed as (pmol x min^{-1} x mg
protein^{-1}).

Determination of protein kinase activity

Protein kinase activity was determined by the method
of Walsh et al. (1968) as modified by Gill and Garren (1971).
The assay system consisted of 50mM sodium glycero-3-phosphate
pH 6.5, 10mM $MgCl_2$, 4mM sodium fluoride, 2mM theophylline,
histone 0.5 mg per ml and 25μM $[\gamma-^{32}P]$ ATP sodium salt
(approx. 10^7 counts per assay). The final volume of the

[$4-^{14}C$] CHOLESTEROL ADDED (c.p.m.)	[$4-^{14}C$] CHOLESTEROL RECOVERED (c.p.m.)	[$4-^{14}C$] CHOLESTEROL RECOVERED %
12,960	11,386	88
15,493	16,003	100
85,765	78,032	91
17,601	15,101	86
26,189	22,400	86
25,865	23,231	90

Table 2.1 The precipitation of cholesterol by digitonin
Experimental procedure as the text. Results are expressed
as the average of two assays

incubation mixture was 0.2mls. The incubations were conducted
for 20 minutes at 30°C along with a control which contained
no histone. The reaction was terminated by the addition
of 2mls of ice cold 10% trichloroacetic acid which precipit-
ated the protein. The protein was isolated by centrifugation
and redissolved in 0.2mls of 1.0 sodium hydroxide. The
protein was reprecipitated by the addition of 2mls of ice
cold 10% trichloroacetic acid and the isolated precipitate
washed once with 10% trichloroacetic acid. The washed
precipitate was then redissolved in 0.2mls of 23 M formic
acid and 0.1mls taken for counting (De Lange et al. (1968).
Counting was carried out on a Packard Tri Carb Liquid
scintillation counter in a dioxane based scintillation
fluid (Bray (1960)).

Estimation of [^{32}P] radioactivity in protein fractions from Sephadex-G200 and Sepharose 4B columns

To 1 ml samples of the fractions from the Sephadex
G-200 and Sepharose 4B columns 1mg of bovine serum albumin
was added in 50 μl of 20mM potassium phosphate buffer pH 7.4.
To each sample was then added 2mls of ice cold 10% trichloro-
acetic acid. The protein precipitate was then again
centrifuged and washed with 10% trichloroacetic the protein
pellet was dissolved in 23 M formic acid and an aliquot
taken for counting in a Packard Tri Carb liquid scintillation
spectrometer using a dioxone based scintillation fluid
(Bray (1966).

Determination of the fatty acid composition of the cholesterol esters in lipid droplets

Steroids were extracted from the lipid droplets
according to the method of Folch and Sloane-Stanley (1957).

The lipid droplets were boiled for 2 minutes with 5mls of
2:1 (v/v) chloroform-methanol and 1ml of water was then
added and the mixture shaken. The aqueous phase was
removed and the precipitated protein removed by centrifugation.
The chloroform extract was then decanted, and the solvent
evaporated under nitrogen, and the residue redissolved in
a small quantity of chloroform (0.3mls) with 15 n moles of
$[4-^{14}C]$ cholesterol oleate added. To separate the steroids
the organic extract was subjected to thin layer chromatography
on a glass plate (20cm x 5cm) coated with silica gel H.
After the organic extract had been applied to the thin layer
plate, a further two washes of 0.1mls of chloroform were
used to transfer the extract to the thin layer plate. The
plates were developed by running them in a solvent consisting
of petroleum ether $60^{\circ}/80^{\circ}C$ boiling fraction, diethyl ether
and glacial acetic acid in the ratio 75:24:1 by volume.
This solvent system has been shown to separate the lipid
extract into its principal constituents of cholesterol, tri-
glycerides and phospholipids (Boyd and Trzeciak (1973)).
The plates were then scanned with a Panax radioactive thin
layer scanner to localise the radioactive peak of $[4-^{14}C]$
cholesterol oleate. The located cholesterol ester fraction
was then scraped from the plate and the cholesterol esters
extracted by washing the powder three times with chloroform
and the chloroform extract was evaporated to dryness in a
water bath under nitrogen.

To each 10mgs of cholesterol ester recovered 2mls of
40% methanolic KOH was added and the solution was refluxed
for 15 minutes to completely hydrolyse the esters. After

this time 2mls of water was added. The solution was then acidified with HCl and extracted twice with 4mls of petroleum ether. The extracts were combined and washed . twice with 4mls of water and the petroleum ether extract dried over anhydrous sodium sulphate.

The petroleum ether extract was then taken to dryness under nitrogen and 2 mls of a Boron trifluoride/methanol complex was added and heated for 5 minutes at $60^{\circ}C$. After this time 2mls of water was added to the sample and the solution extracted three times with petroleum ether and this extract was washed as before (Klopfenstein (1971)).

The composition of the fatty acids isolated from the cholesterol esters of the adrenal lipid droplets by this procedure were then determined by gas liquid chromatography on 12.5% diethylene glycol adipate on a chromosorb W support at $180^{\circ}C$, using a Pye-104 gas liquid chromatograph and a flame ionisation detector.

The separated fatty acids were identified by running standards and the percentage of each fatty acid in the lipid droplets was determined by measuring the area of the peaks

Determination of cholesterol and cholesterol ester concentration in delipidated 105,000 x g supernatant and the lipid droplets

The extraction and chromatography of cholesterol and cholesterol esters was carried out as described in the previous section for the determination of fatty acids. A chromatography solvent of di-isopropyl ether, petroleum ether $60^{\circ}/80^{\circ}C$ boiling fraction and glacial acetic acid in the ratio of 70:30:2 by volume was employed to separate cholesterol from its esters. Prior to the extraction a

known amount of $[7\alpha^3H]$cholesterol and $[4-^{14}C]$ cholesterol

oleate was added to correct for losses of the steroids

during extraction procedures.

When the steroids had been extracted and separated by

thin layer chromatography and the plates scanned for radio-

activity, the areas containing the cholesterol and cholesterol

esters were scraped into separate tubes and the steroids

extracted with chloroform. The cholesterol extract was

taken to dryness and redissolved in 2.2mls of acetone; 0.2mls

of this was then taken for the determination of radioactivity

and thus the recovery of the cholesterol was determined.

The cholesterol in the remainder of the sample was determined

by gas liquid chromatography on a Pye 104 gas chromatograph

using a 5 foot column containing 1% SE-30 Gas Chrom Q100/120

as a support. The column was operated at 245°C and the

chromatograph used a flame ionisation detector. Pregnenolone

acetate was added to the cholesterol sample to act as an

internal standard to correct for losses on gas liquid

chromatography (J. Arthur (1975)). The actual amounts of

cholesterol could be determined by the peak height ratios

from the gas liquid chromatography.

The cholesterol ester fraction was hydrolysed using

40% methanolic KOH as described in the previous section

for the determination of fatty acids. After hydrolysis the

petroleum ether extract was subjected to thin layer chromato-

graphy using the solvent system described previously in

this section. The free cholesterol was then eluted from

the silica gel with 2 x 5mls of chloroform and the combined

extracts taken to dryness under nitrogen. The cholesterol

was then redissolved in 2.2mls of acetone and 0.2mls of
this was used to determine the recovery of the steroid
through the extraction and hydrolysis procedure. This
was generally 55%-65%. The cholesterol content was then
determined by gas liquid chromatography as described
previously in this section.

In some experiments cholesterol and cholesterol esters
were determined using a modified Lieberman Burchard technique
(Kabara (1954)).

Determination of plasma corticosteroids

Measurement of plasma corticosterone in the rat plasma
samples was carried out by the method of Mattingly (1962)
using corticosterone standards.

Protein determinations

Protein concentrations were determined by the method
of Lowry et al. (1951) with bovine serum albumin as a standard.

Disc gel polyacrylamide electrophoresis

Electrophoresis of the native protein was carried out
on 5% polyacrylamide gels (approx. 8cm) at $4^{o}C$ as described
by Maizel (1973) using a high pH discontinuous gel system.
Gels were run at 2 mA per gel until the bromophenol blue
dye marker had reached the end of the gel.

After electrophoresis gels were stained for protein
by the method of Chrombach et al. (1967) using 0.05%
Coomasie Brilliant Blue in 12% trichloroacetic acid.
Destaining was carried out in 7% acetic acid.

Staining of the gel for esterase activity was conducted
by the method of Gomori (1952) using αnapthol propionate
as substrate with Fast blue RR.

After staining the gels were scanned on a Gilford gel scanner at 540 nm.

Electrophoresis of the proteins on an SDS gel system was conducted according to the method of Dunker and Rueckerts (1969) on a 10% gel containing 0.1% SDS. The protein samples were prepared by dissolving them in 1% 2-mercapto-ethanol (v/v), 4 M urea and 1% SDS and then heating them at $100^{\circ}C$ for 1 minute. Protein standards of γ-globulin, ovalbumin, bovine serum albumin and myoglobin were also run. Electrophoresis was carried out at room temperature at 2 mA per gel until the dye marker of bromo-phenol blue had reached the end of the gel. The gels were then soaked for 12 to 18 hours in 50% trichloroacetic acid and stained for 20 minutes in a 1% solution of Coomasie Brilliant Blue in 50% trichloroacetic acid. Gels were destained in 7% acetic acid.

Materials

Chemicals and radiochemicals

ATP disodium salt; cyclic AMP; oleic acid (99% pure); erucic acid (99% pure); cholesterol oleate; histone type II, from calf thymus; bovine serum albumin; cholesterol; EDTA; α napthol propionate; Fast Blue RR; DEAE cellulose and GLC fatty acid standards were purchased from Sigma chemical Co. (St. Louis).

PPO; POPOP and cycloheximide were obtained from Koch-Light Laboratories, Colnbrook, Bucks and silica gel H from E. Merk., Darmstadt.

Pregnenolone acetate; Pregnant Mare Serum Gonadotrophin (Gestyl) and Human Chorionic Gonadotrophin (Pregnyl) were obtained from Organon.

Gas chromatography column packing was obtained from Applied Science Laboratories Inc. LH-S-18 was a gift from N.I.H. Bethesda, U.S.A. Olive oil was purchased locally and the rape seed oil was a gift from Dr. A. Vergroessen, Unilever Research, Vlaardingen, Holland.

All other chemicals and solvents were of analytical grade and purchased from British Drug Houses Ltd., Poole, England.

Sephadex G-25, G-200 and Sepharose 4B were purchased from Pharmacia Fine Chemicals, London. Biogel A 0.5 M and Hydroxylappatite were purchased from Bio Rad Laboratories Ltd., Bromley, Kent.

The sodium salt of adenosine $5'[\alpha-^{32}P]$ and adenosine $5'[\gamma-^{32}P]$ triphosphate (specific activity 500-3000 mCi per m mol, $[4-^{14}C]$ cholesterol (specific activity 55.6 mCi per m mol) and $[7\alpha-^{3}H]$ cholesterol (specific activity 9.4 mCi per m mol) were purchased from the Radiochemical Centre, Amersham.

$[4-^{14}C]$ cholesterol oleate and erucate were prepared by refluxing equimolar concentrations of $[4-^{14}C]$ cholesterol, fatty acid and dicyclohexyl-carbodiimide in dry benzene. The cholesterol esters produced were then purified by thin layer chromatography on Silica Gel G. (Merk, E., Darmstadt) using a di-isopropyl ether, petroleum ether $60^{o}/80^{o}C$ boiling fraction and acetic acid 70:30:2 (v/v) solvent system as were all the other radioactive steroids.

Enzymes and proteins

Cyclic AMP dependent protein kinase isoenzymes I and II were prepared by the method of Cohen (1973).

Cyclic AMP dependent protein kinase inhibitor was prepared from rabbit skeletal muscle by the method of Walsh et al. (1971) and phosphorylase kinase phosphatase was prepared by the method of Antoniw and Cohen (1975).

The kinases and phosphatases and protein kinase inhibitor were donated by Dr. P. Cohen of Dundee University.

Cyclic AMP dependent protein kinase from beef heart and cyclic AMP phosphodiesterase were obtained from Sigma Chemical Co. (St. Louis) as was the beef liver alkaline phosphatase type IV.

CONTENTS

CHAPTER 3

GENERAL PROPERTIES OF BOVINE ADRENAL CHOLESTEROL

ESTER HYDROLASE

Introduction

The cholesterol esters stored in lipid droplets found
in the cell cytoplasm provide cholesterol for steroidogenesis
in the adrenal cortex and corpus luteum (see chapter I).
Hydrolysis of these cholesterol esters liberates free
cholesterol and is effected by the enzyme-cholesterol ester
hydrolase. Cholesterol ester hydrolase activity has been
detected in the adrenal (Dailey et al. (1963)), corpus
luteum (Behrman and Armstrong (1969)), brain (Pritchard and
Nicholson (1964)), placenta (Chen and Morrin (1970)),
pancreas (Vahouney et al. (1965)), liver (Deykin and Goodman
(1962)) and aorta (Kritchevsky and Himanshu (1973)).

Injections of ACTH (Behrman and Greep (1972)), or
ether anaesthesia stress (Trzeciak and Boyd (1973)), which
is known to increase plasma ACTH levels (Matsayuma et al.(1971)),
significantly increased the activity of cholesterol ester
hydrolase in the rat adrenal gland. Haynes (1958) has
shown that cyclic AMP accumulated in the adrenal gland in
response to a stimulation by ACTH. He proposed that cyclic
AMP was the obligatory intermediate in the ACTH induced
rise in corticosteroidogenesis in the adrenal gland.
Boyd and Trzeciak (1973) have shown that upon the induction
of ether anaesthesia there is an immediate rise in the
concentration of rat adrenal cyclic AMP.

Additions in vitro of cyclic AMP per se will not
stimulate cholesterol ester hydrolase activity in the rat

adrenal 105,000 x g supernatant, however it has been shown that in the presence of ATP cyclic AMP could stimulate cholesterol ester hydrolase activity in the 105,000 x g supernatant (Trzeciak and Boyd (1973)). A similar activation of cholesterol ester hydrolase by ATP and cyclic AMP has been shown in the bovine cortical 105,000 x g supernatant (Trzeciak and Boyd (1974)).

The method for the determination of cholesterol ester hydrolase activity is described in chapter 2. The material presented in this chapter is concerned with preliminary investigations into the bovine adrenal cortex cytosol cholesterol ester hydrolase. The subcellular distribution and the optimum incubation conditions for measuring cholesterol ester hydrolase activity in the bovine adrenal cortical 105,000 x g supernatant are investigated and discussed.

The effect of metal ions and nucleotides on cholesterol ester hydrolase activity and the general properties of the bovine adrenal cortical 105,000 x g cholesterol ester hydrolase are also considered.

Subcellular distribution of cholesterol ester hydrolase activity in the bovine adrenal cortex.

It has been reported that most of cholesterol ester hydrolase activity in the rat liver (Deykin and Goodman (1962)), human placenta (Chen and Morrin (1971)) and the rat adrenal cell (Trzeciak and Boyd (1973)) is located in the 105,000 x g supernatant. However in the rat corpus luteum conflicting reports have been published. Coutts and Stansfield (1968) have reported that cholesterol ester hydrolase activity resides in the 5,000 x g pellet of the luteal homogenate, while

Behrman and Armstrong (1969) have reported a similar distri-
bution in the corpus luteum to that found in the rat adrenal.
Naghshineh et al. (1974) have reported that most of the
cholesterol ester hydrolase in the bovine adrenal cortex
occurs in the 105,000 x g pellet. The subcellular distri-
bution of cholesterol ester hydrolase in the bovine adrenal
cortex was therefore studied. Subcellular fractions were
prepared from bovine adrenal cortex as described in Figure 2.1.
Each fraction was washed and assayed for cholesterol ester
hydrolase activity as described in chapter 2.

The results are summarised in table 3.1. Under the
assay conditions specified in table 3.1 most of the cholest-
erol ester hydrolase activity was located in the 105,000 x g
fraction (65%). The mitochondrial and microsomal fractions
however contained respectively 20% and 14% of the total
activity found in the homogenate.

These results are in conflict with those of Naghshineh et al.
(1974) who reported the distribution of cholesterol ester
hydrolase in the bovine cortical cell mitochondria, micro-
somes and 105,000 x g supernatant as being 23.5%, 36.6%,
and 26.5% respectively.

The work contained in this thesis has concentrated on
the cholesterol ester hydrolase found in the 105,000 x g
cortical supernatant.

The general properties of bovine adrenal cortical 105,000 x g
supernatant

Cholesterol ester hydrolase activity was determined
by the method of Trzeciak and Boyd (1974) (see chapter 2).
After centrifugation at 105,000 x g for an hour the floating

SUBCELLULAR FRACTION	CHOLESTEROL ESTER HYDROLASE % of the total activity
105,000 x g SUPERNATANT	65
MITOCHONDRIA	20
MICROSOMES	14
OTHER CELLULAR BODIES	1

Table 3.1 The subcellular distribution of bovine adrenal cholesterol ester hydrolase.

Subcellular fractionation was carried out as described in Figure 2.1 and cholesterol ester hydrolase activity was determined as described in chapter 2.

lipid layer was carefully removed and the clear infranatant
was desalted by passage through a Sephadex G-25 column
equilibrated with 20mM potassium phosphate buffer pH 7.4
(see chapter 2). This procedure was carried out on the
supernatant fractions used in all the experiments reported
in this chapter, except where otherwise stated.

Cholesterol ester concentration in the delipidated
105,000 x g supernatant fraction did not exceed 12μ g per mg
protein. This value was relatively constant and showed
little variation among different preparations of bovine
adrenal cortex.

Free or unesterified cholesterol was found at a concen-
tration of 10μ g per mg protein. Cholesterol and cholesterol
esters were measured by the Liebermann-Burchardt technique
as described in chapter 2.

The cholesterol ester hydrolase in the crude 105,000 x g
supernatant was stable for 4 to 7 days at 0°C or 1 to 2
months when frozen.

The rate of production of cholesterol from cholesterol oleate
in bovine adrenal cortical supernatant.

The rate of hydrolysis of cholesterol oleate in bovine
adrenal cortical 105,000 x g supernatant is shown in Figure 2.1.
The reaction was initiated by the addition of [4-^{14}C] cholest-
erol oleate at a concentration of 30μ M. Aliquots of 0.5mls
were taken into acetone-ethanol 1:1 at the time intervals
shown and the amount of [4-^{14}C] cholesterol produced was
measured as described in chapter 2. The protein concentra-
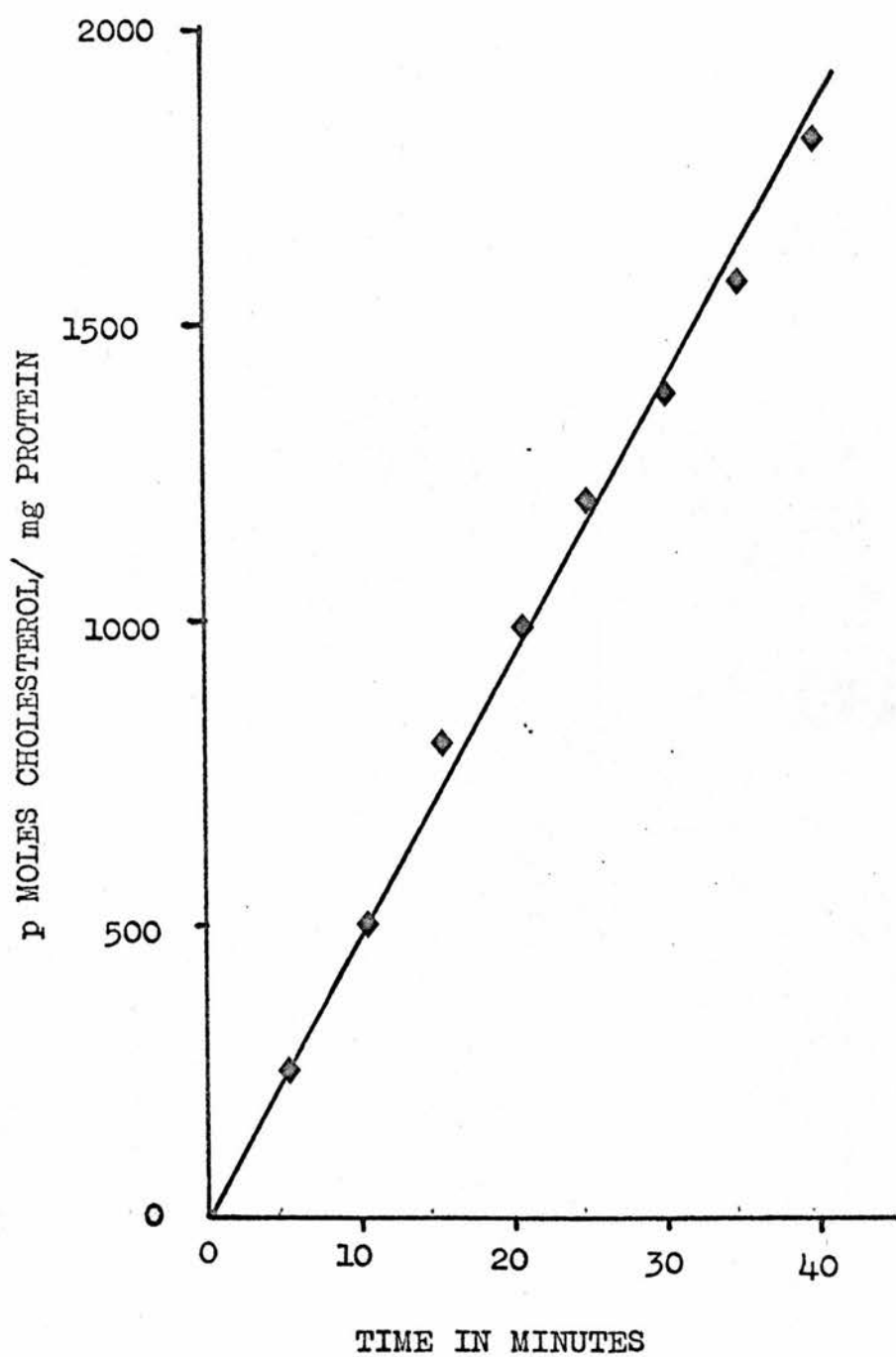tion in the incubations was 2mg per ml.

Figure 3.1 The rate of production of cholesterol
from cholesterol oleate in bovine cortical supernatant.
Samples (0.5 mls) were taken at the times indicated
and assayed for [^{14}C] cholesterol as described in
chapter 2.

It can be seen from Figure 3.1 that the rate of hydroly-
sis of cholesterol oleate was linear over the 40 minutes
of incubation.

The effect of pH on cholesterol ester hydrolase activity in bovine adrenal 105,000 x g supernatant

105,000 x supernatant was prepared in the usual way
(see chapter 2) and desalted by passage through a Sephadex
G-25 column equilibrated with 5mM potassium phosphate buffer
pH 7.4. Potassium phosphate 1.0 M or triethanolamine 1.0 M
buffer at the required pH was then added to the desalted
supernatant to obtain a concentration of 0.1 M and the pH
of the solution was checked after the addition of the buffer
was used over the range pH 6.4 to pH 7.6 and triethanolamine
over the range pH 7.6 to 8.7.

The results of the pH/activity experiment are shown
in Figure 3.2. Cholesterol ester hydrolase was found to
have a broad pH optimum between pH 7.3 and 7.8. Incubations
in subsequent experiments were carried out at pH 7.4. The
pH curve did not appear to be symmetrical. Cholesterol
ester hydrolase activity decreased more rapidly at high pH
values than at low pH values; the reason for this however
is unclear.

The effect of increasing concentration of cholesterol oleate on cholesterol ester hydrolase activity in bovine adrenal cortical supernatant.

The effect of increasing the concentration of the sub-
strate cholesterol oleate on the cholesterol ester hydrolase
activity is shown in Figure 3.3. Incubations were carried
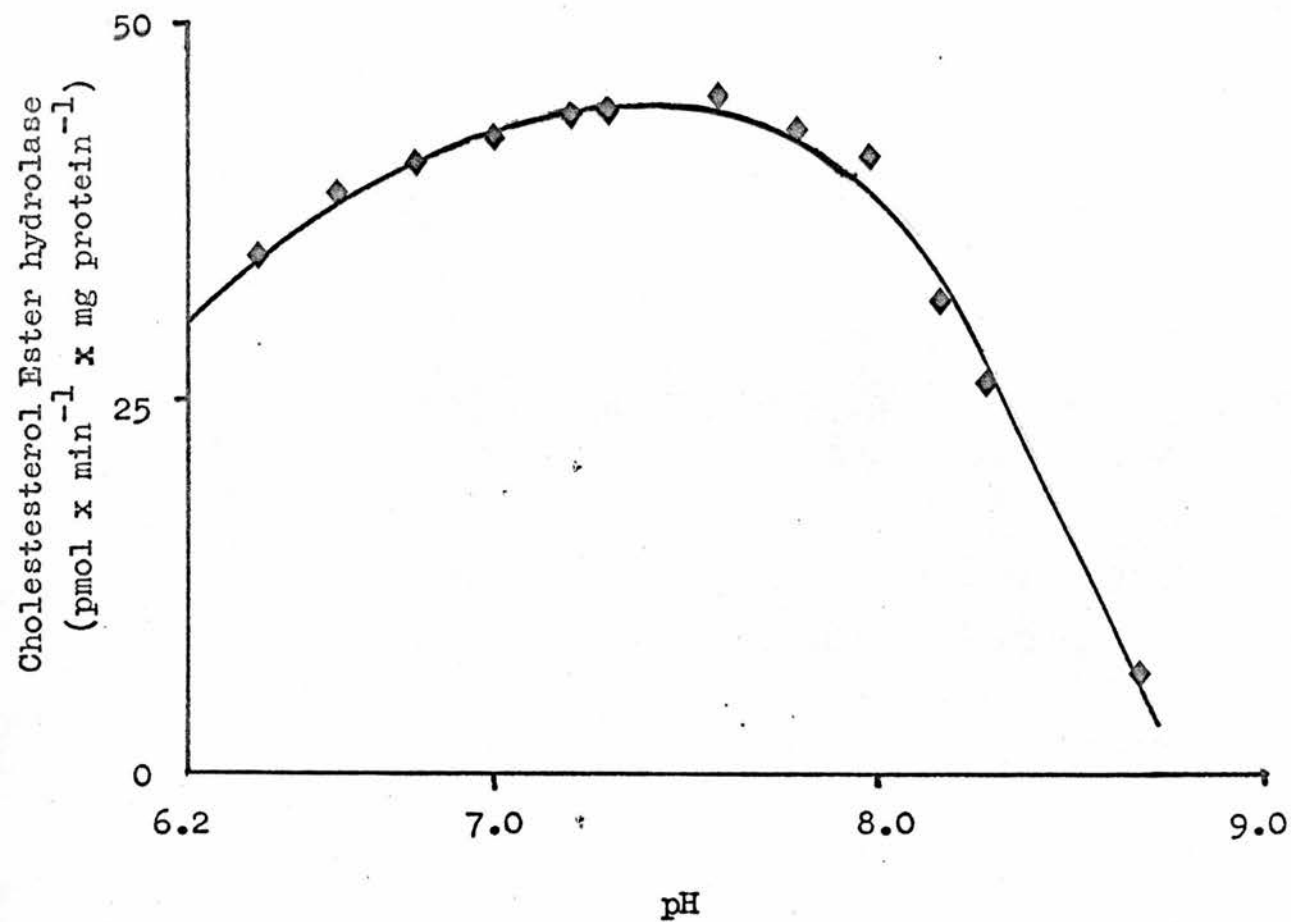out on desalted supernatant in the presence of 20mM

Figure 3.2 The effect of pH on cholesterol ester

hydrolase activity in bovine adrenal cortical

supernatant. Assay for cholesterol ester hydrolase

was carried out in a final volume of 1 ml as

described in chapter 2.

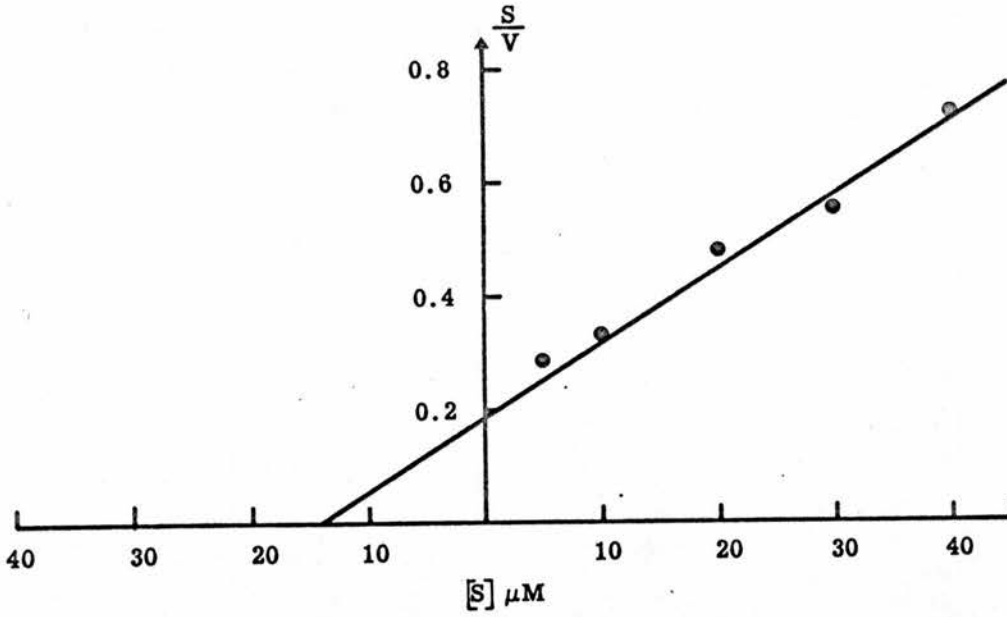$\frac{S}{V}$ v/s S PLOT FOR BOVINE ADRENAL
CHOLESTEROL ESTER HYDROLASE

Figure 3.3   The effect of increasing concentration

of cholesterol oleate on cholesterol ester hydrolase

activity in bovine adrenal cortical supernatant.

Cholesterol ester hydrolase activity was determined

as is described in chapter 2 in the presence of

increasing concentrations of cholesterol oleate as

indicated above and in the text.

potassium phosphate buffer pH 7.4. $[4-^{14}C]$cholesterol oleate was added at increasing concentrations to 1 ml incubations in 10 μl of acetone. The total number of counts added at each concentration was constant; the amount of "cold" cholesterol oleate was altered. Cholesterol ester hydrolase activity was determined as is described in chapter 2.

It can be seen from Figure 3.3 that if S v/s $\frac{S}{V}$ is plotted (Hanes (1932)) for the bovine adrenal cortical cholesterol ester hydrolase a Km value of 14.3μ M for cholesterol oleate is given. This compares with the Km value of 13.9μ M found for the rat adrenal 105,000 x g supernatant cholesterol ester hydrolase determined by the same method in chapter 7.

The effect on cholesterol ester hydrolase activity in bovine adrenal cortical 105,000 x g supernatant with varying concentrations of:-

Divalent cations

There are few reports on the effect of divalent cations on cholesterol ester hydrolase activity in the bovine adrenal cortex. Trzeciak and Boyd (1974) have reported that preincubation of desalted bovine adrenal cortical supernatant with 5mM $MgCl_2$ resulted in a decrease in cholesterol ester hydrolase activity. The effect of magnesium and calcium concentration on cholesterol ester hydrolase activity in the bovine adrenal cortical supernatant was therefore investigated.

Figure 3.4 shows the effect of magnesium and calcium ion concentrations on cholesterol ester hydrolase activity.
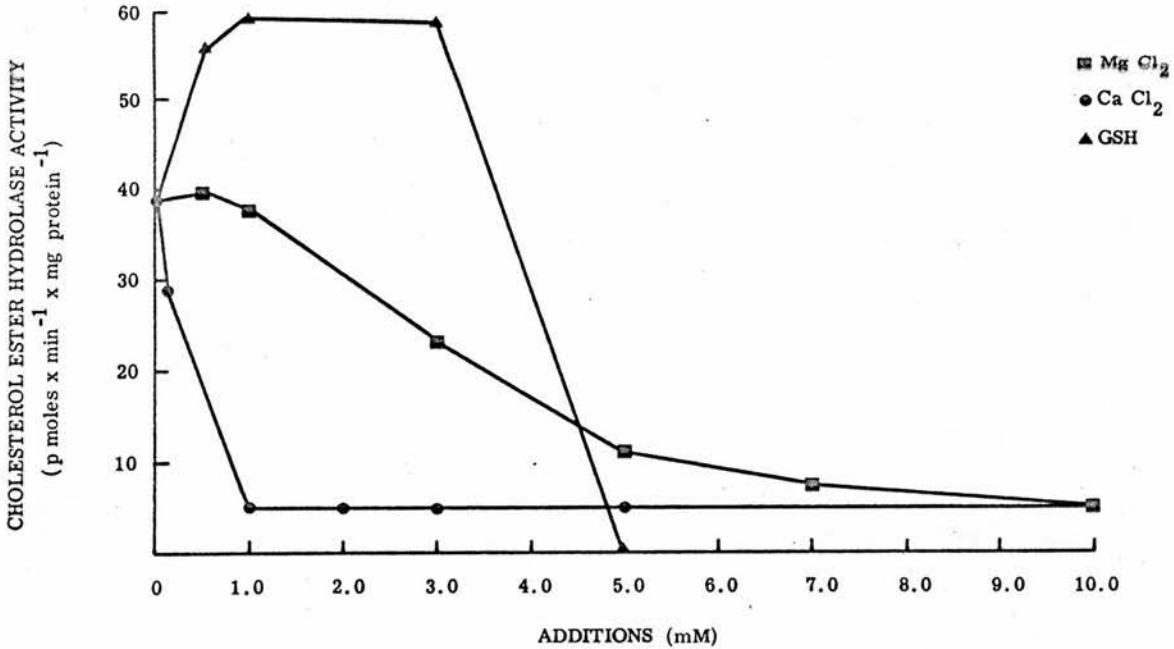
Figure 3.4   The effect of magnesium, calcium and

glutathione addition on cholesterol ester hydrolase

activity in bovine cortical supernatant.

Cholesterol ester hydrolase activity was determined

as described in chapter 2 in the presence of $MgCl_2$,

$CaCl_2$ and glutathione at the concentrations shown

in the Figure.

$MgCl_2$ and $CaCl_2$ were added at concentrations ranging from 0 to 10mM and cholesterol ester hydrolase activity determined. $MgCl_2$ added at concentrations of 1mM or less had no effect on cholesterol ester hydrolase activity. At higher concentrations however $MgCl_2$ became increasingly inhibitory until at a concentration of 10mM cholesterol ester hydrolase activity was only 12% of its original activity. As will be shown later in this chapter magnesium ions are required for the activation of cholesterol ester hydrolase by ATP and cyclic AMP. For this reason 5mM $MgCl_2$ was added to incubations in experiments where an activation of cholesterol ester hydrolase by ATP and cyclic AMP was required.

The inhibitory effect of $CaCl_2$ added to a desalted supernatant at concentrations as low as 0.2mM was demonstrated and at 1.0mM only 12% of the original cholesterol ester hydrolase activity was retained. For this reason $CaCl_2$ was not included in the incubation medium for cholesterol ester hydrolase assay.

Ascorbic acid

There is in the adrenal gland a large quantity of ascorbic acid (Sayers et al. (1946)). It was thought therefore that ascorbic acid may have a direct effect on the activity of cholesterol ester hydrolase. Ascorbic acid was added to a desalted supernatant at concentrations up to 10mM. It can be seen from Fig. 3.5 that ascorbic acid is slightly stimulatory at concentrations up to 3mM after which it inhibits cholesterol ester hydrolase activity.
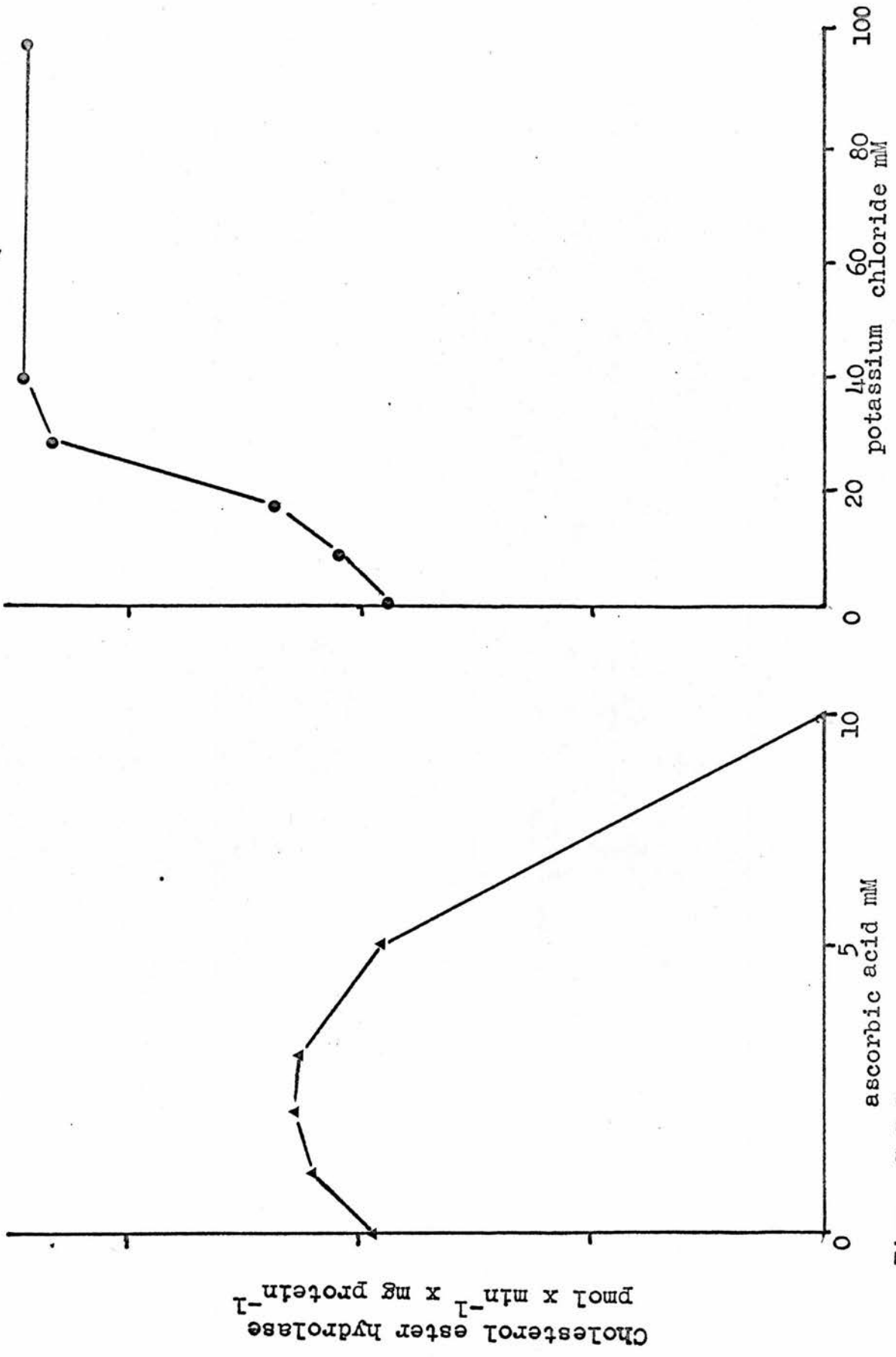
Figure 3.5 The effect of ascorbic acid and potassium chloride addition on cholesterol ester hydrolase activity in bovine adrenal cortical supernatant.

Potassium chloride

When potassium chloride was added at concentrations
up to 0.1 M cholesterol ester hydrolase activity was stim-
ulated. (Fig. 3.5). For this reason KCl was added to
cholesterol ester hydrolase assays at a concentration of 30mM.

Glutathione

The adrenal is rich in glutathione and is known to
contain as much as 204mg/100gm wet weight (Bonerjee et al.
(1955)). The effect of glutathione addition on cholesterol
ester hydrolase activity was therefore investigated.

It can be seen from Fig. 3.4 that glutathione caused
a marked stimulation of cholesterol ester hydrolase at
concentrations up to 3mM. At a concentration of 5mM glut-
athione completely inhibited cholesterol ester hydrolase
activity. Glutathione however was not generally added to
incubations of cholesterol ester hydrolase. The reason
for the omission of glutathione will be shown later in
this chapter because glutathione appears to inhibit the
activation of cholesterol ester hydrolase by ATP and cyclic AMP.

Stimulation of cholesterol ester hydrolase activity by ATP and cyclic AMP addition.

It has been reported by Boyd and Trzeciak (1973) that
addition of cyclic AMP and ATP in vitro to rat adrenal super-
natant significantly stimulated cholesterol ester hydrolase
activity. In a later publication (Trzeciak and Boyd (1973))
it was shown that cholesterol ester hydrolase in the rat
adrenal supernatant was not stimulated by cyclic AMP added
per se. The addition of 5mM ATP alone caused a 15% stimula-
tion of cholesterol ester hydrolase. When 5mM ATP was added

together with 0.1 μM cyclic AMP a 40% stimulation of cholesterol ester hydrolase activity was observed. In all their incubations 5mM $MgCl_2$ was added.

Trzeciak and Boyd (1974) have reported a similar stimulation of cholesterol ester hydrolase by dibutyryl cyclic AMP and ATP in bovine adrenal cortical supernatant. Naghshineh et al. (1974), have recently confirmed the results of Trzeciak and Boyd (1974), however in their experiments the addition of ATP and $MgCl_2$ without dibutyryl cyclic AMP addition gave no stimulation of the enzyme.

Figure 3.6 shows the effect of cyclic AMP and ATP addition on cholesterol ester hydrolase activity in the bovine cortical supernatant. Incubations were carried out as described in chapter 2 with additions of 10 μM cyclic AMP, 5mM ATP and 5mM $MgCl_2$ as indicated in Figure 3.6. It can be seen that 5mM $MgCl_2$ addition caused an inhibition of cholesterol ester hydrolase activity. The addition of 10 μM cyclic AMP had no significant effect on cholesterol ester hydrolase activity. When 5mM ATP was added to bovine cortical supernatant with 5mM $MgCl_2$ cholesterol ester hydrolase activity was markedly stimulated. The addition of 10 μM cyclic AMP with 5mM ATP and 5mM $MgCl_2$ produced an even greater stimulation.

It should be noted that in some experiments, it was found that 5mM ATP and 5mM $MgCl_2$ addition to the 105,000 x g supernatant in the absence of cyclic AMP also gave a marked stimulation of cholesterol ester hydrolase activity. Cyclic AMP added to these preparations with ATP and $MgCl_2$ could not further stimulate cholesterol ester hydrolase.
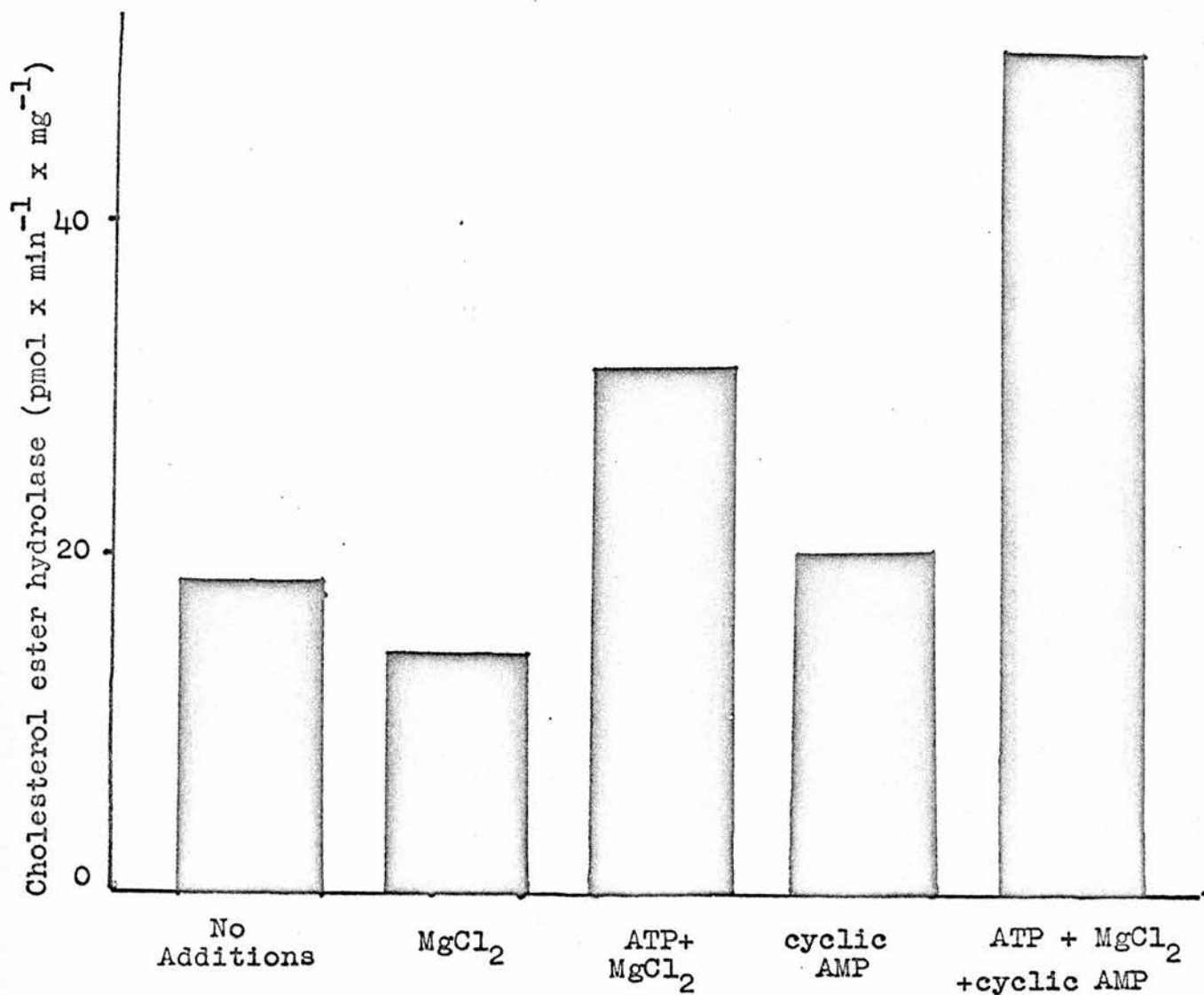
Figure 3.6 The stimulation of cholesterol ester hydrolase in bovine adrenal cortical supernatant, by ATP, cyclic AMP and magnesium addition.

Cholesterol ester hydrolase activity was determined as described in chapter 2 with the addition of 5 mM ATP, 10 μM Cyclic AMP and 5 mM magnesium as indicated in the Figure. Incubations were carried out in duplicate in a final volume of 1 ml.

The inability of cyclic AMP to stimulate cholesterol ester hydrolase activity in some preparations will be discussed in chapter 10.

The requirement for Magnesium ions for the activation of cholesterol ester hydrolase by ATP and cyclic AMP.

Activation of cholesterol ester hydrolase by ATP and cyclic AMP required 5mM $MgCl_2$ in the incubation mixture. It can be seen from Table 3.2 that if ATP and cyclic AMP are added without $MgCl_2$ a much lower stimulation of cholesterol ester hydrolase is produced. Again it can be seen that $MgCl_2$ added alone inhibited cholesterol ester hydrolase.

It is not surprising that magnesium ions are required for the activation of cholesterol ester hydrolase if as postulated by Trzeciak and Boyd (1974) a protein kinase is involved. ATP is known to exist in solution and especially in biological fluids as a mixture of variously ionised, metal complexed and structurally conformed species (Hers (1952)); (Neuberg and Mandl (1949)) and (Spicer (1952)). It has been found in studies on the mechanism of action of creatine kinase that a $MgATP^{2-}$ complex is the substrate for this enzyme and not ATP (Noda et al. (1960)). Pyruvate kinase has been shown to utilise $MgADP^-$ as a substrate (Melchior (1965)).

The effect of cyclic AMP concentration on cholesterol ester hydrolase activity in bovine adrenal cortical supernatant.

Incubations of bovine cortical supernatant in the presence of ATP and cyclic AMP described earlier in this chapter have used cyclic AMP concentrations of $10 \mu M$. This is in contrast to Trzeciak and Boyd (1974) who used $0.1 \mu M$ dibutyrl cyclic AMP.

Additions	Cholesterol Ester activity pmol x min^{-1} x mg protein^{-1}
none	20
5mM MgCl$_2$	16
10 μM cyclic AMP	20
5mM ATP+5mM MgCl$_2$	35
5mM ATP + 10 μM cyclic AMP	28
5mM ATP + 10 μM cyclic AMP + 5mM MgCl$_2$	45

Table 3.2 The requirement for magnesium ions for the activation of cholesterol ester hydrolase by ATP and cyclic AMP.

Incubations (1ml) were carried out as described in chapter 2 with the addition of ATP, cyclic AMP and MgCl$_2$ as shown in the Table.

The reason for the use of 10μ M cyclic AMP is shown
in Figure 3.7. Estimations of cholesterol ester hydrolase
activity were carried out in the presence of 5mM ATP and
5mM $MgCl_2$. Cyclic AMP concentrations were varied from
0.01μM to 100 μM. It was found that cholesterol ester
hydrolase activity increased with increasing concentration
of cyclic AMP up to 10μ M. A cyclic AMP concentration of
10 μM was therefore employed in experiments requiring the
addition of this compound. It is possible that the differ-
ences in the concentration of cyclic AMP required to give
maximal stimulation between the values shown in Figure 3.7
and the concentrations used by Trzeciak and Boyd (1974) are
concerned with the use by them of dibutyrl cyclic AMP and
not cyclic AMP.

The effects of reducing agents on cholesterol ester hydro-
lase and its stimulation by ATP and cyclic AMP.

Table 3.3 shows the effect of the addition of various
reducing agents in vitro on cholesterol ester hydrolase in
the desalted 35% saturated ammonium sulphate fraction from
cortical supernatant prepared as described in chapter 4.
The results show that while many of the reducing agents used
stimulated cholesterol ester hydrolase activity, the stimul-
ation produced by the addition of ATP with cyclic AMP was
not additive with the stimulation produced by the reducing
agents. For this reason reducing agents were not added to
cholesterol ester hydrolase incubations.

Polyacrylamide disc gel electrophoresis of bovine cortical
supernatant followed by esterase staining.

Cortical supernatant was subjected to polyacrylamide
disc gel electrophoresis on 5% gels as described in chapter 2.

EFFECT OF CYCLIC AMP CONCENTRATION ON
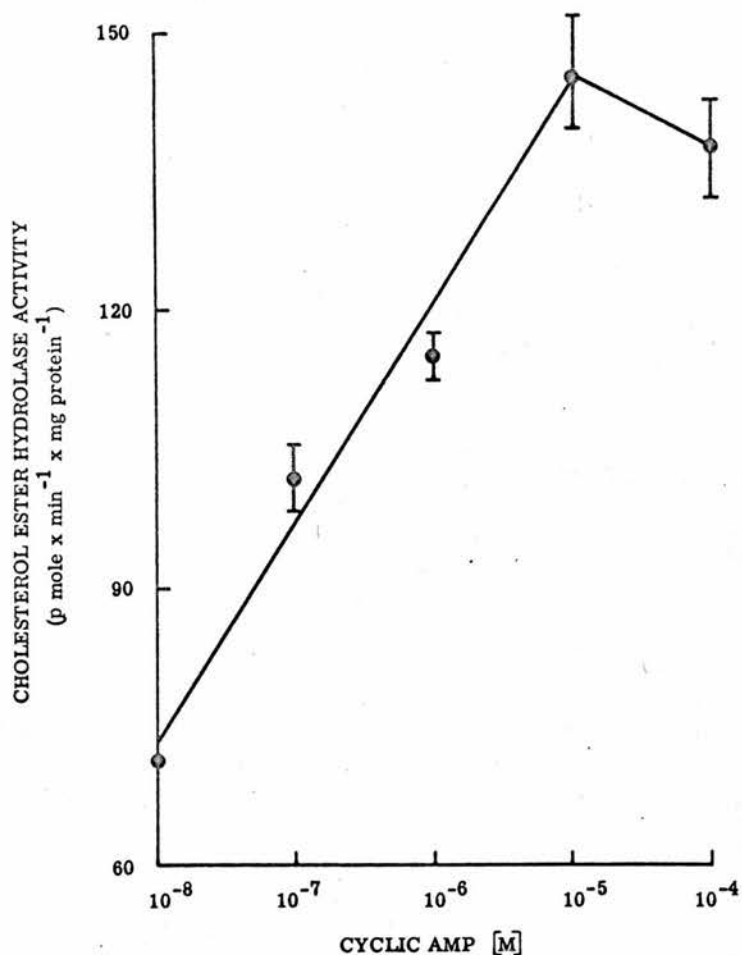CHOLESTEROL ESTER HYDROLASE ACTIVITY

Figure 3.7 The effect of cyclic AMP concentration
on cholesterol ester hydrolase activity in bovine adrenal
cortical supernatant. 1ml incubations were carried out
in duplicate as described in Chapter 2 in the presence
of 5mM ATP and 5mM $MgCl_2$ and increasing concentration
of cyclic AMP. The results are shown as the average of
the two incubations.

Additions	No Cofactors	5mM ATP + 10 μM cyclic AMP
	Cholesterol ester hydrolase pmol x min x mg protein	
None	84	98
1mM Ascorbic acid	90	97
1mM Glutathione	101	97
1mM Mercapto-ethanol	102	95
1mM Dithio-threitol	84	98

Table 3.3 The effects of reducing agents on cholesterol ester hydrolase and its stimulation by ATP and cyclic AMP Incubations (1ml) were carried out as described in chapter 2 with the additions indicated in the text. The incubations were carried out on an ammonium sulphate fraction of bovine adrenal cortical supernatant prepared as described in the text (Figure4.2)

After running at 2mA per gel until the dye marker had reached
the end of the gel, the gel was stained for esterase activity
using the histochemical method of Gomori (1952). Staining
for activity was carried out as is described in chapter 2
and the results are shown in Figure 3.8.

It can be seen that on staining for esterase activity
in bovine cortical supernatant at least 12 bands of esterase
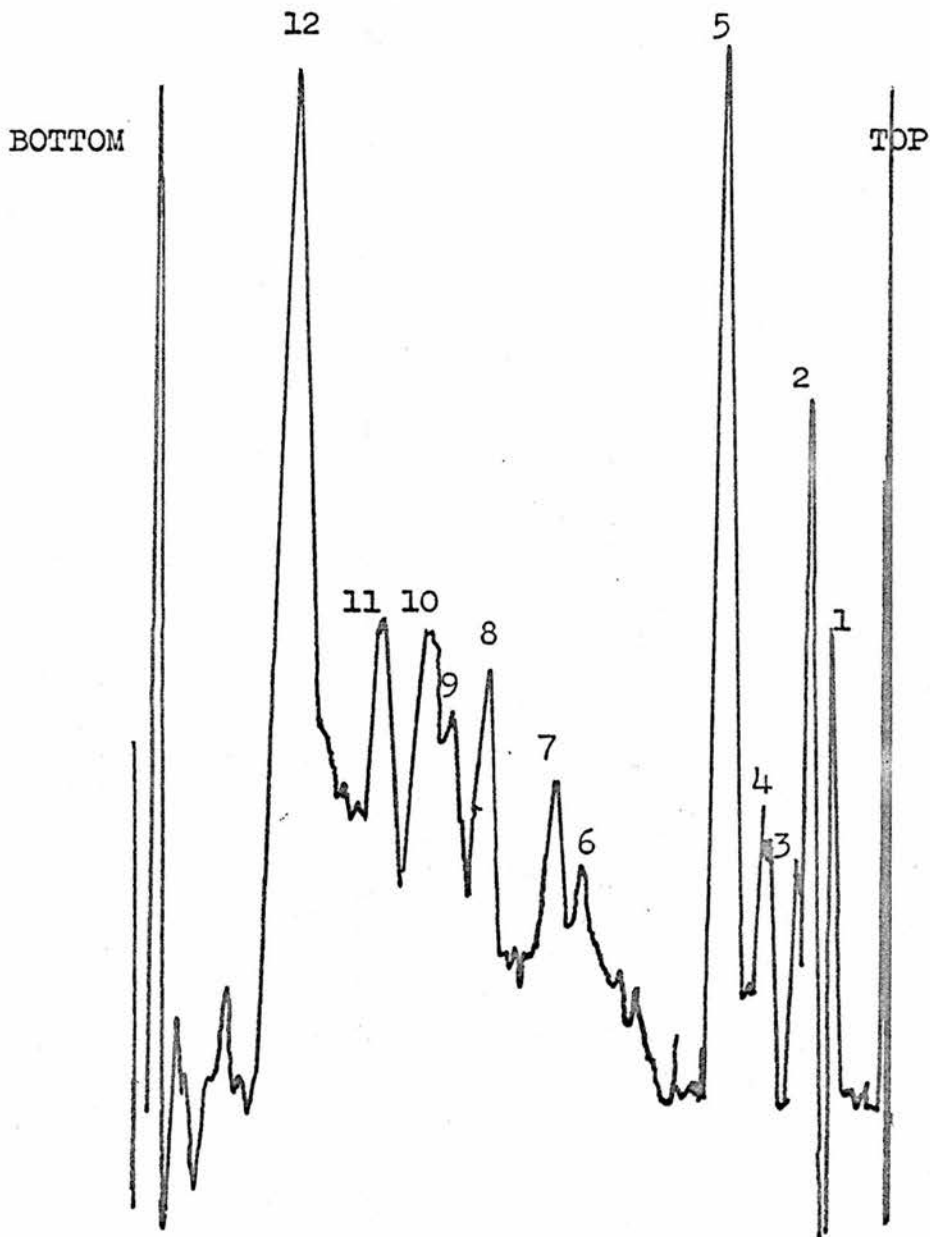activity were observed.

Figure 3.8 Esterase activity stain of bovine adrenal
cortical supernatant subjected to polyacrylamide gel
electrophoresis. Electrophoresis and staining was carried
out as described in chapter 2 and the gel scanned at
540nm. At least 12 bands of esterase activity were seen.

Summary

The general properties of bovine adrenal cholesterol ester hydrolase have been investigated with the following findings:-

a) Most of the cholesterol ester hydrolase activity was found in the 105,000 x g supernatant.

b) Cholesterol ester hydrolase activity was found to be directly proportional to the amount of protein present.

c) Under the conditions employed in the assay the rate of hydrolysis of cholesterol oleate was linear over 40 minutes of incubation.

d) Cholesterol ester hydrolase exhibited its maximum activity over the pH range 7.3 to 7.8.

e) The Km of cholesterol ester hydrolase was found to be 14.3 μM using cholesterol oleate as substrate.

f) Magnesium and calcium ions inhibited cholesterol ester hydrolase while ascorbic acid and glutathione at low concentrations stimulated the enzyme but inhibited at higher concentrations. Potassium chloride was found to stimulate cholesterol ester hydrolase at all the concentrations used.

g) Cholesterol ester hydrolase was stimulated by in vitro addition of ATP and cyclic AMP.

h) The optimal cyclic AMP concentration was found to be 10 μM.

i) Reducing agents stimulated cholesterol ester hydrolase activity but inhibited the activation of cholesterol ester hydrolase by ATP and cyclic AMP addition.

j) At least 12 proteins with esterase activity were found in bovine cortical 105,000 x g supernatant.

CONTENTS

CHAPTER 4

THE PURIFICATION AND PROPERTIES OF CHOLESTEROL ESTER

HYDROLASE FROM BOVINE ADRENAL CORTEX

Introduction

A partial purification of cholesterol ester hydrolase
from bovine adrenal cortex has been reported by Trzeciak and
Boyd (1974). Using a purification procedure involving
ammonium sulphate protein precipitation, calcium phosphate
gel adsorption and gel filtration Trzeciak and Boyd (1974)
obtained a seven fold purification of cholesterol ester
hydrolase. If the ammonium sulphate fraction was preincubated
with $[\gamma -^{32}P]$ ATP before the gel filtration step,
Trzeciak and Boyd (1974) found that the $[^{32}P]$ radioactivity
was associated with the fraction from the gel eluate
containing cholesterol ester hydrolase activity. It was
postulated from this evidence that phosphorylation of the
cholesterol ester hydrolase had taken place.

No other evidence has been presented to show that it
is cholesterol ester hydrolase which is phosphorylated in
this partially purified preparation, or that the $[^{32}P]$
radioactivity is covalently linked to the protein. It
cannot be excluded therefore that the phosphorylated
protein in the purified preparation of Trzeciak and Boyd (1974)
may be another protein which stimulates or plays a key
role in the hydrolysis of cholesterol esters.

Naghshineh et al. (1974) have reported a partial
purification of cholesterol ester hydrolase from bovine
adrenal cortex involving ammonium sulphate protein fraction-
ation and DEAE cellulose chromatography. From their

published results however it is not possible to determine
what degree of purification of cholesterol ester hydrolase
was obtained. No experiments involving the preincubation
of cholesterol ester hydrolase with $[\gamma-^{32}P]$ ATP followed
by purification have been reported by Naghshineh et al. (1974)
and no evidence for a phosphorylation of cholesterol ester
hydrolase has been presented by them.

This chapter presents two procedures for the purification
of cholesterol ester hydrolase from bovine adrenal cortex.
Experiments involving the preincubation of a crude ammonium
sulphate fraction with $[\gamma-^{32}P]$ ATP followed by purification
of cholesterol ester hydrolase are described. Evidence
is also presented for the phosphorylation of cholesterol
ester hydrolase by ATP. Some of the properties of purified
cholesterol ester hydrolase are reported in this chapter
and a comparison presented with the properties of the
enzyme in the 105,000 x g cortical supernatant.

Procedure A for the purification of cholesterol ester hydrolase from bovine adrenal cortex

The purification procedure for cholesterol ester
hydrolase from bovine adrenal cortex is summarised in
Figures 4.1, 4.2, 4.3. Using the purification procedure
to be described the specific activity of cholesterol
ester hydrolase was increased 57 fold.

a) Preparation of subcellular fractions

A 20% homogenate of bovine adrenal cortex was prepared
in 0.25 M sucrose as described in chapter 2.

Subcellular fractionation of the homogenate was then
carried out by the procedure summarised in Figure 4.1.

<u>Purification Procedure for Cholesterol Ester</u>
<u>Hydrolase from Bovine Adrenal Cortex</u>

Adrenal cut longitudinally
Medulla removed

↓

Cortex scraped off the capsule

↓

Cortex in 20 per cent homogenate with
ice-cold 0.25 M Sucrose

Cell debris ◄————————— Homogenate centrifuged
600 x g for 10 minutes

↓

Mitochondria ◄————————— Supernatant centrifuged
10,000×g for 15 minutes

↓

Microsomes ◄————————— Supernatant centrifuged
105,000×g for 60 minutes

↓

Infranatant removed from
lipid floating layer

↓

1M potassium phosphate
buffer pH 7.4 added to
0.15M

Figure 4.1 Purification procedure for cholesterol
ester hydrolase from bovine adrenal cortex.

Purification Procedure for Cholesterol
Ester Hydrolase from Bovine Adrenal Cortex

Solid ammonium sulphate
added at 35% saturation

Solution centrifuged 10,000 x g
for 5 mins

Pellet dissolved in 20 mM
potassium phosphate

Solution desalted on
Sephadex G-25 equilibrated
with 50 mM sodium glycerophosphate
pH 6.5, 1 mM $MgCl_2$, 4 mM NaF and
2 mM theophylline

Desalted fractions pooled and
incubated in the presence of
10 μM cyclic AMP and 1 mM ATP
containing 125 μCi of $\gamma-^{32}P$
ATP for 30 mins at 30°C

Desalted on Sephadex G-25
equilibrated with 20 mM
potassium phosphate
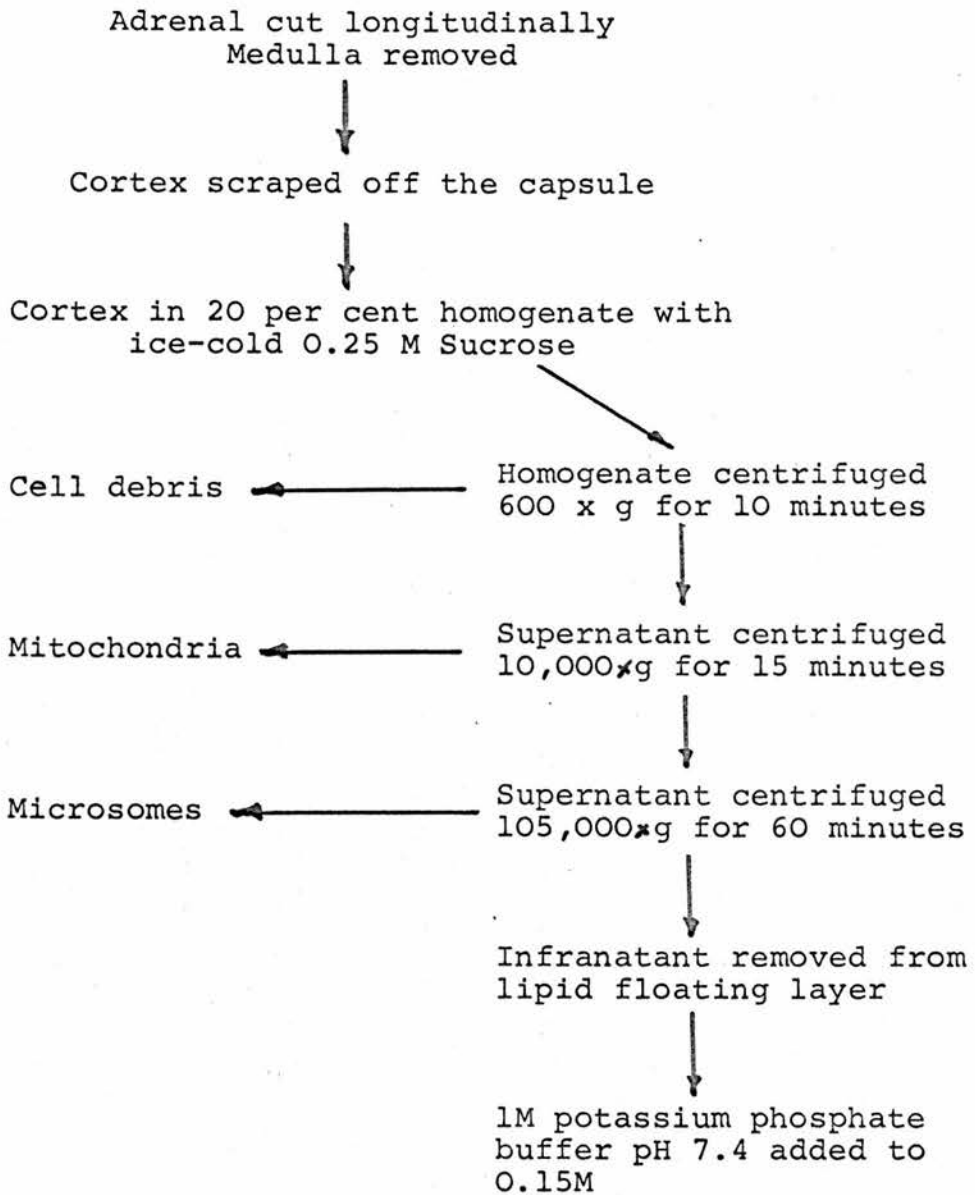buffer pH 7.4 and eluted
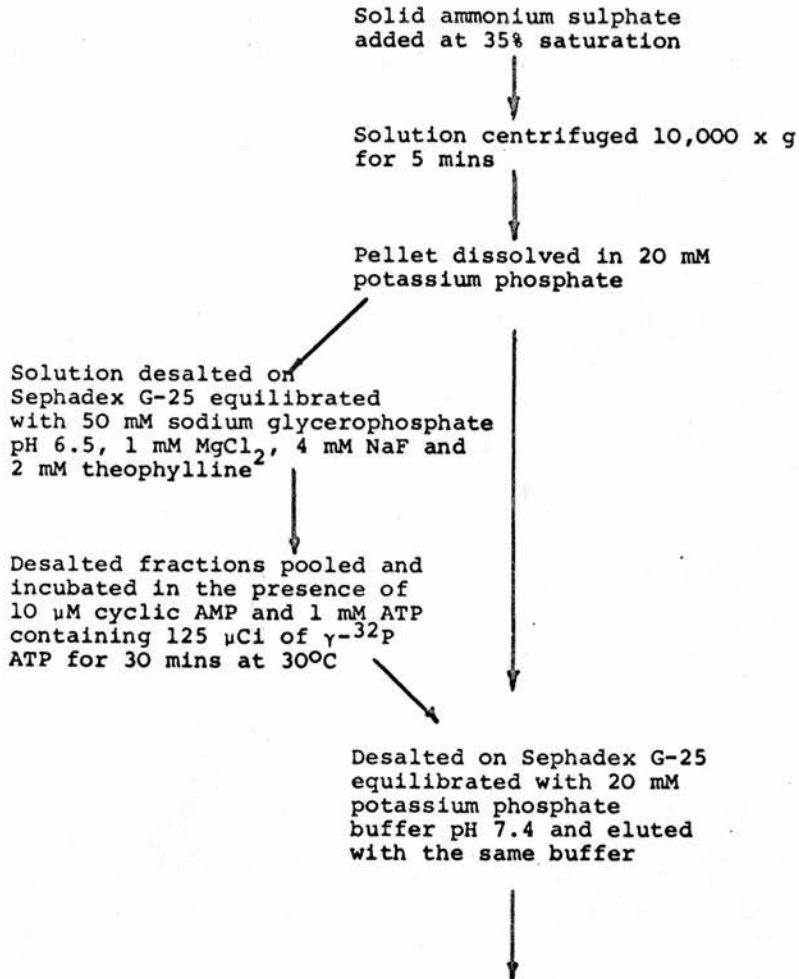with the same buffer

Figure 4.2 Purification procedure for cholesterol
ester hydrolase from bovine adrenal cortex.

<u>Purification Procedure for Cholesterol Ester</u>
<u>Hydrolase from Bovine Adrenal Cortex</u>

Desalted protein fractions pooled
and applied to Sephadex G-200
equilibrated with 20 mM potassium
phosphate and eluted with the
same buffer

Void volume fractions containing
cholesterol ester hydrolase activity
pooled and applied to Sepharose 4B
equilibrated with 20 mM potassium
phosphate buffer pH 7.4 and eluted
with the same buffer

Void volume fractions containing
cholesterol ester hydrolase activity
pooled - centrifuged 105,000 x g for
30 mins

Infranatant removed and applied to
hydroxylapatite equilibrated with
20 mM potassium phosphate pH 7.4

Hydroxylapatite eluted with 50 mM,
100 mM, 200 mM, 300 mM, 400 mM and
500 mM potassium phosphate buffer
pH 7.4 and the protein fractions
eluted 200 mM-500 mM pooled

Pooled protein fractions desalted
on Sephadex G-25 equilibrated with
50 mM potassium phosphate and eluted
with the same buffer

Figure 4.3 Purification procedure for cholesterol
ester hydrolase from bovine adrenal cortex.

The homogenate was centrifuged at 600 x g for 10 minutes to remove nuclei, erythrocytes and unbroken cells which were discarded. The supernatant was then centrifuged at 8,500 x g for 15 minutes to produce a mitochondrial pellet. The post mitochondrial supernatant was centrifuged at 105,000 x g for 60 minutes to remove any remaining mito- chondria or lysosomes and produce a microsomal pellet. The 105,000 x g centrifugation produced a pellet, a clear infranatant and a floating yellow lipid layer. This floating lipid layer was carefully removed with a syringe and the clear infranatant was carefully decanted from the microsomal pellet. Potassium phosphate buffer 1.0 M pH 7.4 was then added to the infranatant to a concentration of 0.15 M.

b) Ammonium sulphate protein fractionation

Table 4.1 shows the degree of purification of cholesterol ester hydrolase obtained in ammonium sulphate precipitated protein pellets. Solid ammonium sulphate was added slowly to 105,000 x g cortical supernatant with constant stirring. Time was allowed between each addition for the ammonium sulphate to completely dissolve. Additions were kept small to minimise local concentration effects and the operation was carried out at 0°C. Ammonium sulphate was added up to 60% saturation at increments of 10% saturation. When the required concentration of ammonium sulphate had been reached the solution was left at 0°C for 30 minutes. The protein precipitated was removed by centrifugation at 10,000 x g for 5 minutes. The supernatant was decanted from the pellet and solid ammonium sulphate was added to

Ammonium Sulphate % saturation	Cholesterol ester hydrolase $\text{pmol} \times \text{min}^{-1} \times \text{mg protein}^{-1}$
0	18
0 - 10	51
10 - 20	105
20 - 30	153
30 - 40	94
40 - 50	7
50 - 60	7
60% supernatant.	6

Table 4.1 Ammonium sulphate fractionation of
bovine adrenal cortical supernatant.
Experimental procedure as text.

the supernatant to produce a further 10% saturation. The isolation of the precipitated protein was then carried out as before and in this way a series of protein pellets were isolated.

Each pellet was dissolved in 20 mM potassium phosphate buffer pH 7.4 and desalted by passage through a sephadex G-25 column (30cm x 2cm) which was equilibrated with the phosphate buffer. This procedure was employed to remove any ammonium sulphate remaining in the pellet. Proteins desalted by this procedure have been shown to contain no sulphate ions (Trzeciak and Boyd (1974)).

The pooled protein fractions from the Sephadex G-25 column were assayed for cholesterol ester hydrolase activity as is described in chapter 2. The 60% ammonium sulphate saturated supernatant was also desalted and assayed for cholesterol ester hydrolase activity. From Table 4.1 it can be seen that most of the cholesterol ester hydrolase activity was precipitated when the ammonium sulphate reached 40% saturation. When a 30% to 40% saturated ammonium sulphate precipitation was carried out the specific activity of this fraction was lower than that of the 20% to 30% fraction. On examining the ammonium sulphate fractions taken from 30% to 35% saturation and 35% to 40% saturation for cholesterol ester hydrolase activity, it was decided to use the protein precipitated at an ammonium sulphate saturation of 35% for further purification. This protein was redissolved in 20mM potassium phosphate buffer pH 7.4 and desalted by passage through a Sephadex G-25 column (30cm x 2cm) equilibrated with 20mM potassium phosphate buffer pH 7.4. The protein fractions were pooled.

c) Sephadex G-200 gel filtration

The pooled protein fractions from the Sephadex G-25 column were applied to a Sephadex G-200 column (50cm x 3cm) equilibrated with 20mM potassium phosphate buffer pH 7.4 and the protein eluted with the same buffer. Fractions (5 ml) were collected and each fraction was assayed for cholesterol ester hydrolase activity. The fraction collector was equipped with a Uvicord to monitor the absorption at 284nm of the fractions. These fractions were also assayed for protein by the method of Lowry et al. (1951).

Figure 4.4 shows a typical elution profile for the chromatography of the ammonium sulphate protein fraction on G-200 Sephadex. Most of the cholesterol ester hydrolase activity was eluted in the void volume fraction of the Sephadex G-200 column. The specific activity of the cholesterol ester hydrolase eluted in the void volume fractions of the Sephadex G-200 for separate preparations was relatively constant.

As well as the cholesterol ester hydrolase activity found in the void volume fraction of the Sephadex G-200 column there was also another cholesterol ester hydrolase eluted later from the column. The ratio of the amounts of the two cholesterol ester hydrolases varied from preparation to preparation. If one compares the elution profile from Sephadex G-200 in Figure 4.4 with that of Figure 4.8, it can be seen that in Figure 4.4 there is little of the second cholesterol ester hydrolase present while in Figure 4.8 a large percentage of the total cholesterol ester hydrolase activity is associated with the second cholesterol

ester hydrolase. There are three possible origins of the second cholesterol ester hydrolase.

The enzyme could be a disaggregated or degraded form of the cholesterol ester hydrolase found in the void volume fractions of the Sephadex G-200 column. The second cholesterol ester hydrolase could be an enzymic contamination of the cholesterol ester hydrolases found in the microsomes and mitochondria. The 105,000 x g cortical supernatant however has been found to be free of iron sulphur protein (J.I. Mason personal communication). It seems unlikely therefore that the second cholesterol ester hydrolase originates from a mitochondrial contamination. It is possible that the second cholesterol ester hydrolase is a non-specific esterase which is situated in the cell cytosol. This however does not explain the difference in ratios of the two esterases found in different preparations. The nature of the second esterase will be disucssed in chapter 10.

The void volume fractions from the Sephadex G-200 column containing most of the cholesterol ester hydrolase activity were pooled and carried forward to the next purification step.

d) Sepharose 4B gel filtration

The pooled void volume fractions from the Sephadex G-200 column were applied to a Sepharose 4B column (35cm x 2.5cm) which had been equilibrated with 20mM potassium phosphate buffer pH 7.4 and the protein was eluted with the same buffer. Fractions were collected of 5ml and each fraction assayed for cholesterol ester hydrolase activity and protein concentration.

Figures 4.6 and 4.10 show typical elution profiles
obtained from Sepharose 4B filtration. Only one peak of
cholesterol ester hydrolase activity was obtained from
Sepharose 4B gel filtration and again this was eluted in
the void volume fractions. Sepharose 4B has an exclusion
limit molecular weight of 20×10^6, it is unlikely however
that cholesterol ester hydrolase has a molecular weight
greater than this in its native cellular environment. The
void volume eluate from the Sepharose 4B column was noticably
turbid with lipid. It is   likely that as the lipid contains
cholesterol esters then cholesterol ester hydrolase associ-
ates with the lipid and aggregates with it. These large
aggregates would then be eluted in the void volume of the
Sepharose 4B column. It may be a property of cholesterol
ester hydrolase to aggregate with the lipid and thus facili-
tates a good purification on Sepharose 4B. Proteins which
do not associate with the lipid fraction are retarded by
the gel and eluted well after the aggregated proteins. The
specific activity of the cholesterol ester hydrolase after
the gel filtration step was relatively constant from prepar-
ation to preparation.

As stated previously the void volume eluate from the
Sepharose 4B column was turbid with lipid. The lipid could
be removed in two ways involving centrifugation or hydroxyl-
apatite chromatography.

If the void volume fractions were pooled and centrifuged
for 30 minutes at 105,000 x g a pellet and a floating lipid
layer was obtained with an optically clear infranatant.
The pellet was composed of protein, phospholipid and

cholesterol ester. The pellet was found to have the same specific activity of cholesterol ester hydrolase as was found in the supernatant.

The alternative method for the removal of the turbidity was by hydroxylapatite chromatography.

e) Purification of cholesterol ester hydrolase on hydroxylapatite

Hydroxylapatite was prepared by washing the powder three times with water followed by two washes in 20mM potassium phosphate buffer. A small column of the hydroxylapatite was then prepared of approximately 10mls.

The void volume fractions from the Sepharose 4B column were pooled and applied to the hydroxylapatite column (approximately 25mls at a protein concentration of 0.1mg per ml). The column was then eluted with two and a half column volumes of 20mM potassium phosphate pH 7.4.

Table 4.2 shows the specific activity of cholesterol ester hydrolase found in each fraction by the elution of the hydroxylapatite with increasing concentrations of potass- ium phosphate pH 7.4. The effect of the addition of 5mM ATP and $10\mu M$ cyclic AMP on cholesterol ester hydrolase activity in each fraction is also shown. Most of cholesterol ester hydrolase activity was eluted by a potassium phosphate concentration of 300mM to 500mM. It can be seen from Table 4.2 however that the cholesterol ester hydrolase obtained by elution of the column with 400mM to 500mM potassium phos- phate buffer was not stimulated by ATP and cyclic AMP addit- ions indicating that the fractions were free from protein kinase activity. When these fractions were assayed for protein kinase activity as described in chapter 2 no protein kinase activity could be found. The fractions obtained by

Potassium phosphate (mM)	Cholesterol ester hydrolase	
	No cofactors	5mM ATP, 10 μM cyclic AMP
	pmol x min^{-1} x mg protein^{-1}	
0 - 20	-	-
20 - 50	18	15
50 - 100	120	120
100 - 150	153	173
150 - 200	260	258
200 - 300	450	450
300 - 400	662	640
400 - 500	1000	900
500 - 1000	689	600

Table 4.2 The purification of bovine adrenal
cholesterol ester hydrolase on hydroxylapatite.
Experimental details as text

the elution of the hydroxylapatite column with 400mM to 500mM potassium phosphate buffer were optically clear. On centrifugation of these fractions at 105,000 x g for 30 mins no pellet or floating lipid layer was formed.

From these observations a scheme for the purification of cholesterol ester hydrolase from the Sepharose 4B void volume fractions was evolved.

The void volume from the Sepharose 4B column were pooled and applied to a hydroxylapatite column prepared in the way described earlier. The protein was then eluted from the column with stepwise elutions of two and a half column volumes of potassium phosphate buffer pH 7.4. The protein eluted at a potassium phosphate concentration of 400mM to 500mM was pooled and desalted by passage through a Sephadex G-25 column (30cm x 2cm) equilibrated with 50mM potassium phosphate buffer pH 7.4 and eluted with the same buffer.

No significant increase in the specific activity of cholesterol ester hydrolase was obtained by hydroxylapatite chromatography but the step was carried out to remove all traces of endogenous protein kinase activity.

A summary of the purification of bovine adreno-cortical cholesterol ester hydrolase based on specific activity is shown in Table 4.3. It can be seen that a fifty-seven fold purification of cholesterol ester hydrolase was achieved by this purification procedure.

Evidence for the phosphorylation of bovine adrenal cortical cholesterol ester hydrolase by $[\gamma-^{32}P]$ ATP

The evidence for the phosphorylation of bovine adrenal cortical cholesterol ester hydrolase presented in this

Fraction	Cholesterol ester hydrolase pmol x min xmg protein	Purification
Supernatant	14	
Ammonium sulphate precipitation	55	4
Sephadex G 200	436	31
Sepharose 4B	800	57
Hydroxylappatite	750	53

Table 4.3 Purification of bovine adrenal
cortex cholesterol ester hydrolase.

section consists of the preincubation of the ammonium sulphate fraction with $[\gamma-^{32}P]$ ATP followed by purification of the cholesterol ester hydrolase. The ammonium sulphate fraction was prepared from the adrenal cortical 105,000 x g supernatant by the method described earlier in this chapter. The ammonium sulphate fraction was then desalted by passage of the protein solution through a Sephadex G-25 column (30 x 2cm) equilibrated with 50mM sodium glycerophosphate buffer pH 6.5, 1mM $MgCl_2$, 4mM NaF and 2mM theophylline and the protein fractions were pooled. This procedure removed endogenous nucleotides and gave the optimal conditions for protein kinase activity as reported by Ichii (1972). At this stage of the purification the pooled desalted protein fractions were preincubated at $30^{\circ}C$ for 20 mins with 5mM $[\gamma-^{32}P]$ ATP, 1mM $MgCl_2$ 10µM cyclic AMP, 50mM KCl, 2mM theophylline and 4mM NaF. After preincubation the protein solution was desalted by passage through a Sephadex G-25 column (30cm x 2cm) equilibrated with 20mM potassium phosphate buffer pH 7.4 and the protein fractions were pooled. This preincubation procedure is summarised in Figure 4.2.

The pooled protein fractions were then subjected to the same purification procedures as were described previously and summarised in Figure 4.3.

Fractions from the Sephadex G-200 and Sepharose 4B columns were assayed for cholesterol ester hydrolase activity and total and protein bound $[^{32}P]$ radioactivity. Assays for $[^{32}P]$ radioactivity in the column fractions were carried out by counting 100 µl aliquots from each fraction in 10mls of dioxane based scintillation fluid on a Packard Tri Carb liquid scintillation spectrophotometer. The assay of the

[^{32}P] radioactivity bound to the protein in the chromatogram fractions was carried out as described in Chapter 2.

The elution profiles obtained from the Sephadex G-200 and Sepharose 4B columns are shown in Figures 4.4, 4.5, 4.6, and 4.7. Fig. 4.4 and 4.5 show apparently two cholesterol ester hydrolases are present in the ammonium sulphate fraction. These activities can be separated on a Sephadex G-200 column. Most of the cholesterol ester hydrolase activity is eluted in the void volume fractions and, after protein precipitation with trichloroacetic acid, [^{32}P] radioactivity remained bound to the protein in these fractions. The second cholesterol ester hydrolase enzyme was eluted after the elution of the first enzyme and in this case no [^{32}P] radioactivity was incorporated into the protein after precipitation with tri-chloroacetic acid. This evidence gives support the concept that the cholesterol ester hydrolases are different enzymes. The possibility cannot be excluded however that the second cholesterol ester hydrolase has been phosphorylated in vivo. If this were the case obviously no [^{32}P] radioactivity would be incorporated into the protein. It is still possible therefore that this second cholesterol ester hydrolase is a disaggregated form of the esterase eluted in the void volume.

Figs. 4.6 and 4.7 show the elution profile from the Sepharose 4B column. Most of the cholesterol ester hydrolase is eluted in the void volume fractions of the column and again after precipitation of protein with trichloroacetic acid [^{32}P] radioactivity remained bound to the protein in these fractions. The [^{32}P] radioactivity also remained bound to the protein eluted in later fractions after the protein was precipitated with trichloroacetic acid.

ELUTION PROFILE OF BOVINE ADRENAL CORTEX 105,000 x g SUPERNATANT PROTEIN
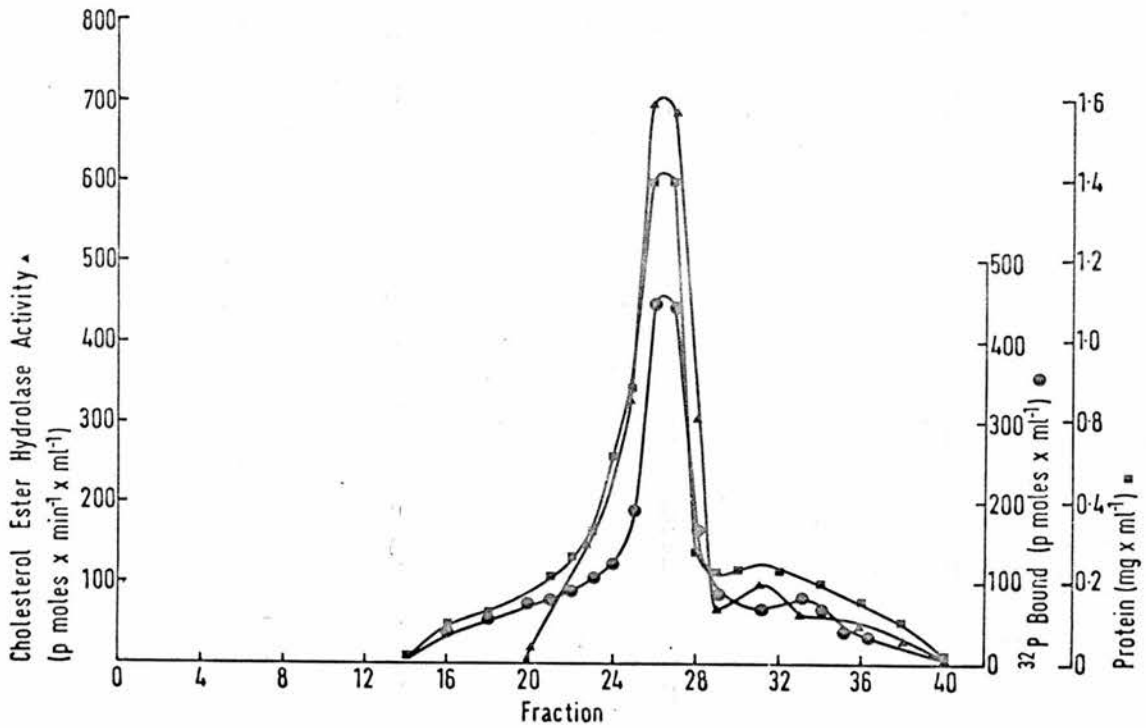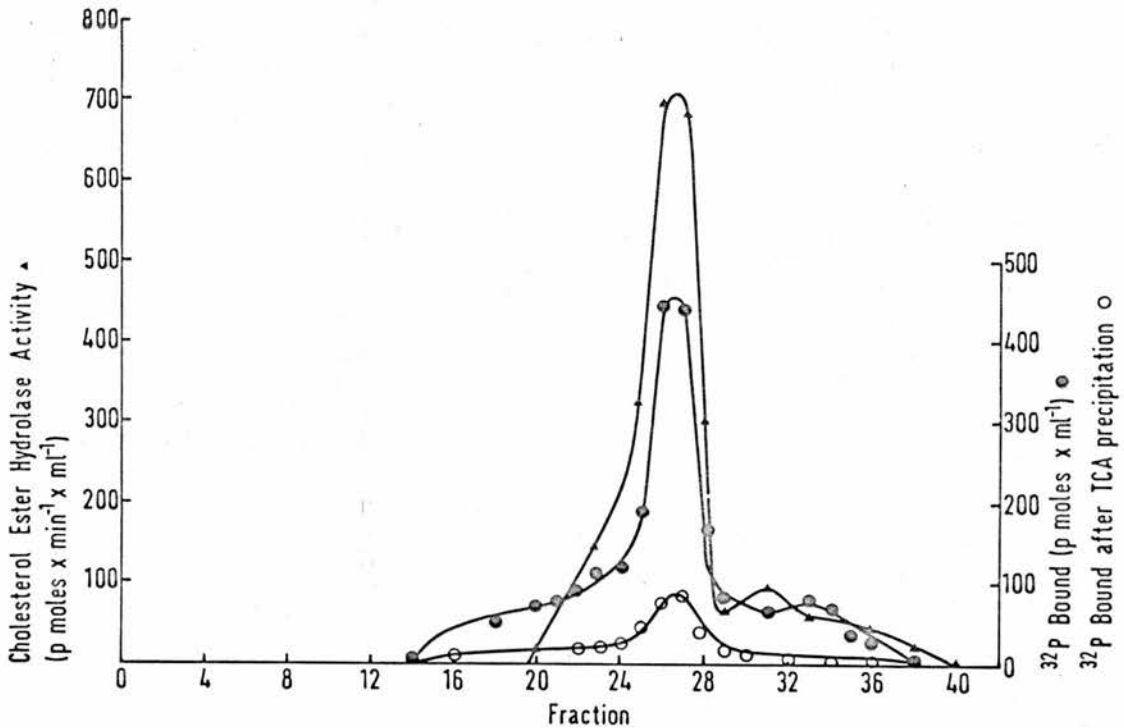FROM SEPHADEX G200 AFTER INCUBATION WITH [γ^{32}P]ATP

Figure 4.4   The elution profile of a protein fraction
obtained from bovine adrenal cortical supernatant and
subjected to gel filtration on Sephadex G 200.
The protein was firstly preincubated with [γ^{32}P] ATP
under the conditions described in the text. The
fractions obtained from the column were assayed for
cholesterol ester hydrolase, [^{32}P] radioactivity and
protein as described in chapter 2.

ELUTION PROFILE OF BOVINE ADRENAL CORTEX 105,000 x g SUPERNATANT PROTEIN
FROM SEPHADEX G200 AFTER INCUBATION WITH [γ^{32}P] ATP

Figure 4.5 The elution profile of a protein fraction
obtained from bovine adrenal cortical supernatant and
subjected to gel filtration on Sephadex G 200.
The protein was firstly preincubated with [γ^{32}P] ATP
under the conditions described in the text. The fractions
obtained from the column were assayed for cholesterol ester
hydrolase, protein bound [^{32}P] radioactivity and
total [^{32}P] radioactivity as described in chapter 2.

ELUTION PROFILE FROM SEPHAROSE 4B OF THE SEPHADEX G200 FRACTIONS
CONTAINING CHOLESTEROL ESTER HYDROLASE ACTIVITY AFTER INCUBATION WITH
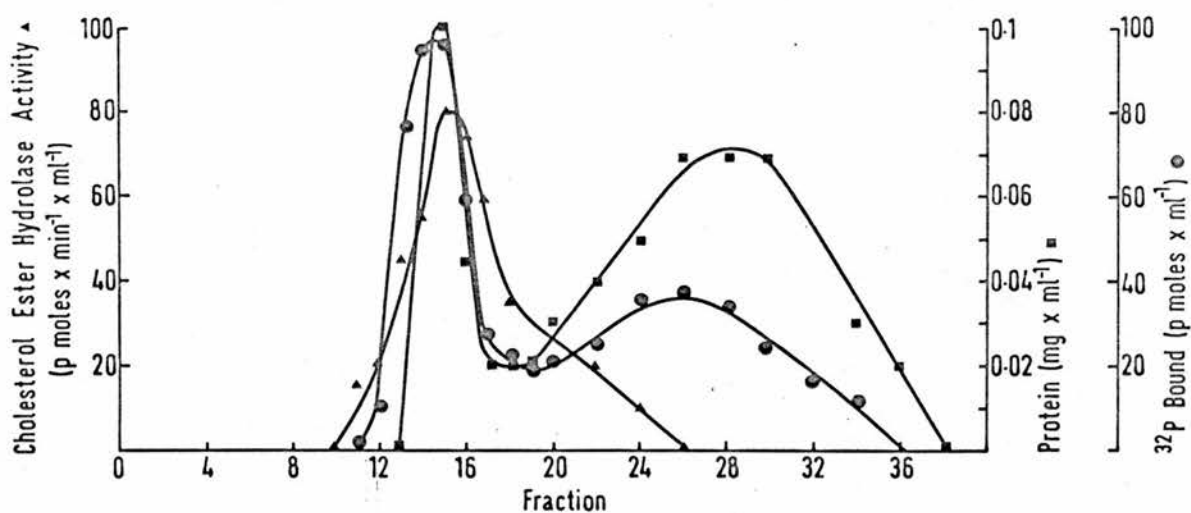$[\gamma\text{-}^{32}P]$ ATP

Figure 4.6 The elution profile from Sepharose 4B of
the void volume Sephadex G 200 fractions containing
cholesterol ester hydrolase activity.

The void volume fractions of protein from the Sephadex
G 200 column shown in Figures 4.4 and 4.5 were subjected
to gel filtration on a Sepharose 4B column . Fractions
were assayed for cholesterol ester hydrolase, protein
and [^{32}P] radioactivity as described in chapter 2.

ELUTION PROFILE FROM SEPHAROSE 4B OF THE SEPHADEX G200 FRACTIONS CONTAINING CHOLESTEROL ESTER HYDROLASE ACTIVITY AFTER INCUBATION WITH [γ-^{32}P] ATP
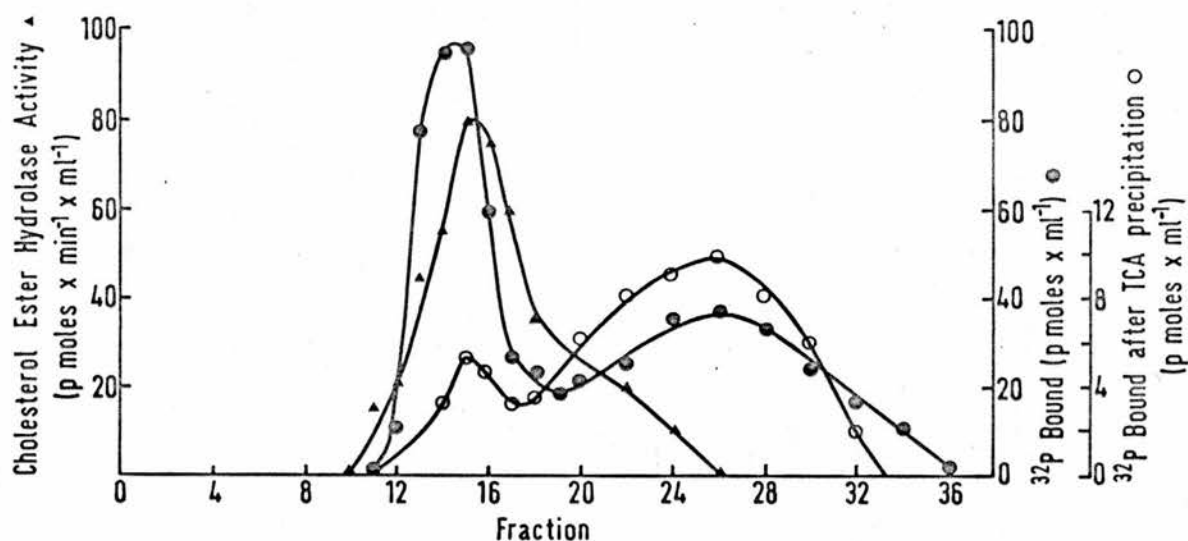
Figure 4.7 The elution profile from Sepharose 4B of the void volume fractions from Sephadex G 200 containing cholesterol ester hydrolase activity.

The procedure was carried out as is described in Figure 4.6 Fractions were assayed for cholesterol ester hydrolase activity, protein bound [^{32}P] radioactivity and total [^{32}P] radioactivity as described in chapter 2.

These fractions did not contain cholesterol ester hydrolase activity. It is possible that other proteins as well as cholesterol ester hydrolase have undergone a phosphorylation involving the transfer of the terminal phosphate of ATP to the protein. These results lend support to the view that cholesterol ester hydrolase can undergo covalent modification by phosphorylation of the enzyme.

Preincubation of the ammonium sulphate precipitated protein fraction with $[\alpha-^{32}P]$ ATP followed by purification of cholesterol ester hydrolase

It is possible, but unlikely, that the $[^{32}P]$ radioactivity which remained bound to the protein fractions in the previous experiment was due to $[\gamma-^{32}P]$ ATP binding tightly to the protein even after trichloroacetic acid treatment. For this reason the previous experiment was repeated using a preincubation of the ammonium sulphate fraction with $[\alpha-^{32}P]$ ATP followed by purification of the cholesterol ester hydrolase on Sephadex G-200 and Sepharose 4B.

Figures 4.8, 4.9 and 4.10, 4.11 show the elution profiles from Sephadex G-200 and Sepharose 4B columns. It can be seen that $[^{32}P]$ radioactivity occurs in all the protein fractions from the columns before protein precipitation with trichloroacetic acid. After protein precipitation with trichloroacetic acid practically all the $[^{32}P]$ radioactivity is lost. Preincubation of the ammonium sulphate fraction with $[\alpha-^{32}P]$ ATP followed by purification resulted in only 12 pmol of $[^{32}P]$ bound per mg protein in the Sepharose 4B purified cholesterol ester hydrolase fraction after protein precipitation with trichloroacetic acid. When $[\gamma-^{32}P]$ ATP

ELUTION PROFILE OF BOVINE ADRENAL CORTEX 105,000 x g SUPERNATANT PROTEIN
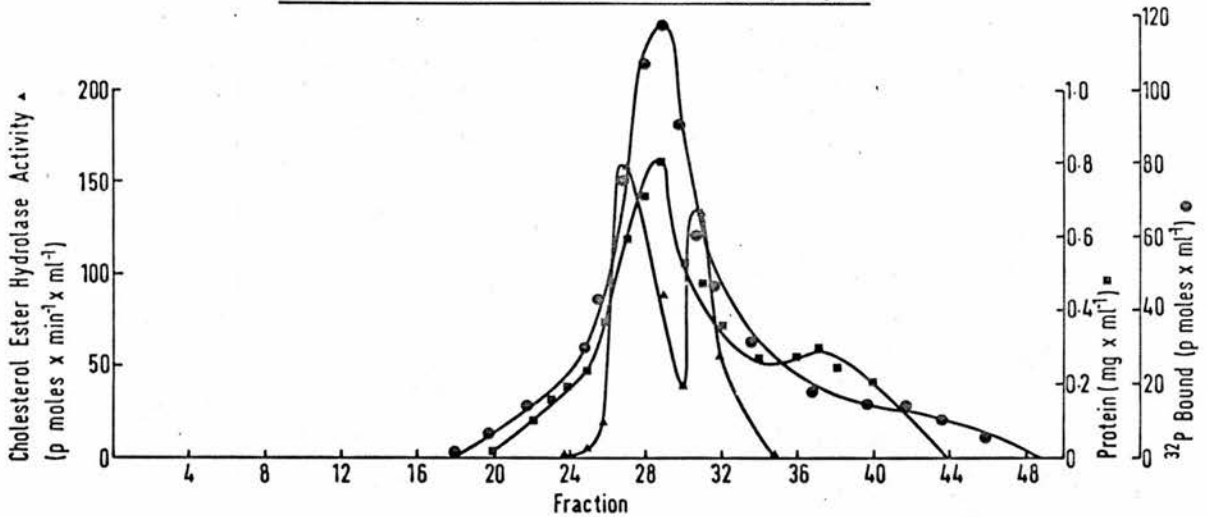FROM SEPHADEX G200 AFTER INCUBATION WITH [α^{32}P] ATP

Figure 4.8   The elution profile of a protein fraction
obtained from bovine adrenal cortical supernatant and
subjected to gel filtration on Sephadex G 200.
The protein was firstly preincubated with [a^{32}P]
ATP   under the conditions described in the text.
The fractions obtained from the column were assayed for
cholesterol ester hydrolase , [^{32}P] radioactivity and
protein as described in chapter 2.

ELUTION PROFILE OF BOVINE ADRENAL CORTEX 105,000 x g SUPERNATANT PROTEIN
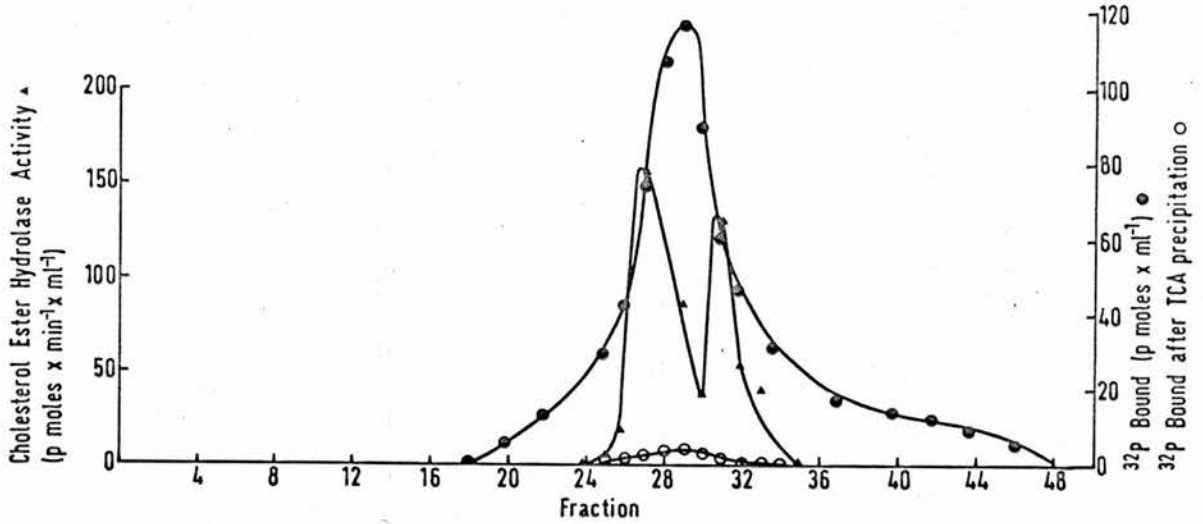FROM SEPHADEX G200 AFTER INCUBATION WITH [α³²P] ATP

Figure 4.9 The elution profile of a protein fraction
obtained from bovine adrenal cortical supernatant and
subjected to gel filtration on Sephadex G 200.
The protein was firstly preincubated with [α ³²P] ATP
under the conditions described in the text. The fractions
obtained from the column were assayed for cholesterol
ester hydrolase, protein bound[³²P] radioactivity and
total [³²P] radioactivity as described in chapter 2.

ELUTION PROFILE FROM SEPHAROSE 4B OF THE SEPHADEX G200 FRACTIONS
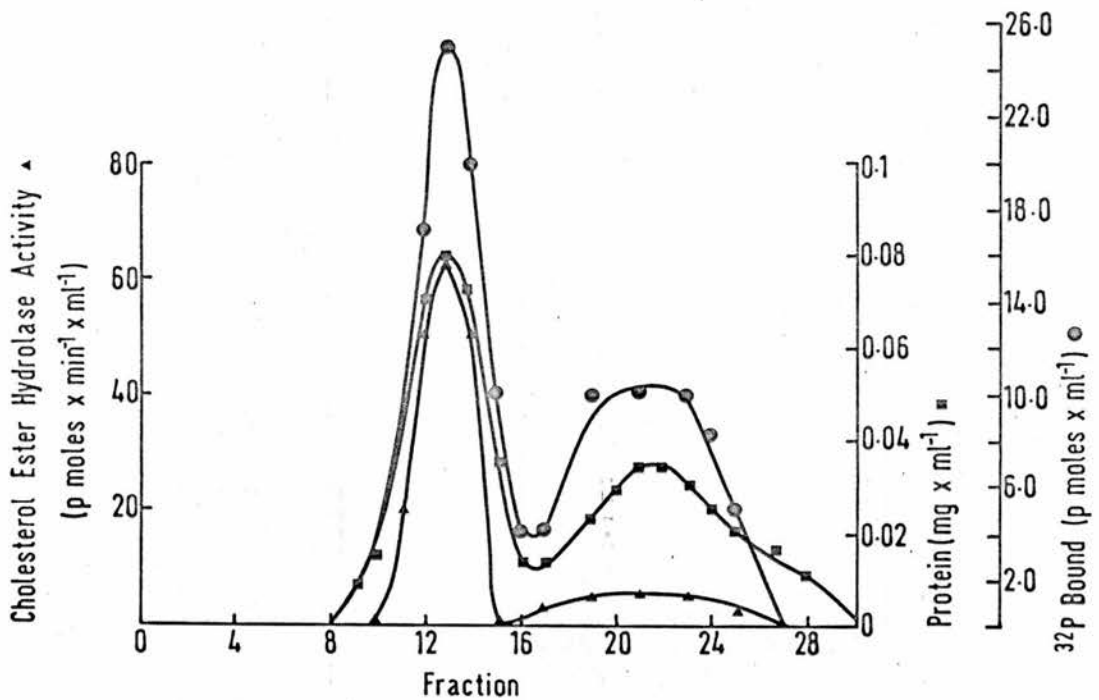CONTAINING CHOLESTEROL ESTER HYDROLASE ACTIVITY WITH [α-^{32}P] ATP

Figure 4.10 The elution profile from Sepharose 4B of the
void volume Sephadex G 200 fractions (shown in Figure 4.9)
containing cholesterol ester hydrolase activity. The
void volume fractions of protein from the Sephadex G 200
column shown in Figures 4.9 and 4.8 were subjected to
gel filtration on a Sepharose 4B column . Fractions were
assayed for cholesterol ester hydrolase, protein and
[^{32}P] radioactivity as described in chapter 2.

ELUTION PROFILE FROM SEPHAROSE 4B OF THE SEPHADEX G200 FRACTIONS CONTAINING CHOLESTEROL ESTER HYDROLASE ACTIVITY AFTER INCUBATION WITH $[\alpha ^{32}P]$ ATP
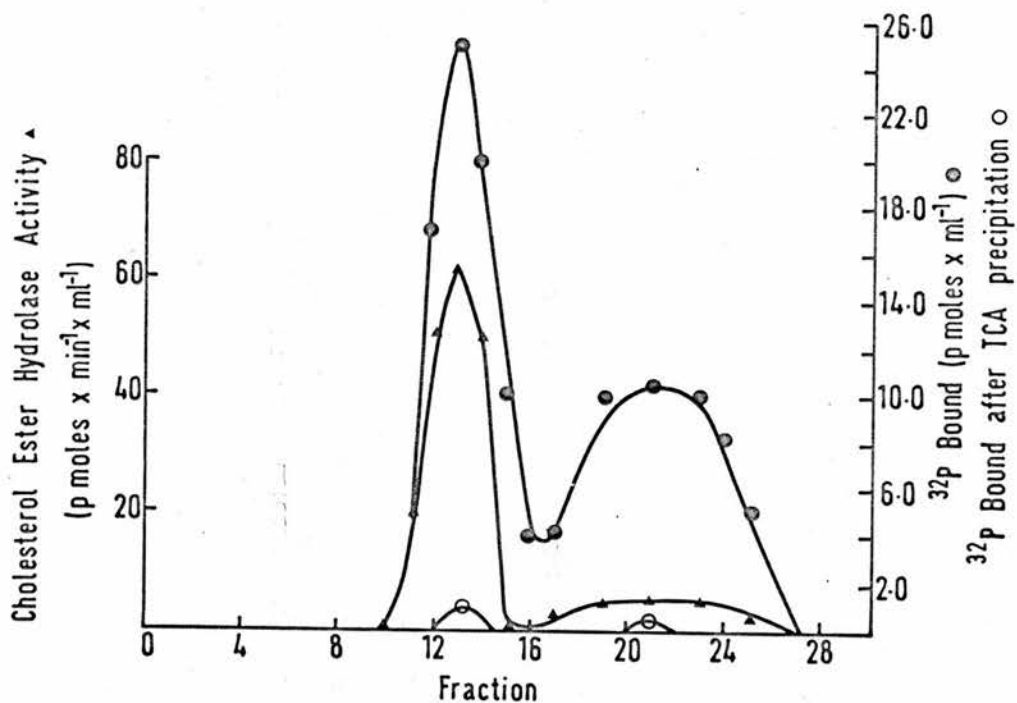
Figure 4.11 The elution profile from Sepharose 4B of the void volume fractions from Sephadex G 200 containing cholesterol ester hydrolase

The procedure was carried out as described in Figure 4.10 Fractions were assayed for cholesterol ester hydrolase activity, protein bound [^{32}P] radioactivity and total [^{32}P] radioactivity as described in chapter 2.

was used, 60 pmol of $[^{32}P]$ per mg protein was bound in the Sepharose 4B void volume fractions after protein precipitation with trichloroacetic acid.

These results indicate a phosphorylation of cholesterol ester hydrolase has taken place and not an adenylation. It is still possible however that it is another protein in the Sepharose 4B void volume fraction and not cholesterol ester hydrolase which is phosphorylated. To determine which of the proteins in the Sepharose 4B void volume fraction were phosphorylated gel electrophoresis was carried out on the Sepharose 4B void volume fraction.

Polyacrylamide gel electrophoresis of purified cholesterol ester hydrolase on a native protein gel system

The protein concentration of the void volume fraction from the Sepharose 4B column was too dilute for gel electrophoresis. The pooled void volume fractions were therefore freeze dried and the solid redissolved in a small volume of water up to a protein concentration of 1mg per ml.

This solution was subjected to gel electrophoresis on a 5% polyacrylamide gel. The method used was that of Maizel (1969) which is described in detail in chapter 2. After electrophoresis duplicate gels were stained for protein with Coomassie Blue and stained for esterase activity with Fast Blue R.R. as described in chapter 2. Figure 4.12 shows the results from the stains. Two bands were observed when the gel was stained for protein and only the protein with the higher mobility showed esterase activity.

When the ammonium sulphate protein fraction was pre-incubated with $[\gamma-^{32}P]$ ATP and the resulting void volume
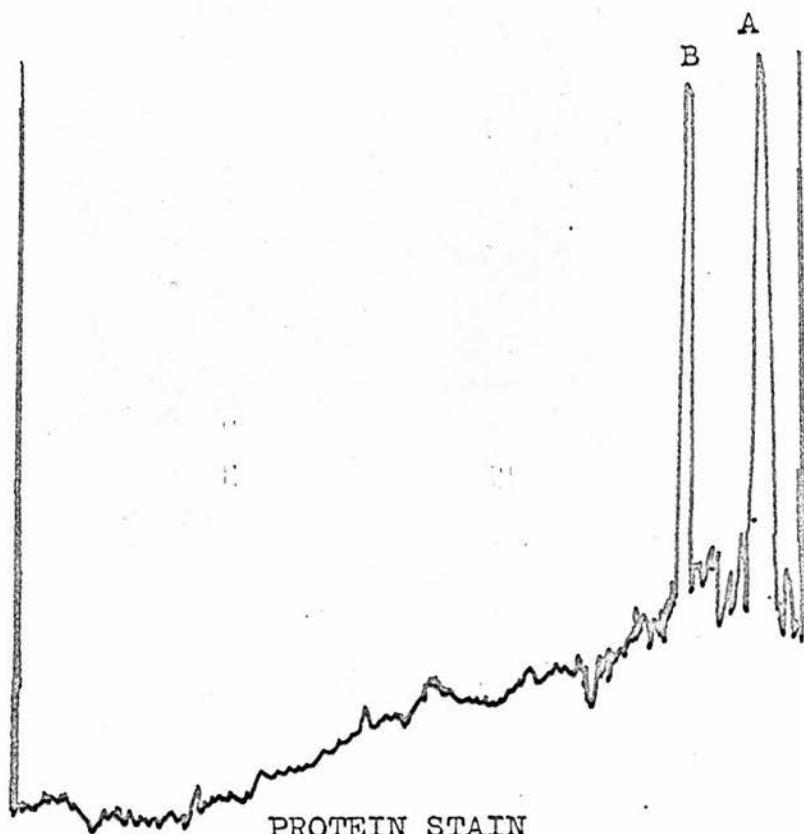
Figure 4.12. polyacrylamide gel scan of a purified,
phosphorylated fraction of bovine adrenal cortex cholesterol
ester hydrolase stained for esterase activity and protein.
The phosphorylated Sepharose 4B void volume fraction of
cholesterol ester hydrolase was prepared for electrophoresis
as descibed in the text and electrophoresis of the fraction
was carried out at $0^{\circ}C$ at 2ma per gel. The gells were stained
for protein and esterase activity and sliced and counted
for [^{32}P] radioactivity. Only protein B exibited esterase
activity and [^{32}P] radioactivity.

fraction from the Sepharose 4B column subjected to gel electro-
phoresis and staining, again two proteins were observed.
When the gel was sliced and counted for $[^{32}P]$ radioactivity,
radioactivity was found to be incorporated only in the
protein which exhibited esterase activity.

It was found however that on the surface of the gel,
after electrophoresis, an insoluble yellow film had formed.
On slicing and counting the gel slices for $[^{32}P]$ radio-
activity it was found that some of the $[^{32}P]$ radioactivity
resided on the surface of the gel. The colour of the insol-
uble film on the top of the gel was indicative of the float-
ing lipid layer and pellet obtained from the 105,000 x g
centrifugation of the Sepharose 4B void volume fraction.

The supernatant from the 105,000 x g centrifugation
of the Sepharose 4B void volume fraction was therefore
subjected to gel electrophoresis and staining on 5% gels.
Gel electrophoresis of this fraction showed no insoluble
film on top of the gel. Again when the gel was stained
for protein with Coomasie Blue two proteins were observed
(Figure 4.13). The mobility of the proteins however were
found to be approximately twice that observed in the Sepha-
rose 4B void volume fraction. When these gels were stained
for esterase activity three bands of activity were found
in close proximity with each other. The first of these
eaterase bands had the same mobility as the second protein band.

The production of two extra bands of esterase activity
on centrifugation together with the increased mobility of
the proteins on removal of the lipid from the Sepharose 4B
void volume fraction by centrifugation can be explained by
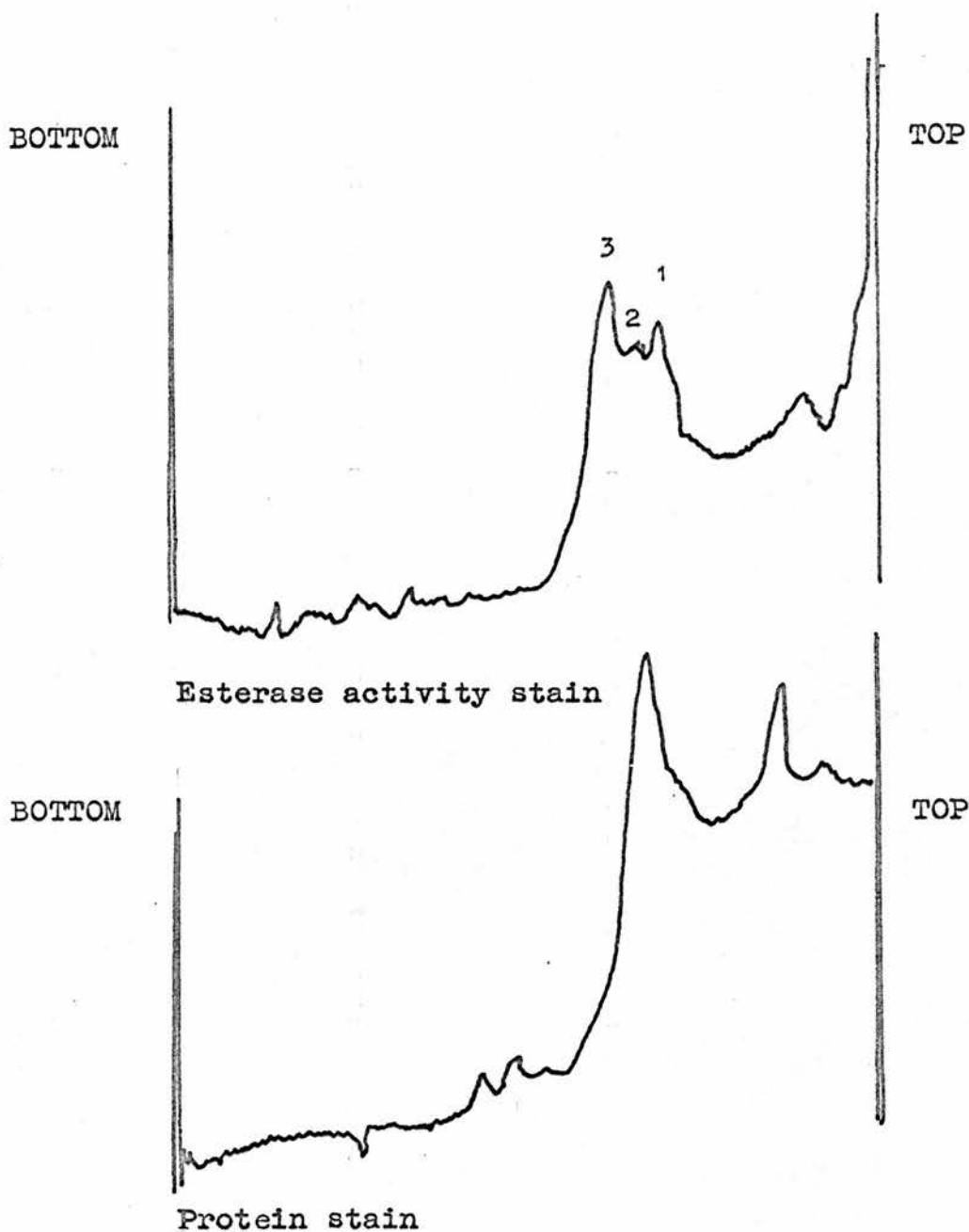aggregation. As stated previously the void volume fraction

Figure 4.13. Scan of the purified preparation of bovine adrenal cortex cholesterol ester hydrolase which had been centrifuged at 105, 000 x g for 30 minutes and subjected to gel electrophoresis and staining for esterase activity and protein. The Sepharose 4B void volume fraction prepared as described in the text was centrifuged at 105,000 x g for 30 minutes and the floating lipid layer removed. The supernatant was subjected to electrophoresis and staining as described in the text and Figure 4.12. Three bands of esterase activity were found the first of which (1) had the same mobility as the second protein band.

of the Sepharose 4B column is turbid with lipid. It is
probably the association of cholesterol ester hydrolase
with this lipid which facilitates its elution with the lipid
in the void volume fraction of the Sepharose 4B column.
If this is true then the removal of the lipid would cause
a disruption of the cholesterol ester hydrolase-lipid complex
with a resulting change in the mobility of the proteins on
the polyacrylamide gels. The three bands of esterase activ-
ity are probably due to different aggregates of the same
enzyme. Experimental evidence to support this postulate
is presented later in this chapter.

The experimental evidence presented in this section
suggests that cholesterol ester hydrolase can be phosphory-
lated by the transfer of the terminal phosphate from ATP
to the enzyme.

Polyacrylamide gel electrophoresis of purified cholesterol ester hydrolase on an S.D.S. gel system

Electrophoresis of the phosphorylated protein in the
Sepharose 4B void volume fraction was carried out on 10%
polyacrylamide S.D.S. gels. Again the protein concentration
of the void volume fraction from the Sepharose 4B column
was not sufficiently high for electrophoresis. The fraction
was therefore desalted by passage through a Sephadex G-25
column equilibrated with 2mM sodium phosphate buffer and
freeze dried. The solid was redissolved in a small volume
of water up to a protein concentration of 1mg per ml. The
sample was then treated with S.D.S., urea and mercaptoethanol
as described in chapter 2 and 100 μl of the sample was then
applied to a 10cm 10% polyacrylamide gel containing 0.1% S.D.S.

Electrophoresis was carried out by the method described in chapter 2 at 2ma per gel until the bromophenol blue dye marker had reached the end of the gel. Protein markers of bovine serum albumin, γglobulin, ovalbumin and myoglobin were also run to enable the molecular weight of cholesterol ester hydrolase to be determined. After electrophoresis the gels were stained for protein with Coomasie Blue (see chapter 2), scanned, sliced and counted for $[^{32}P]$ radioactivity.

Figure 4.14 shows the scan obtained of the proteins on electrophoresis of the Sepharose 4B void volume fraction which had been phosphorylated. Two major proteins were found in close proximity to one another. These two proteins were presumably the two proteins found on electrophoresis of the Sepharose 4B void volume fraction.

When the S.D.S. gel was sliced into 2mm slices and counted for $[^{32}P]$ radioactivity the majority of the radio-activity was associated with the protein band of the higher mobility. From the electrophoresis of the native protein described in the previous section it was shown that the protein with the higher mobility, in that system, had esterase activity and this protein was also associated with the majority of the $[^{32}P]$ radioactivity. For this reason the calculation of the molecular weight of cholesterol ester hydrolase on S.D.S. gels has assumed that the protein which is associated with $[^{32}P]$ radioactivity is the cholesterol ester hydrolase.

A scan of the marker proteins is shown in Figure 4.15. The relative mobility of each protein compared to myoglobin was calculated and plotted against the log. of the molecular weight (Figure 4.16). The graph was found to be linear as
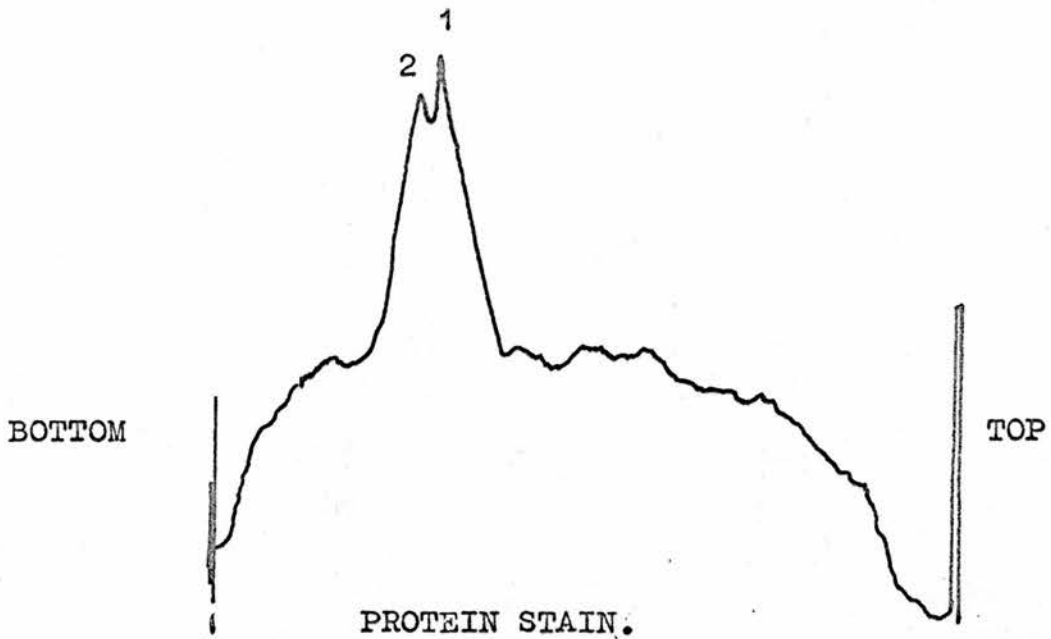
BOTTOM　　　　　　　　　　　　　　　　　　　　　TOP

PROTEIN STAIN.

Figure 4.14　　Gel scan of a purified preparation of
bovine adrenal cortex cholesterol ester hydrolase
subjected to S.D.S gel electrophoresis and stained
for protein.　The phosphorylated Sepharose 4B void
volume fraction of cholesterol ester hydrolase was
prepared for elctrophoresis as is described in the text
and electrophoresis was carried out at room temperature
on 5% polyacrylamide gels at 2ma per gel. The gel was
stained for protein　and sliced and counted for $[^{32}P]$
radioactivity as described in chapter 2. The second
protein (2) was found to incorporate $[^{32}P]$ radioactivity.
A gel of marker proteins was run at the same time
(figure 4.15.)
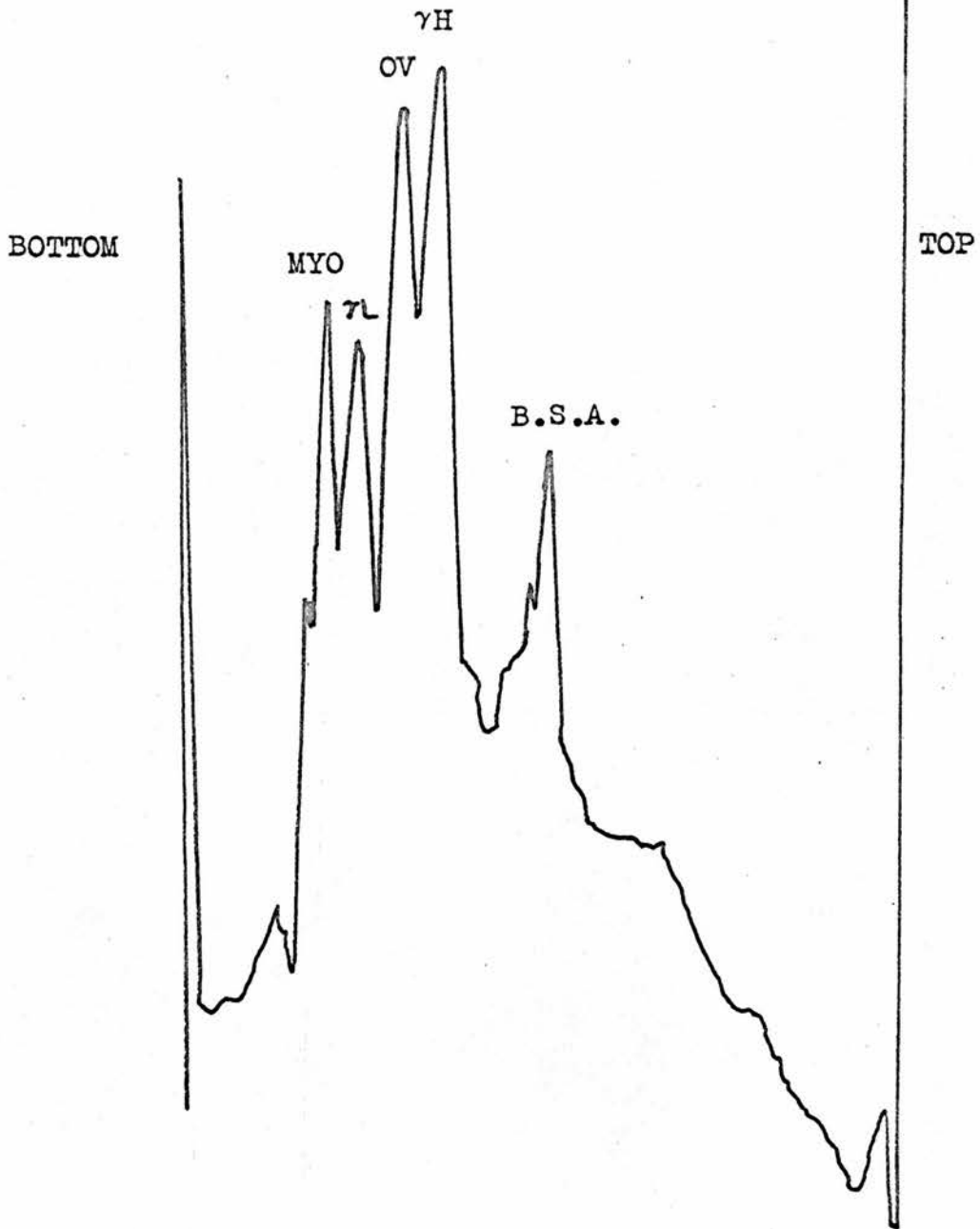
Figure 4.15 gel scan of marker proteins on an S.D.S gel. Experimental procedure as described in Figure 4.14. B.S.A = Bovine serum albumin, γH=γglobulin heavy, OV = Ovalbumen, γL = γ globulin light MYO = Myoglobin.
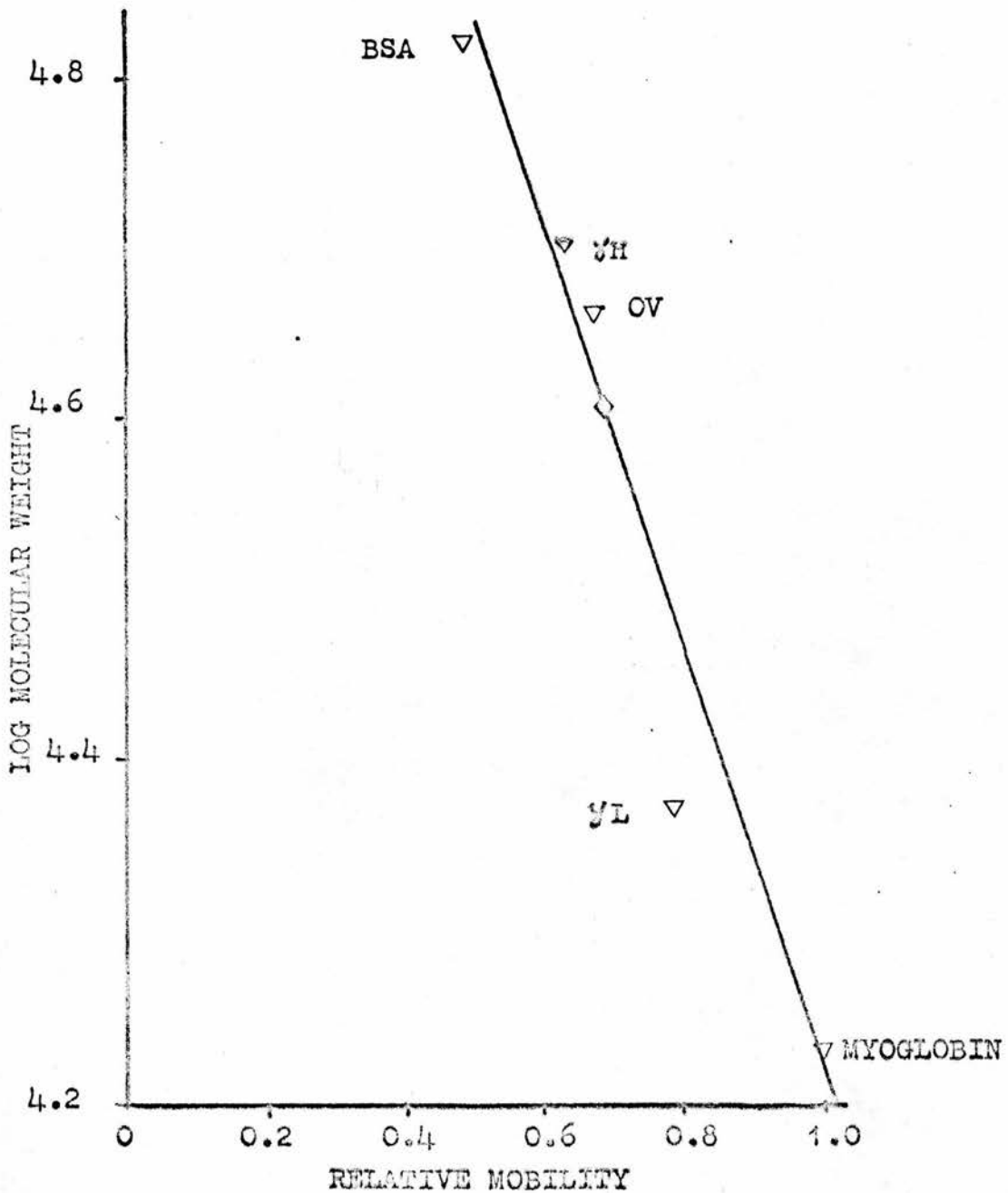
Figure 4.16 Estimation of the molecular weight of
cholesterol ester hydrolase using S.D.S polyacrylamide
gel electrophoresis. The relative mobilities of each
protein was determined from the data presented in
Figures 4.15 and 4.14 and calculated as described in
the text. From this data the molecular weight of (◇)
cholesterol ester hydrolase was calculated as 41,000.
BSA = bovine serum albumin, ɣL and ɣH = ɣ globulin
(Light and Heavy), OV= ovalbumen.

reported by Shapirol et al. (1967). From this graph the
molecular weight of cholesterol ester hydrolase has been
determined as approximately 41,000.

Procedure B for the purification of cholesterol ester hydro-
lase from bovine adrenal cortex using DEAE cellulose chromato-
graphy and gel filtration.

A second method for the purification of cholesterol
ester hydrolase was attempted and the purification procedure
adopted is summarised in Figure 4.17. Using this method a
20 fold purification of cholesterol ester hydrolase was achieved.

Bovine adrenal cortical 105,000 x g supernatant was
prepared as was described earlier in this chapter and the
ammonium sulphate fractionation was carried out in the normal way.
After desalting the ammonium sulphate protein fraction by
passage through a Sephadex G-25 column equilibrated with
20mM potassium phosphate buffer, the pooled desalted protein
fractions were applied to a DEAE cellulose column (14cm x
2cm). Stepwise elution of the column was then carried out
with 0.05M, 0.15M, 0.3M and 0.5M potassium phosphate buffer
pH 7.4. The major fraction of the cholesterol ester hydro-
lase activity was eluted by the 0.3M buffer. If the ammonium
sulphate protein fraction was preincubated with $[\gamma-^{32}P]$ATP
as described previously the majority of the $[^{32}P]$ radioactivity
was also eluted from the DEAE cellulose column by the 0.3M
buffer addition.

The protein eluted from the DEAE cellulose column by
the 0.3M buffer was applied to a Biogel A 0.5M column (50cm x
3cm) equilibrated with 20mM potassium phosphate buffer and
eluted with the same buffer. Again the cholesterol ester

Figure
4.17

DEAE Purification Procedure for Cholesterol
Ester Hydrolase from Bovine Adrenal Cortex

Solid ammonium sulphate added at
35% saturation

Solution centrifuged 10,000 x g
for 5 mins

Pellet dissolved in 20 mM potassium
phosphate

Desalted on Sephadex G-25 equilibrated
with 20 mM potassium phosphate buffer
pH 7.4 and eluted with the same buffer

Pooled protein fractions applied to
DEAE cellulose equilibrated with 20 mM
potassium phosphate buffer pH 7.4

DEAE eluted stepwise with potassium
phosphate buffer pH 7.4

Protein fractions eluted between 0.15 M
and 0.3 M potassium phosphate were
pooled and applied to Biogel equilibrated
with 50 mM potassium phosphate buffer
pH 7.4 and eluted with the same buffer

Fractions containing cholesterol ester
hydrolase activity - pooled and applied
to hydroxylapatite

Hydroxylapatite eluted stepwise with
potassium phosphate buffer pH 7.4 and
the protein eluted at 100 mM-200 mM
pooled

Pooled protein fractions desalted on
Sephadex G-25 equilibrated with potassium
buffer pH 7.4 and eluted with the same
buffer

hydrolase was eluted in the void volume of the column and this was associated with the $[^{32}P]$ radioactivity. The pooled void volume fractions were applied to a hydroxylapatite column (10mls) and eluted stepwise with two and a half column volumes of 0.1M, 0.2M and 0.3M potassium phosphate buffer pH 7.4. The protein eluted by the 0.2M buffer addition was desalted by passage through a Sephadex G-25 column (30 cm x 2 cm) equilibrated with 20mM potassium phosphate.

The specific activity was determined at each stage of the purification and the results are shown in Table 4.4. It can be seen that no increase in specific activity was achieved after the Biogel purification step. Thus despite the removal of protein which did not exhibit any cholesterol ester hydrolase by the hydroxylapatite column, the specific activity remained unchanged. This could be due to the denaturation of the cholesterol ester hydrolase on the hydroxylapatite. The desalted fraction eluted from the hydroxylapatite was freeze dried and redissolved in water to a protein concentration of 1 mg per ml and subjected to gel electrophoresis.

Polyacrylamide gel electrophoresis of purified cholesterol ester hydrolase

The purified cholesterol ester hydrolase was applied to a 10cm 5% polyacrylamide gel and electrophoresis at 2mA per gel was conducted as described in chapter 2. Duplicate gels were run and stained for protein and esterase activity. The gels were also sliced and counted for $[^{32}P]$ radioactivity.

On staining the gels for protein, nine proteins were observed (Figure 4.18). When a duplicate was stained for esterase activity with α napthol proprionate and Fast Blue RR (chapter 2), two bands of esterase activity were observed

Fraction	Cholesterol ester hydrolase pmol min x mg	Purification
Supernatant	26	-
Ammonium sulphate	59	2
DEAE cellulose	180	7
Biogel A 0.5M	250	10
Hydroxylappatite	252	10

Table 4.4 Purification of bovine adrenal cortex cholesterol ester hydrolase.

BOTTOM   TOP

2
1
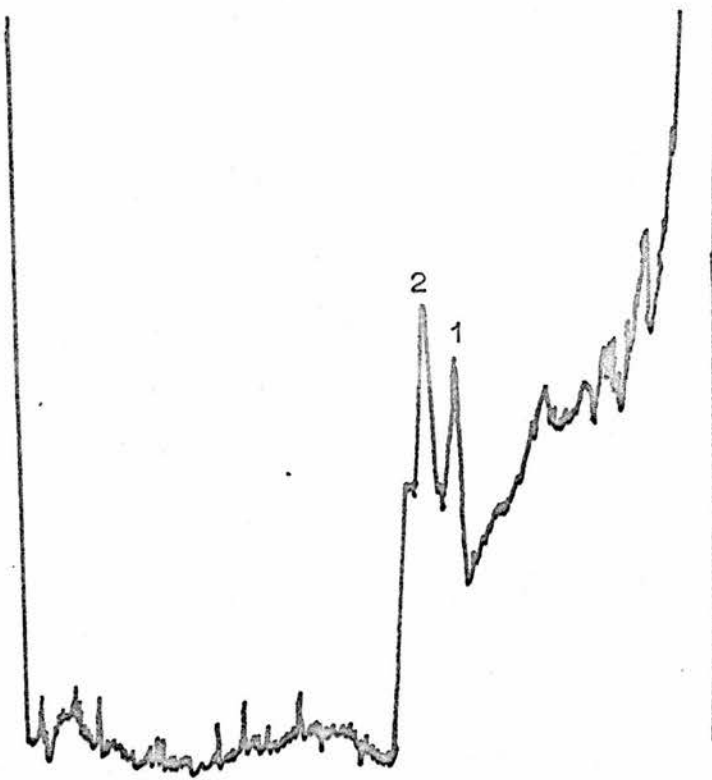
ESTERASE ACTIVITY STAIN

BOTTOM   TOP

8
9
6
7   5   2
3
4   1

PROTEIN STAIN

Figure 4.18 Gel electrophoresis of purified bovine
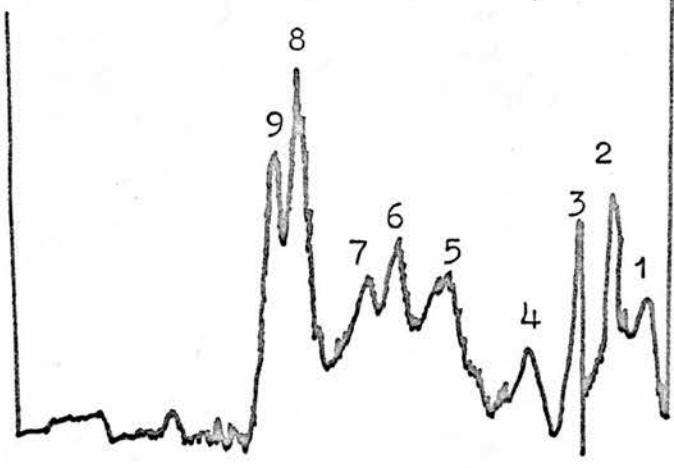adrenal cortical cholesterol ester hydrolase. Cholesterol
ester hydrolase was purified by the method shown in
Figure 4.17. Experimental procedure as Figure 4.12.
Nine protein bands were observed on staining and
two bands of esterase activity were observed.

in close proximity to each other. The mobility of these
two bands corresponded to the mobility of two of the protein
bands on the duplicate gel. When the gel was sliced and .
counted for $[^{32}P]$ radioactivity, both the proteins which
exhibited esterase activity were found to contain $[^{32}P]$ radio-
activity (Figure 4.19). Again it is possible that the two
esterases observed on the gel were the same enzyme in differ-
ent degrees of aggregation. S.D.S. polyacryamide gel electro-
phoresis was therefore used on the same purified phosphorylated
preparation.

S.D.S. polyacryalmide gel electrophoresis of purified cholest- erol ester hydrolase

The phosphorylated protein fraction eluted from the
hydroxylapatite column was desalted by passage through a
Sephadex G-25 column (30cm x 2cm) equilibrated with 3mM
sodium phosphate pH 7.4. The desalted fraction was freeze
dried and the solid redissolved in water up to a protein
concentration of 1 mg per ml. The sample was then heated
with 4M urea, 1% S.D.S. and 1% 2-mercaptoethanol as described
in chapter 2. The sample was applied to a 10cm, 10% poly-
acrylamide gel containing 0.1% S.D.S. together with marker
proteins of globulin, myoglobin, ovalbumin and bovine serum
albumin. Electrophoresis was carried out at 2ma per gel
and the gels were stained for protein and sliced and counted
for $[^{32}P]$ radioactivity. The results of the protein stain
and $[^{32}P]$ radioactivity counting are shown in Figure 4.20
and 4.21. The protein from the hydroxylapatite fraction
was masked by the protein markers but it can clearly be seen
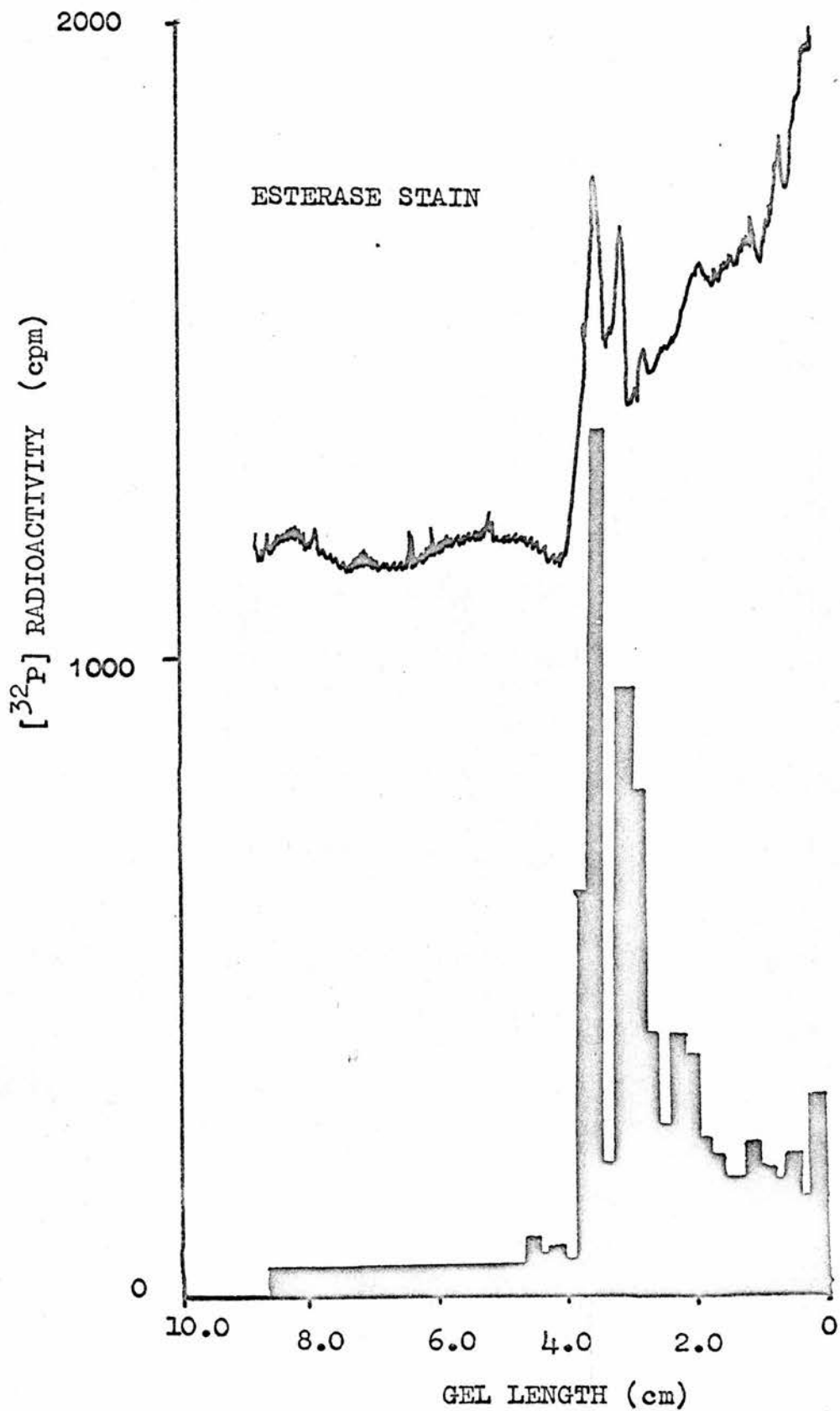from Figure 4.21 that only 1 major peak of $[^{32}P]$ radioactivity

Figure 4.19 Gel electrophoresis of phosphorylated
purified cholesterol ester hydrolase. Cholesterol
ester hydrolase was phosphorylated under the conditions
described in the text and purified as described in
Figure 4.17. Experimental proceedure as text.
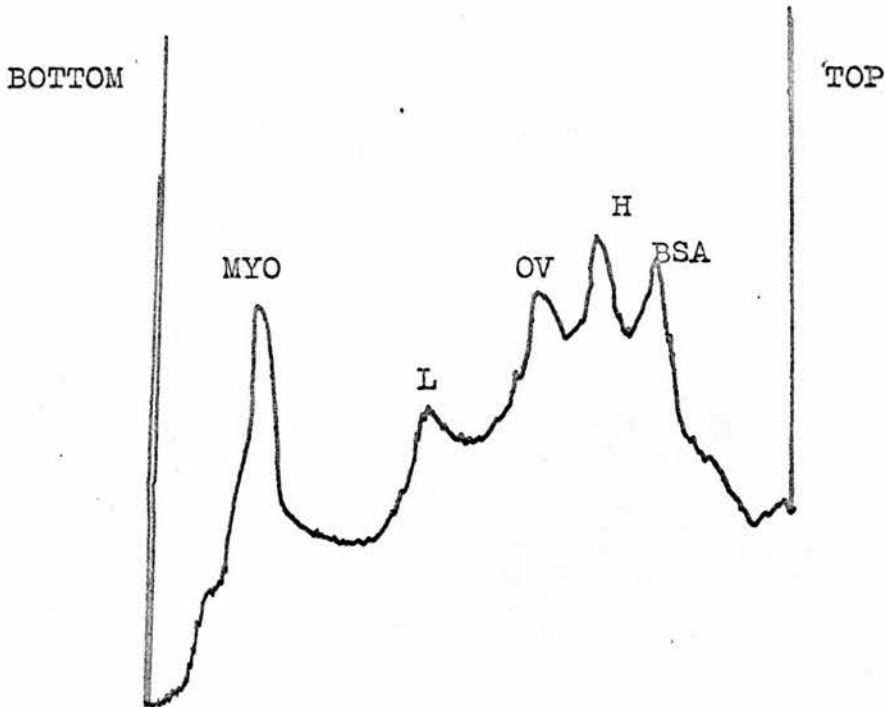
Figure 4.20 Gel scan of a purified preparation of
phosphorylated bovine adrenal cortex cholesterol
ester hydrolase subjected to S.D.S. gel electrophoresis
in the presence of marker proteins. Phosphorylation
of cholesterol ester hydrolase was carried out as
described in the text and purification of cholesterol
ester hydrolase was performed as shown in Figure 4.17.
Experimental procedure as Figure 4.14 using a 10%
polyacrylamide gel. The proteins originating from the
adrenal cortex in the purified preparation and shown
in Figure 4.18 were obscured by the marker proteins.
The phosphorylated cholesterol ester hydrolase was
therefore detected by slicing and counting 2mm gel
slices (Figure 4.21.)

Figure 4.21 Gel electrophoresis of purified

phosphorylated cholesterol ester hydrolase.

Experimental procedure as Figure 4.20.

was produced on the gel in contrast to the two produced in the native system. This is suggestive that the two esterases found on the native gel system may be the same enzyme which have undergone different degrees of aggregation.
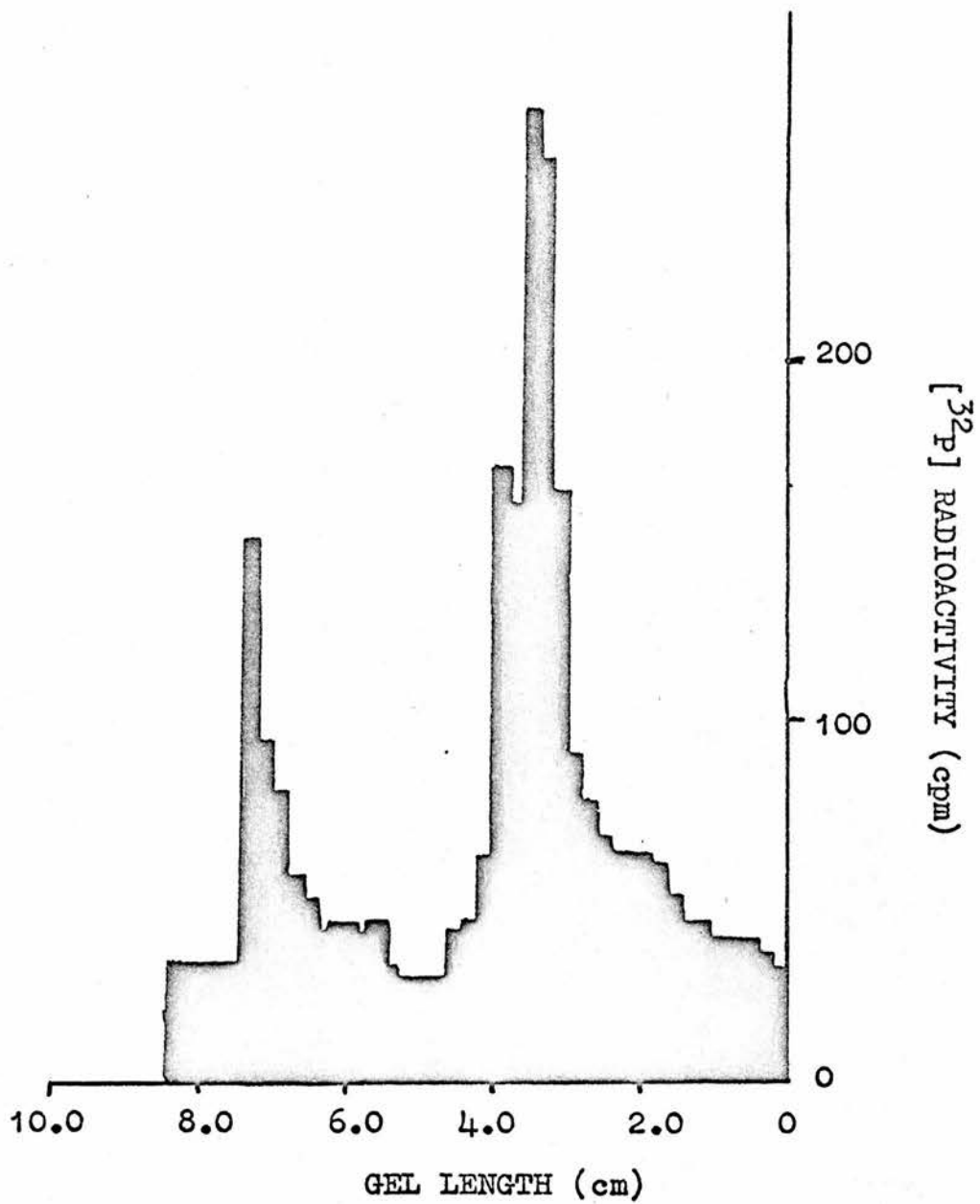
The relative mobilities of the marker proteins and the [^{32}P] radioactivity peak were compared to the mobility of myoglobin and plotted against the molecular weight (Figure 4.22). Again assuming that the [^{32}P] radioactivity is associated with the cholesterol ester hydrolase the molecular weight of the cholesterol ester hydrolase obtained from the graph was found to be approximately 41,000. This result agrees with the molecular weight determined for cholesterol ester hydrolase purified by the first procedure A.

The subunit structure of cholesterol ester hydrolase

The native molecular weight of the purified cholesterol ester hydrolase was determined from its Kav on a 40ml (35cm x 1.2cm) column of Sepharose 4B. Cholesterol ester hydrolase was purified by the method shown in Figure 4.17 and applied to the Sepharose 4B column equilibrated with 20mM potassium phosphate buffer and its elution volume was determined by assaying 2ml fractions eluted from the column for cholesterol ester hydrolase activity. The void volume of the column was determined from the elution volume of blue dextran and the Kav was determined from the following formulae:-

$$Kav = \frac{Ve - Vo}{Vt - Vo}$$ (Sephadex gel filtration in theory and practice-(1973) Pharmacia fine chemicals).

where Vo = void volume of the gel   =12mls

Vt = total volume of the gel   =40mls

Ve = elution volume   =26mls

Kav = the partion coefficient between the liquid and the gel phase
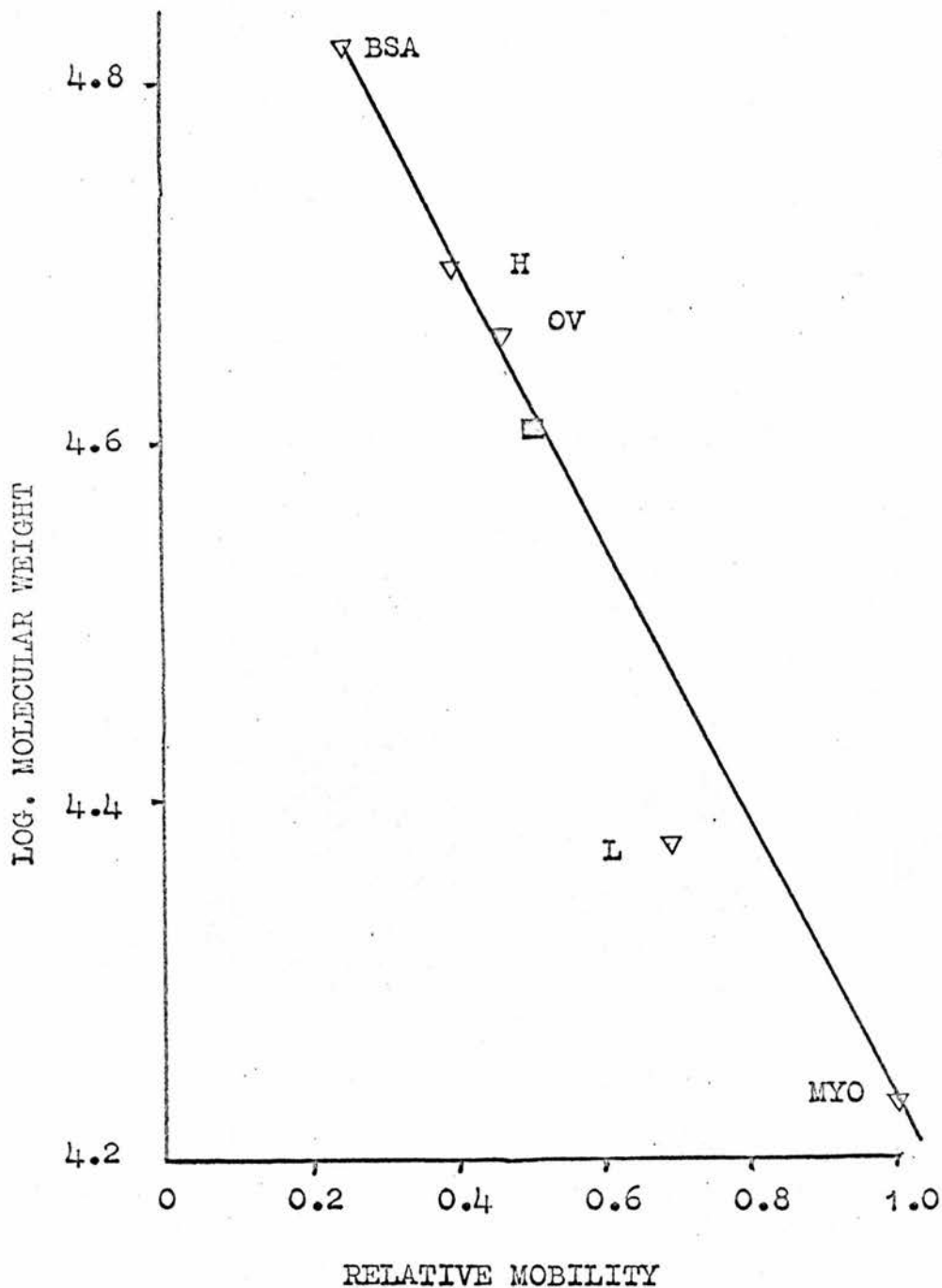
Figure 4.22 Estimation of the molecular weight
of cholesterol ester hydrolase using S.D.S polyacrylamide
gel electrophoresis. The relative mobilities of each
protein was determined from the data presented in
Figure 4.20 and calculated as described in the text.
From this data the molecular weight of cholesterol ester
hydrolase (□) was calculated as 41,000.
BSA= bovine serum albumin, L and H=γglobulin (Light
and Heavy), OV= Ovalbumen.
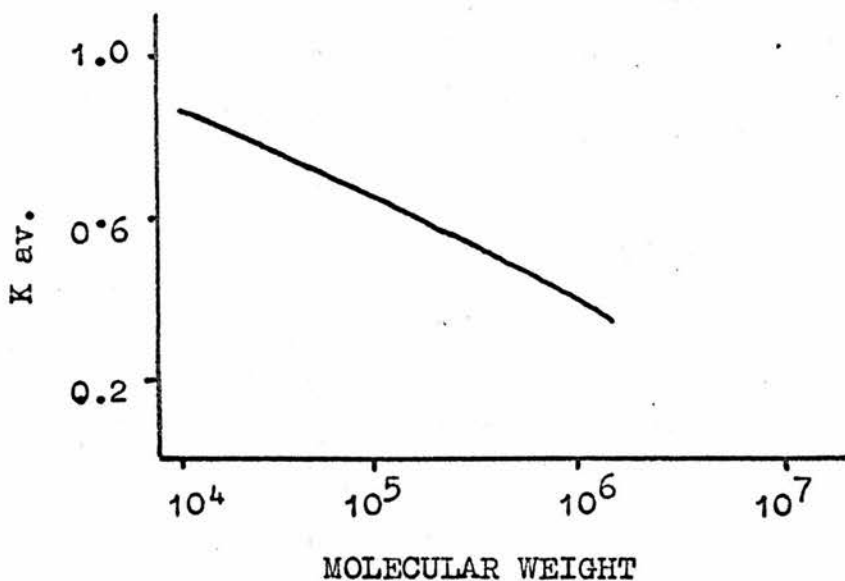
Figure 4.23 The protein molecular weight selectivity
curve of Sepharose 4B determined with Ribonuclease
ovalbumen, transferrin, glucose oxidase, thyroglobulin
and α-crystallin. (From 'Beaded Sepharose 2B-4B-6B'.
Pharmacia Fine Chemicals, Uppsala, Sweden.)

therefore from the results we have

$$Kav = \frac{26-12}{40-12} = 0.5$$

From Figure 4.23 and the calculated Kav the molecular weight of the cholesterol ester hydrolase was found to be about 320,000. This estimation of molecular weight however can only be regarded as approximate. It is possible that the cholesterol ester hydrolase in this preparation is still aggregated in which case the molecular weight determined by this method will be too high.

If the molecular weight of the cholesterol ester hydrolase is of the order of 320,000 then as the subunits appear to have a molecular weight of 41,000 it would seem that the active cholesterol ester hydrolase is composed of eight identical subunits.

The effect of calcium and magnesium ion additions on the activity of purified cholesterol ester hydrolase

The effect of in vitro addition of calcium and magnesium ions on cholesterol ester hydrolase activity in 105,000 x g supernatant was described in chapter 3. It was decided therefore to investigate whether these additions had the same effect on purified cholesterol ester hydrolase (Figure 4.24).

The desalted fraction of cholesterol ester hydrolase from the hydroxylapatite column was assayed for cholesterol ester hydrolase activity in the presence of increasing concentrations of $CaCl_2$ and $MgCl_2$. Figure 4.24 shows the results obtained. It was found that $MgCl_2$ when added at concentrations up to 5mM had no effect on cholesterol ester hydrolase activity. This is in contrast to the inhibitory effect of $MgCl_2$ on cholesterol ester hydrolase observed in the 105,000 x g supernatant reported in chapter 3 Figure 3.4.

EFFECT OF Mg^{++} Ca^{++} AND GSH ON PURIFIED
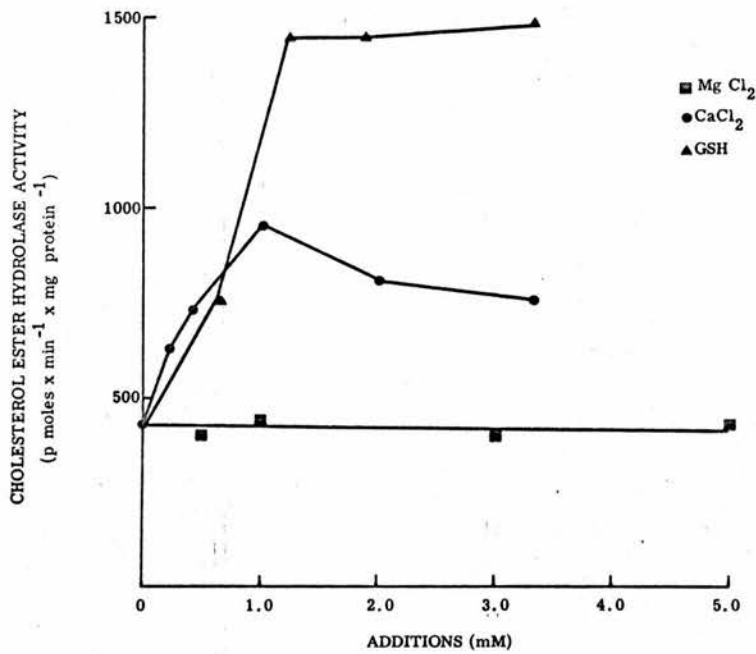CHOLESTEROL ESTER HYDROLASE

Figure 4.24 The effect of magnesium, calcium and glutathione addition on purified cholesterol ester hydrolase activity. Incubations were carried out in duplicate as described in chapter 2 in the presence of $MgCl_2$, $CaCl_2$ and glutathione at the concentrations shown in the Figure.

CaCl$_2$ added to the purified preparation of cholesterol ester hydrolase at concentrations up to 3mM stimulated cholesterol ester hydrolase activity with a maximal stimulation being observed at a concentration of 1mM. Again this is in contrast to the effect of CaCl$_2$ on cholesterol ester hydrolase activity in the desalted supernatant (Figure 3.4). In this crude preparation the addition of 1mM CaCl$_2$ produced practically a total inhibition of cholesterol ester hydrolase activity.

It seems therefore that the inhibitory effect of magnesium and calcium ions on cholesterol ester hydrolase must be mediated through another protein. This protein is absent in the purified preparation and therefore magnesium and calcium ions are not inhibitory in this preparation. It is suggested later in chapter 6 that deactivation of cholesterol ester hydrolase takes place by dephosphorylation of the enzyme involving a calcium or magnesium ion dependent phosphoprotein phosphatase.

The effect of glutathione on the activity of purified cholesterol ester hydrolase

The effect of _in vitro_ addition of glutathione on purified cholesterol ester hydrolase is shown in Figure 4.24. Glutathione stimulated cholesterol ester hydrolase activity which is similar to the effect of glutathione addition shown in Figure 3.4 where glutathione was added to 105,000 x g supernatant. It seems therefore that glutathione has a direct stimulatory effect on cholesterol ester hydrolase.

Summary

(a) Two methods for the purification of cholesterol ester hydrolase are described.

(b) Preincubation of the ammonium sulphate fraction with $[\gamma-^{32}P]$ ATP followed by purification resulted in the isolation of a phosphorylated protein fraction which contained cholesterol ester hydrolase activity.

(c) Purified cholesterol ester hydrolase was not significantly stimulated by in vitro addition of ATP and cyclic AMP.

(d) Polyacrylamide gel electrophoresis of the purified phosphorylated protein fraction showed that the protein which was phosphorylated also had esterase activity.

(e) The phosphorylated protein which had esterase activity existed in different degrees of aggregation.

(f) Polyacrylamide gel electrophoresis on S.D.S. gels showed that cholesterol ester hydrolase had a molecular weight of 41,000.

(g) The molecular weight of the active cholesterol ester hydrolase was tentatively determined as 320,000.

(h) Calcium and magnesium ions exhibited no inhibitory effect on cholesterol ester hydrolase activity in the purified preparation.

CONTENTS

CHAPTER 5

EVIDENCE FOR CYCLIC AMP DEPENDENT PROTEIN KINASE ACTIVATION
OF BOVINE ADRENAL CHOLESTEROL ESTER HYDROLASE

Introduction

The results presented in the last chapter indicate
activation of cholesterol ester hydrolase by phosphorylation
of the enzyme. Phosphorylation occurred by the transfer
of the terminal phosphate from ATP to the protein. Protein
kinases have been shown to catalyse the transfer of the
terminal phosphate of ATP to a variety of proteins (Greengard
and Kuo (1970)). It has been demonstrated that cyclic AMP
activates protein kinase by complexing with a regulatory
subunit and causing the release of a catalytic subunit in
the active state (Garren et al. (1971)). Protein kinases
have been shown to be required for the phosphorylation and
activation of hormone sensitive lipase (Khoo et al. (1972))
and phosphorylase kinase (Cohen (1973)).

This chapter describes experiments carried out to eluc-
idate the role of cyclic AMP dependent protein kinase in
the activation of cholesterol ester hydrolase.

Activation of cholesterol ester hydrolase by cyclic AMP
dependent protein kinases from different sources

Figure 5.1 shows the effect of in vitro addition of
the cofactors 5mM ATP/MgCl$_2$ and 10 μM cyclic AMP on the
purified preparation of cholesterol ester hydrolase isolated
from the Sepharose 4B column as described in chapter 3.
Cofactor addition produced a 70% stimulation of cholesterol
ester hydrolase activity.

The effect of cyclic AMP dependent protein kinase addition on the activity of purified cholesterol ester hydrolase in the presence and absence of cofactors was investigated. Three cyclic AMP dependent protein kinases were employed in the experiment. The kinases were isolated from rabbit skeletal muscle (PKI and PKII) and beef heart (PKIII) as is described in chapter 2. PKI and PKII are thought to be isoenzymes (Cohen (1973)). PKI (28mg per ml) and PKII (75 mg per ml) were used at a final concentration of 0.28mg per ml and 0.25 mg per ml respectively. PKIII from heart muscle was used at a final concentration of 0.25 mg per ml. After addition of the cofactors and protein kinase cholesterol ester hydrolase activity was determined as described in chapter 2.

None of the kinases added per se gave a significant stimulation of cholesterol ester hydrolase activity when added in vitro. PKIII gave an inhibition of cholesterol ester hydrolase activity when added in vitro. (Figure 5.1). The addition of each of the cyclic AMP dependent protein kinases to the purified cholesterol ester hydrolase preparation together with cofactors resulted in approximately a 130% stimulation of cholesterol ester hydrolase activity (Figure 5.1). The 70% stimulation of cholesterol ester hydrolase activity by ATP and cyclic AMP addition alone could be due to some endogenous protein kinase activity. However protein kinase activity was not detectable using the protein kinase assay involving the phosphorylation of histone (chapter 2).

Increase in cholesterol ester hydrolase activity on incubation with $[\gamma - ^{32}P]$ ATP and cyclic AMP dependent protein kinase PKI was accompanied by a transfer of the terminal

phosphate from $[\gamma-^{32}P]$ATP to the protein. The $[^{32}P]$ radio-
activity remained bound after precipitation of the protein
with trichloroacetic acid (chapter 2). After trichloro-.
acetic acid precipitation of the protein 68 pmoles of phos-
phate per mg protein remained bound. This compares to the
53 pmoles of phosphate bound to the protein in the void volume
fraction from the Sepharose 4B column described in chapter 4
and shown in Figure 4.7.

It is quite possible that much of the cholesterol ester
hydrolase recovered from the Sepharose 4B void volume fraction
is in the active phosphorylated state. If this was the case
then of course $[^{32}P]$ radioactivity from the $[\gamma-^{32}P]$ ATP could
not be incorporated into the enzyme. This would therefore
give a molar ratio of $[^{32}P]$ radioactivity incorporation into
the cholesterol ester hydrolase lower than the actual phos-
phate to protein ratio.

Time course of activation and phosphorylation of cholesterol ester hydrolase

Estimation of activation and phosphorylation of cholest-
erol ester hydrolase over a 20 minute incubation period was
carried out on the purified preparation of the enzyme obtained
from the void volume fractions of the Sepharose 4B column.

It has been shown by Trzeciak and Boyd (1974) that incubation
of an ammonium sulphate protein fraction from the bovine
105,000 x g supernatant results in a loss of phosphate from
the protein accompanied by a concomitant decrease in cholesterol
ester hydrolase activity. The protein from the Sepharose 4B
column used in this experiment was derived from an ammonium
sulphate fraction which had been incubated with 5mM $MgCl_2$

EFFECT OF PROTEIN KINASE ON THE ACTIVATION OF CHOLESTEROL ESTER HYDROLASE
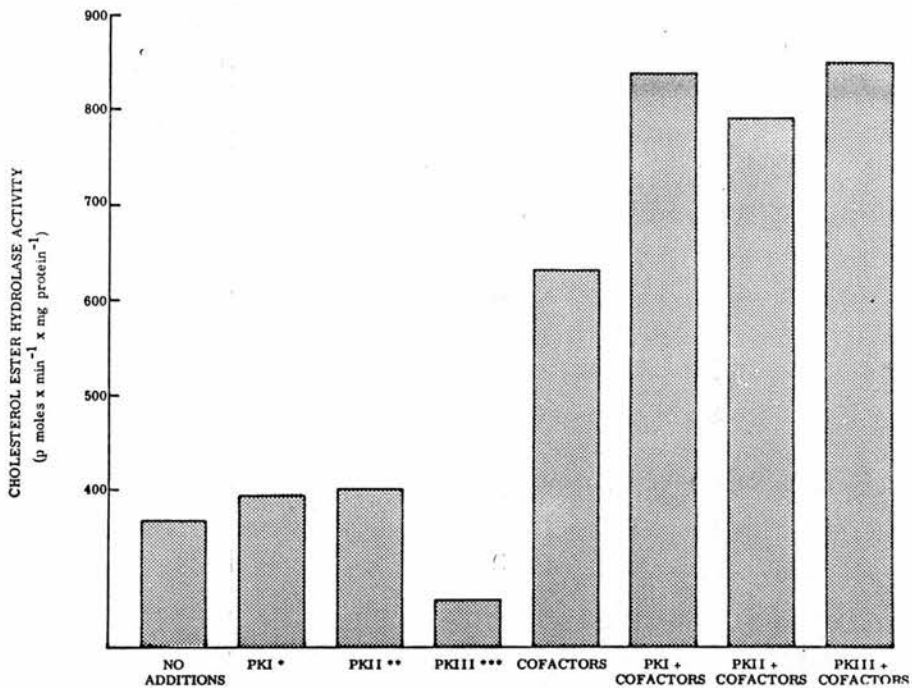
Figure 5.1 Activation of cholesterol ester hydrolase

by ATP, cyclic AMP and cyclic AMP dependent protein

kinase, Incubations were carried out in duplicate

and ATP, cyclic AMP and cyclic AMP dependent protein

kinase was added as indicated. Cholesterol ester hydrolase

was purified by the procedure shown in Figure 4.3

up to the Sepharose 4B gel filtration.

Cofactors = 5mM ATP + 10 μM cyclic AMP + 5mM Magnesium chloride

PKI and PKII= Rabbit skeletal muscle protein kinase

isoenzymes I and II. PKIII = Beef heart protein kinase

at $30^{\circ}C$ for ten minutes. The purpose of this procedure was to obtain a preparation of cholesterol ester hydrolase which had been as far as possible deactivated and dephosphorylated. Using this preparation two identical incubations were carried out at $30^{\circ}C$. Each incubation contained 10mls of purified cholesterol ester hydrolase (0.7mgs protein per ml) 10 μM cyclic AMP, 1mM $MgCl_2$, 30mM KCl.

One incubation was started by the addition of 20 μl of 5mM ATP and the other by the addition of 20μ l of 5mM ATP containing 0.1mCi of $[\gamma-^{32}P]$ ATP. 1ml samples were taken from the incubation containing $[\gamma-^{32}P]$ ATP into 2mls of ice cold 10% trichloroacetic acid at the time intervals shown in Figure 5.2. The determination of $[^{32}P]$ radioactivity bound to the protein was then carried out by the method described in chapter 2.

At the same time intervals 1ml samples were taken from the duplicate incubation into EDTA at a final concentration of 4mM to stop the phosphorylation and activation. The activity of cholesterol ester hydrolase was then determined in each sample in the usual way.

The results of the experiment are presented in Figure 5.2. It can be seen that after 15 minutes, activation of cholesterol ester hydrolase was complete. For the first 4 minutes, phosphorylation closely paralleled cholesterol ester hydrolase activity. However when the cholesterol ester hydrolase had plateaued at fifteen minutes a slow incorporation of $[^{32}P]$ radioactivity into the protein still seemed to be taking place. This is consistent with the observations of Cohen (1973) concerning the phosphorylation of phosphorylase kinase. This may be indicative of a second site phosphorylation of cholesterol ester hydrolase similar to that of phosphorylase kinase.

ACTIVATION AND PHOSPHORYLATION OF CHOLESTEROL
ESTER HYDROLASE
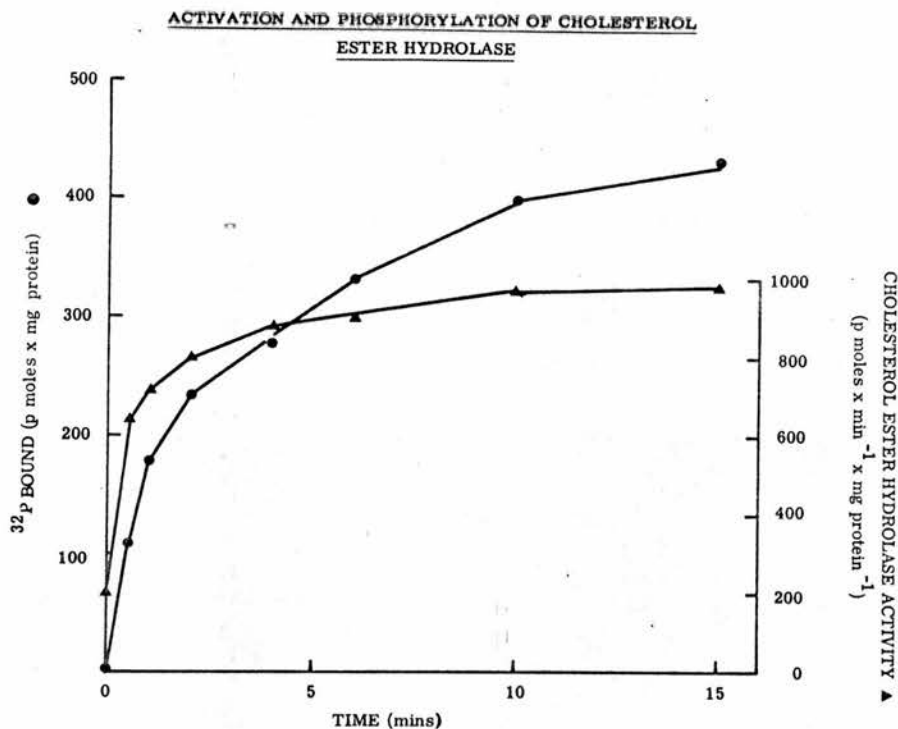
Figure   5.2 Time course of activation and phosphorylation

of cholesterol ester hydrolase. A purified preparation

of bovine adrenal cortex cholesterol ester hydrolase

prepared as shown in Figure 4.3 up to and including

the Sepharose 4B gel filtration step was incubated

under the conditions described in the text. Duplicate

samples (1ml) were taken at the times indicated and

assayed for cholesterol ester hydrolase activity and

protein bound [^{32}P] radioactivity as described in

chapter 2.

Using the purified preparation of cholesterol ester
hydrolase derived from an ammonium sulphate fraction incubated
with $MgCl_2$ a much greater molar incorporation of $[^{32}P]$ radio-
activity was found. After 15 minutes 430 pmol of phosphate
per mg protein was found to be incorporated compared to 68 pmol
per mg protein found in the previous experiment. This
indicates that much of the cholesterol ester hydrolase
reocvered from the Sepharose 4B column is already in the
active phosphorylated form.

Activation of cholesterol ester hydrolase by cyclic AMP
dependent protein kinase in a purified preparation from a
hydroxylapatite column

It can be seen from Figure 5.1 that when ATP and cyclic
AMP are added to the Sepharose 4B void volume fraction a
70% stimulation of cholesterol ester hydrolase activity
occurs. In chapter 4 it was shown that elution of the
Sepharose 4B void volume from hydroxylapatite resulted in
a fraction of the cholesterol ester hydrolase being obtained
that was not stimulated by ATP and cyclic AMP additions.
The effect of cofactor and cyclic AMP dependent protein
kinase addition on cholesterol ester hydrolase activity in ·
the purified preparation obtained from hydroxylapatite
chromatography (chapter 4) is shown in Figure 5.3. Cyclic
AMP dependent protein kinase PKIII from beef heart was
used in the incubations at a concentration of 0.25mg per ml.
After addition of cofactors and protein kinase, cholesterol
ester hydrolase activity was determined.

Cyclic AMP or cyclic AMP plus ATP gave no significant
stimulation of cholesterol ester hydrolase activity when
added in vitro. PKIII added in the presence of cofactors
produced a two fold stimulation of cholesterol ester hydro-
lase activity.

ACTIVATION OF PURIFIED CHOLESTEROL
ESTER HYDROLASE BY PROTEIN KINASE
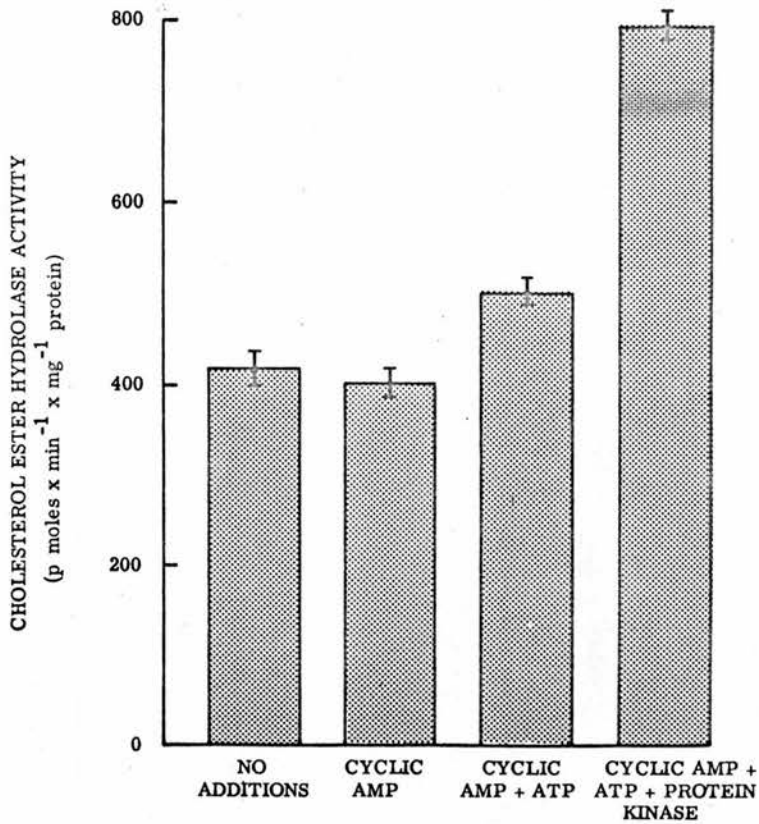
Figure 5.3 Activation of purified cholesterol ester

hydrolase by cyclic AMP dependent protein kinase.

Duplicate incubations were carried out on a preparation

of bovine adrenal cortex cholesterol ester hydrolase

purified as described in Figure 4.3, in the presence

of 5mM ATP, 10 uM cyclic AMP and cyclic AMP dependent

protein kinase PKIII as indicated. Cholesterol ester

hydrolase activity was determined as described in chapter 2.

<u>The effect of cyclic AMP dependent protein kinase inhibitor</u>
<u>protein on cholesterol ester hydrolase activity in the de-</u>
<u>salted 105,000 x g supernatant</u>

The stimulatory effect of ATP and cyclic AMP on cholest-
erol ester hydrolase activity in 105,000 x g supernatant
is shown in Figure 5.4. If the activation of cholesterol
ester hydrolase by ATP and cyclic AMP addition is mediated
by a cyclic AMP dependent protein kinase then the cyclic
AMP dependent protein kinase inhibitor protein isolated by
Walsh <u>et al</u>. (1971) should inhibit this activation. Cyclic
AMP dependent protein kinase inhibitor when added to the
105,000 x g desalted supernatant at a final concentration
of 100μ g per ml had no effect on cholesterol ester hydrolase
activity. When the inhibitor was added prior to the addition
of 5mM ATP and 10μ M cyclic AMP, the stimulation produced
by the addition of these cofactors was completely inhibited.

THE EFFECT OF PROTEIN KINASE INHIBITOR ON THE ACTIVATION
OF CHOLESTEROL ESTER HYDROLASE
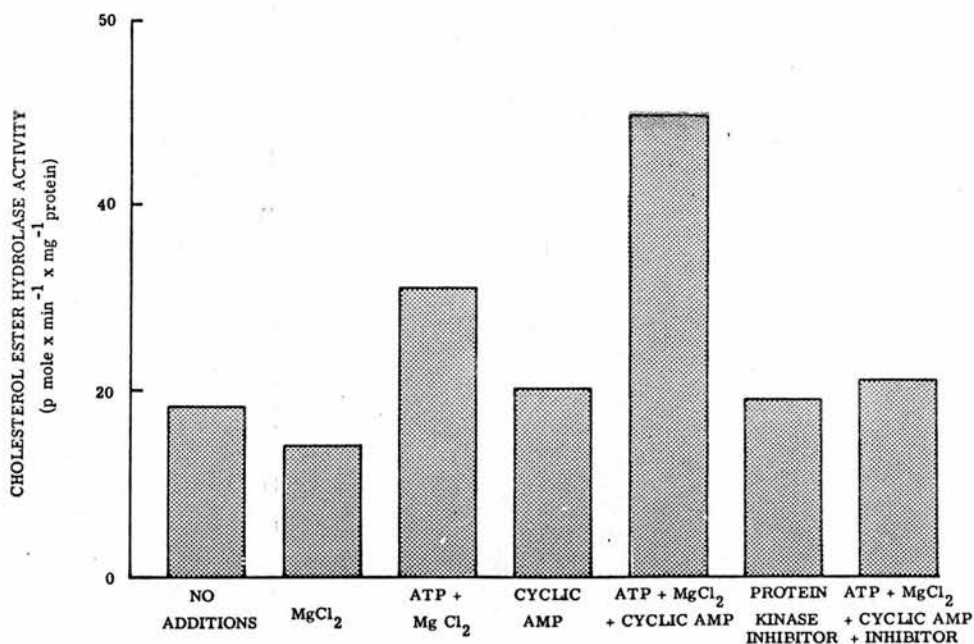
Figure 5.4 The effect of protein kinase inhibitor on

the activation of cholesterol ester hydrolase. Assay

for cholesterol ester hydrolase was carried out as

described in chapter 2 with the addition of 5mM ATP,

10 uM cyclic AMP and 100 ug of protein kinase

inhibitor as indicated. The incubations employed (1ml)

of crude adrenal cortical supernatant.

SUMMARY

a) Cyclic AMP dependent protein kinases from different sources were capable of stimulating cholesterol ester hydrolase activity in the presence of ATP and cyclic AMP.

b) Activation of cholesterol ester hydrolase by these protein kinases resulted in the transfer of $[^{32}P]$ radioactivity from $[\gamma - {^{32}P}]$ ATP to the protein.

c) The rate of activation of cholesterol ester hydrolase by protein kinase closely paralleled the phosphorylation of the protein.

d) Cyclic AMP dependent protein kinase inhibitor protein abolished the activation of cholesterol ester hydrolase in the 105,000 x g cortical supernatant usually observed on the addition of cofactors.

CONTENTS

CHAPTER 6

EVIDENCE FOR THE DEACTIVATION OF CHOLESTEROL ESTER HYDROLASE BY A MECHANISM INVOLVING A PHOSPHOPROTEIN PHOSPHATASE

Introduction

The work presented in chapters 4 and 5 has indicated that activation of cholesterol ester hydrolase occurs by phosphorylation. This phosphorylation occurred by the transfer of the terminal phosphate from ATP to the inactive cholesterol ester hydrolase. This transfer was facilitated by a cyclic AMP dependent protein kinase.

Phosphorylase kinase is known to catalyse the conversion of phosphorylase b to phosphorylase a, by a mechanism involving the transfer of the terminal phosphate group of ATP to a specific serine residue in phosphorylase (Fischer and Krebs (1955)); (Krebs and Fischer (1956)); (Krebs et al. (1958)) and (Nolan et al. (1964)). Phosphorylase kinase is known to be activated by a similar mechanism involving a cyclic AMP dependent protein kinase (Delange et al. (1967)).

Deactivation of both phosphorylase a, (Cori and Green (1943)) (Graves et al. (1960)) and phosphorylase kinase (Cohen and Antoniw (1973)) is accomplished by a dephosphorylation of the protein catalysed by a phosphoprotein phosphatase.

In chapter 3 the effect of in vitro addition of $MgCl_2$ and $CaCl_2$ to desalted 105,000 x g adrenal supernatant was investigated. It was shown that $MgCl_2$ and $CaCl_2$ when added at millimolar concentrations produced an inhibition of cholesterol ester hydrolase activity (Figure 3.4). When the same additions were made to a purified preparation of cholesterol ester hydrolase at millimolar concentrations no inhibition of enzymic activity was observed (Figure 4.24). From these

results therefore it seems that the inactivation of cholest-
erol ester hydrolase in the 105,000 x g supernatant is mediated
through another factor which is present in the crude super-
natant but not in the purified preparation. This factor
appears to be magnesium and calcium ion dependent and as
it is not removed from the 105,000 x g supernatant on desalt-
ing by passage through a Sephadex G-25 column it seems that
this factor has a molecular weight greater than 5,000.

It is possible therefore that the magnesium ion depend-
end factor is a phosphoprotein phosphatase. The experiments
carried out in this chapter were designed to discover if
the deactivation of cholesterol ester hydrolase occurred
by a dephosphorylation mechanism and whether the dephosphor-
ylation was catalysed by a phosphoprotein phosphatase.

The effects of preincubation of the bovine 105,000 x g cortical
supernatant with magnesium ions, ATP and cyclic AMP on chol-
esterol ester hydrolase activity

It has been shown in chapter 3 that magnesium ion addition
to crude 105,000 x g supernatant caused an inhibition of
cholesterol ester hydrolase activity. The effects of a prolonged
preincubation of the 105,000 x g supernatant in the presence
of $MgCl_2$ at $37^{o}C$ is shown in Figure 6.1. Incubations of
10mls were set up with the additions shown in Figure 6.1
and 1ml samples were withdrawn at the times indicated and
assayed for cholesterol ester hydrolase activity using a
20 minute incubation.

It can be seen from Figure 6.1 that preincubation of
the 105,000 x g supernatant for 120 minutes gave no significant
change in cholesterol ester hydrolase activity. However
if this supernatant was desalted on a Sephadex G-25 column,

THE EFFECT OF MAGNESIUM AND ATP ON CHOLESTEROL ESTER HYDROLASE
ACTIVITY IN BOVINE ADRENAL 105,000 x g CORTICAL SUPERNATANT

Legend:
- Not desalted
- Desalted
- Desalted + 5×10^{-3} M MgCl$_2$
- Desalted + 5×10^{-3} M MgCl$_2$ + 5×10^{-3} M ATP + 10^{-5} M cyclic AMP

Y-axis: Cholesterol Ester Hydrolase Activity (p moles x min^{-1} x mg Protein^{-1})
X-axis: Time (mins)
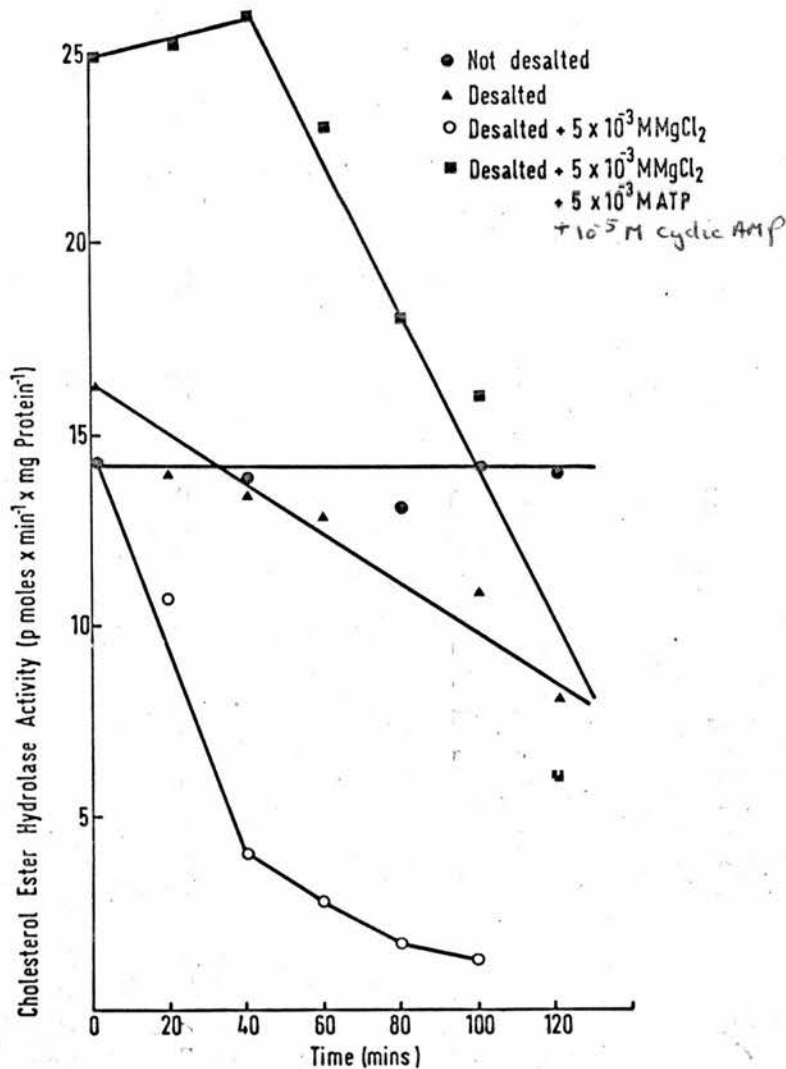
Figure   6.1   The effects on cholesterol ester hydrolase
activity of preincubation of bovine adrenal cortical
supernatant with magnesium ions, ATP and cyclic AMP.
Preincubation of the supernatant was carried out for
120 minutes in the presence of MgCl$_2$ (5mM), ATP(5mM)
and cyclic AMP (10 uM) as indicated in the Figure.
Samples (1ml) were taken every 20minutes and assayed
for cholesterol ester hydrolase activity as described
in chapter 2.

preincubation resulted in a linear decrease in cholesterol
ester hydrolase activity.

The addition of 5mM $MgCl_2$ to the desalted preparation
greatly enhanced the rate of deactivation of cholesterol
ester hydrolase. After 80 minutes preincubation practically
no cholesterol hydrolase activity was detectable.

Preincubation of the 105,000 x g desalted supernatant
in the presence of 5mM $MgCl_2$, 5mM ATP and 10 μM cyclic AMP
resulted in the usual stimulation of cholesterol ester hydro-
lase activity but again after a 60 minute preincubation,
deactivation of cholesterol ester hydrolase took place until
after 120 minutes the activity had fallen to the same value
as that of the desalted control.

When the 105,000 x g desalted supernatant was preincubated
for 60 minutes in the presence of 5mM $MgCl_2$ and then 5mM
ATP and 10 μM cyclic AMP were added, activation of cholesterol
ester hydrolase was achieved (Table 6.1). From the Table
it can be seen that cholesterol ester hydrolase activity
had fallen 50% over the 60 minute preincubation in the presence
of 5mM $MgCl_2$. Addition of 5mM ATP and 10 μM cyclic AMP to
this preincubated preparation gave a stimulation of cholesterol
ester hydrolase activity to the activity found in the control
where ATP and cyclic AMP were present for the whole of the
incubation period. The activity of cholesterol ester hydro-
lase however could not be stimulated to the activity found
in the control at zero time. This could be due to a denatur-
ation of the cholesterol ester hydrolase or the protein kinase
over the 60 minute preincubation period.

Table 6.1 The effect on cholesterol ester hydrolase activity of preincubation of bovine adrenal 105,000 x g supernatant with $MgCl_2$ followed by 5mM ATP and 10 μM cyclic AMP addition.

Time of preincubation	Cholesterol ester hydrolase activity pmol x min^{-1} x mg protein^{-1}		
	0 mins	60mins	60 mins + ATP+cyclic AMP
105,000 x g supernatant + 5mM $MgCl_2$	34	17	27
105,000 x g supernatant +5mM ATP + 10 μM cyclic AMP + 5mM $MgCl_2$	48	24	26

Experimental detail as text

The effect of magnesium ion addition on cholesterol ester
hydrolase activity and on the amount of protein bound [^{32}P]
radioactivity in the Sephadex G-200 void volume protein fraction

As magnesium ions promote the deactivation of cholesterol
ester hydrolase, the effect of magnesium ions on the amount
of [^{32}P] radioactivity protein bound was investigated. A
partially purified preparation of cholesterol ester hydrolase
obtained from the Sephadex G-200 column was phosphorylated
by preincubation of the void volume fraction at 30°C for
20 minutes with 10 μM cyclic AMP, 1mM MgCl$_2$, 30mM KCl, 20mM
potassium phosphate buffer pH 7.4 and 0.1mM ATP containing
125 μCi of [γ -^{32}P] ATP. After 20 minutes the mixture was
desalted by passage through a Sephadex G-25 column equilibrated
with 10mM potassium phosphate buffer pH 7.4. The phosphory-
lated protein was then preincubated for thirty minutes in
the presence of 5mM MgCl$_2$ and the samples were taken at the
times indicated in Figure 6.2 and these samples were assayed
for protein bound [^{32}P] radioactivity and cholesterol ester
hydrolase activity. From Figure 6.2 it can be seen that
deactivation of cholesterol ester hydrolase closely parall-
eled the dephosphorylation of the protein fraction.

The effect of alkaline phosphatase on cholesterol ester hydro-
lase activity

The effect on cholesterol ester hydrolase activity of
preincubating an ammonium sulphate protein fraction with
calf liver alkaline phosphatase is shown in Figure 6.3. The
ammonium sulphate protein fraction was prepared and desalted
on a Sephadex G-25 column as described in chapter 4. The
desalted protein fraction (10mls) was then preincubated for
a period of 80 minutes in the presence of 20 μl of alkaline

EFFECT OF Mg^{++} ON THE DEACTIVATION AND DEPHOSPHORYLATION
OF PARTIALLY PURIFIED CHOLESTEROL ESTER HYDROLASE
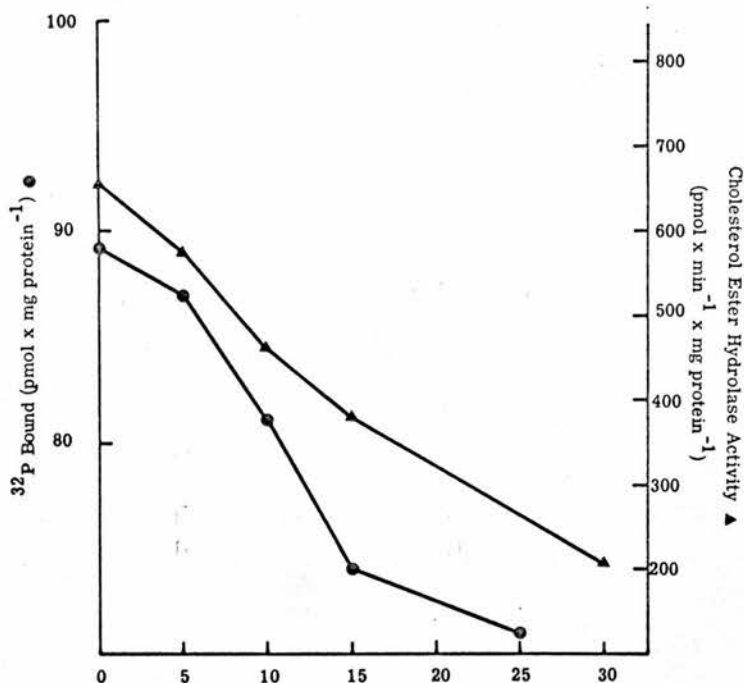
Figure 6.2 The effect of magnesium ion addition on

the deactivation and dephosphorylation of a partially

purified phosphorylated fraction of bovine adrenal

cortex cholesterol ester hydrolase. A partially

purified preparation of cholesterol ester hydrolase

obtained from the Sephadex G 200 column was phosphorylated

under the conditions described in the text. The

phosphorylated protein was then preincubated with (5mM)

MgCl$_2$ and samples taken at the times indicated and

assayed for cholesterol ester hydrolase activity.

DEACTIVATION OF CHOLESTEROL ESTER HYDROLASE ON ADDITION OF ALKALINE PHOSPHATASE
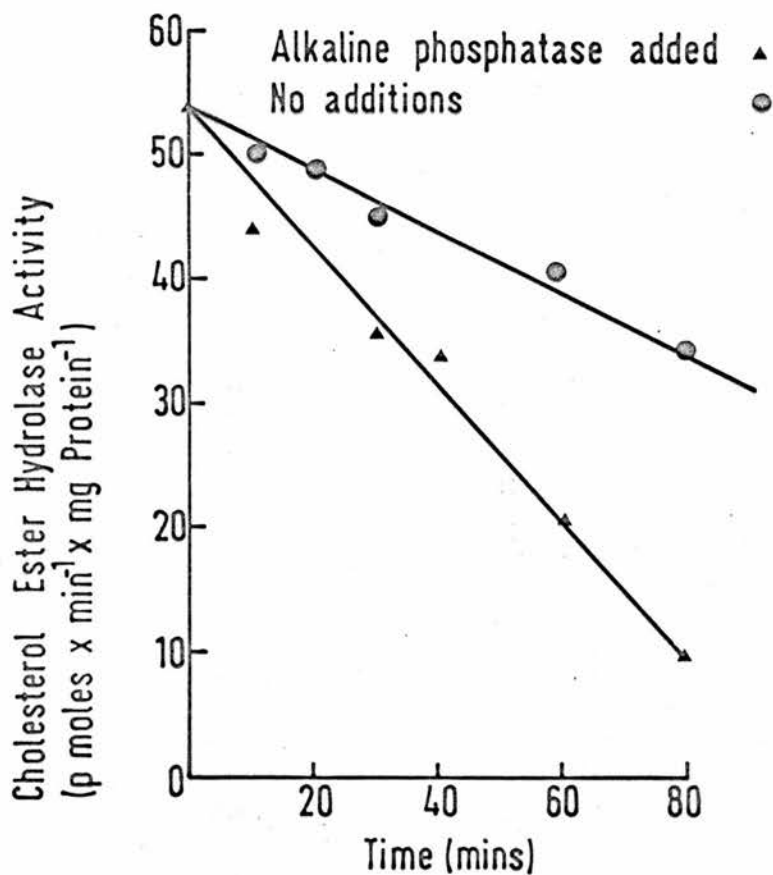
Figure 6.3 The deactivation of bovine adrenal
cholesterol ester hydrolase by beef liver alkaline
phosphatase. Experimental procedure as text. Incubations
were carried out in duplicate at 37°C.

phosphatase and 1ml samples were taken at the times indicated in Figure 6.3 and assayed for cholesterol ester hydrolase activity. A control incubation was also carried out in which no additions were made. It can be seen from Figure 6.3 that the addition of alkaline phosphatase produced a three fold increase in the rate of deactivation of cholesterol ester hydrolase. This would indicate that a phosphatase could be responsible for the deactivation of the enzyme.

The effect of phosphodiesterase on cholesterol ester hydrolase activity in the 105,000 x g supernatant

If a cyclic AMP dependent protein kinase is involved in activation of cholesterol ester hydrolase then preincubation of the 105,000 x g supernatant with cyclic AMP phosphodiesterase should lead to a decrease in cholesterol ester hydrolase activity. The effect of preincubating a 105,000 x g cortical supernatant fraction with phosphodiesterase was therefore investigated. Desalted 105,000 x g cortical supernatant 6mls was preincubated for 80 minutes with the addition of 0.14mg of cyclic AMP phosphodiesterase and 1ml samples were taken from the incubation every 20 minutes and assayed for cholesterol ester hydrolase activity. Control incubations containing no additions, 5mM $MgCl_2$ and beef liver alkaline phosphatase were carried out and the results are shown in Figure 6.4. Phosphodiesterase addition caused an increased rate of deactivation of cholesterol ester hydrolase compared to the control where no additions were made. Both $MgCl_2$ and alkaline phosphatase when added to the same preparation produced an increase in the rate of deactivation of the cholesterol ester hydrolase.
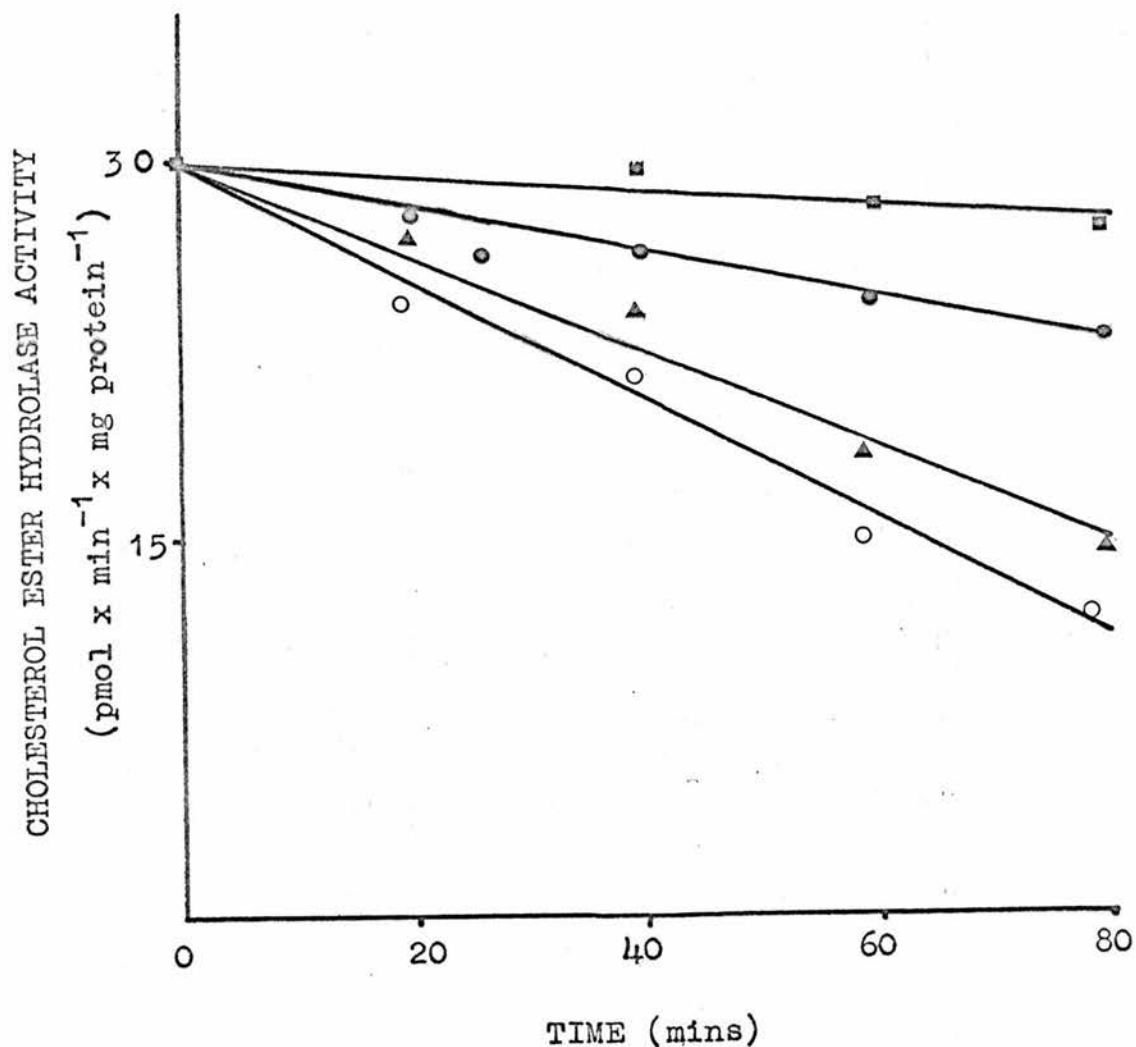
Figure 6.4 The effect of cyclic AMP phosphodiesterase
on cholesterol ester hydrolase activity in bovine adrenal
cortex 105,000 x g supernatant.

The incubations were carried out as described in the
text in the presence of cyclic AMP phosphodiesterase (●)
$MgCl_2$ (5mM) (▲) alkaline phosphatase (O) and no additions
(■).

The deactivation and dephosphorylation of cholesterol ester hydrolase in a purified preparation by α and β phosphorylase kinase phosphatase

The previous experiments in this chapter have indicated that a dephosphorylation of cholesterol ester hydrolase produces an inactivation of the enzyme. However in the previous experiments crude preparations of cortical supernatant were used and it is possible that some other protein was being dephosphorylated in these preparations. For this reason the following experiments were carried out on a purified preparation of cholesterol ester hydrolase obtained from the void volume fraction of a Sepharose 4B column prepared as described in chapter 4. The void volume fraction was subjected to a 30 minute 105,000 x g centrifugation and the lipid layer removed. The clear infranatant was used for the experiment.

10mls of the infranatant protein (0.8mgs per ml) was preincubated for 10 minutes at 30°C in the presence of 50mM sodium glycerophosphate, 1mM $MgCl_2$, 4mM NaF, 2mM theophylline, 10μM cyclic AMP and 0.1mM ATP containing 0.1 mCi of $[\gamma-^{32}P]$ ATP. After preincubation the mixture desalted by passage through a Sephadex G-25 column (30cm x 2cm) equilibrated with 50mM Tris chloride buffer pH 7.0. An addition of EDTA was then made to the phosphorylated protein fraction to a final concentration of 1mM. This preparation was then preincubated for 25 minutes with the addition of α or β phosphorylase kinase phosphatase, prepared by the method of Antoniw and Cohen (1975), at a 200 fold dilution in the presence or absence of 2mM $MgCl_2$. Samples (0.5mls) were taken at the times indicated in Figure 6.5 and assayed for protein bound [^{32}P] radioactivity and cholesterol ester hydrolase activity by the method described
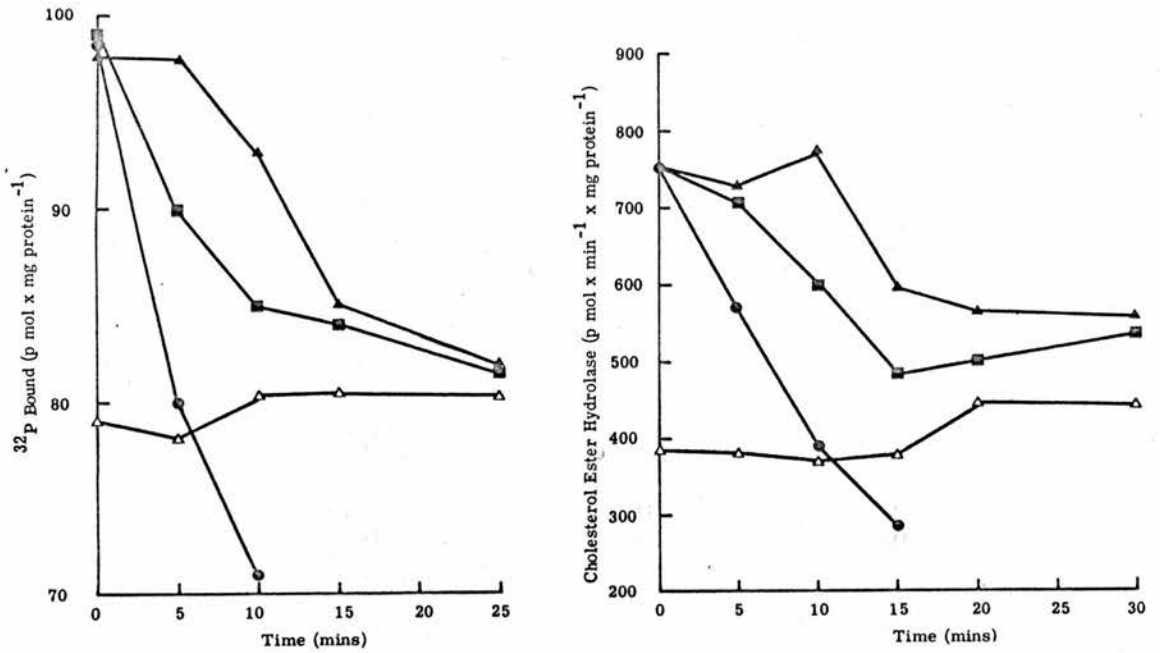
Figure 6.5 The deactivation and dephosphorylation
of bovine adrenal cortex cholesterol ester hydrolase
in a purified preparation by α and β phosphorylase kinase
phosphatase. Experimental procedure as text.
($\blacksquare$) β phosphorylase kinase phosphatase,($\blacktriangle$) α phosphorylase
kinase phosphatase, ($\bullet$) α phosphorylase kinase
phosphatase + $MnCl_2$(2mM) ($\triangle$) alkaline phosphatase

in Chapter 2. The effect of the addition of 20 μl of calf liver alkaline phosphatase at pH 8.4 was also investigated. The results are shown in Figure 6.5. It can be seen that both α and β phosphorylase kinase phosphatase produced a deactivation of cholesterol ester hydrolase activity and in the case of α phosphorylase kinase phosphatase the addition of 2mM $MnCl_2$ caused an increased rate of deactivation of cholesterol ester hydrolase. The α phosphorylase kinase phosphatase preparation however did contain some β phosphorylase kinase phosphatase activity (J. Antoniw personal communication). The addition of alkaline phosphatase caused an immediate deactivation of cholesterol ester hydrolase.

It can be seen that in each case the deactivation of cholesterol ester hydrolase by the phosphatases was closely paralleled by the loss of bound [^{32}P] radioactivity from the protein.As shown in chapter 3 only cholesterol ester hydrolase seemed to be phosphorylated in the Sepharose 4B void volume fraction. It seems therefore that cholesterol ester hydrolase is deactivated by a dephosphorylation process involving a phosphoprotein phosphatase.

The correlation between cholesterol ester hydrolase activity and bound [^{32}P] radioactivity

If cholesterol ester hydrolase activity is related to its phosphorylation it should be possible to obtain a directly proportional relationship between the amount of [^{32}P] radioactivity bound to the protein and the cholesterol ester hydrolase activity.

The results obtained from the previous experiment and
from the deactivation of cholesterol ester hydrolase by magnesium
ions in a crude enzyme preparation was therefore plotted
in this manner. The results shown in Figure 6.6 indicate
that there is a directly proportional relationship between
cholesterol ester hydrolase activity and phosphorylation.
When the graph was extrapolated to zero cholesterol ester
hydrolase activity it was found that 50% of the $[^{32}P]$ radio-
activity remained bound. This could be interpreted as
indicating a deactivation of cholesterol ester hydrolase
by a second site phosphorylation (Cohen and Antoniw (1973))
and this will be discussed in chapter 10. The results presented
in this chapter do indicate that deactivation of cholesterol
ester hydrolase occurs by a dephosphorylation of the active
phosphorylated enzyme.

CORRELATION BETWEEN CHOLESTEROL ESTER
HYDROLASE ACTIVITY AND BOUND ^{32}P
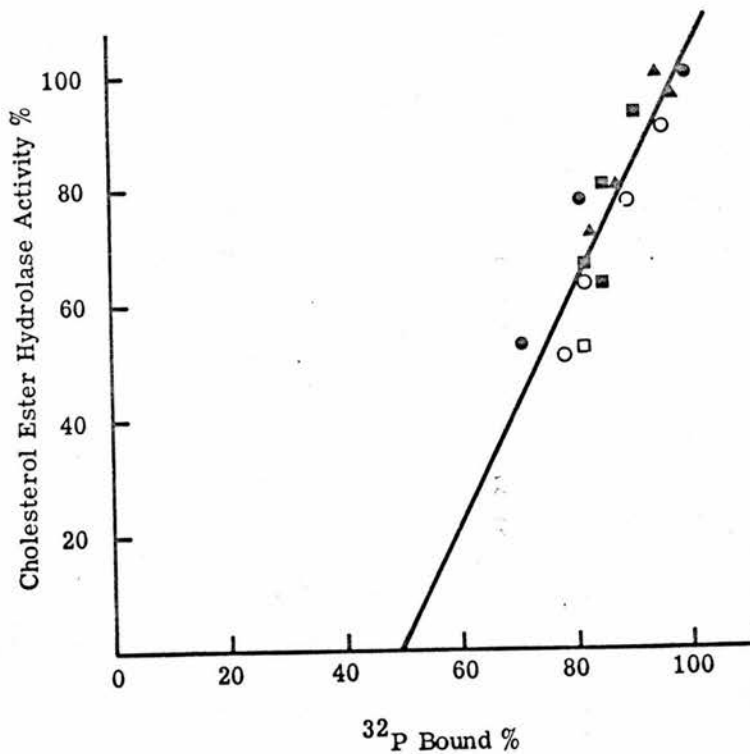
Figure 6.6 The correlation between cholesterol ester
hydrolase activity and protein bound [^{32}P] radioactivity
Experimental procedure as text. Deactivation by alkaline
phosphatase (□), magnesium ions (○), β phosphorylase
kinase phosphatase (■), α phosphorylase kinase phosphatase
(▲), α phosphorylase kinase phosphatase + MnCl$_2$ (●).

Summary

a) In vitro preincubation of a crude preparation of
cholesterol ester hydrolase with 5mM magnesium produced a
deactivation of cholesterol ester hydrolase accompanied by
a loss of [^{32}P] radioactivity from the protein. Cholesterol
ester hydrolase activity could be restored in part by the
addition of cyclic AMP and ATP.

b) Preincubation of a 105,000 x g cortical supernatant
fraction with alkaline phosphatase or phosphodiesterase
inactivated the cholesterol ester hydrolase.

c) Deactivation of purified cholesterol ester hydro-
lase by phosphorylase kinase phosphatase or alkaline phosphatase
was accompanied by a loss of [^{32}P] radioactivity from the protein.

d) Cholesterol ester hydrolase activity was found to
be proportional to the amount of [^{32}P] radioactivity bound
to the protein.

CONTENTS

CHAPTER 7

STUDIES ON RAT ADRENAL CHOLESTEROL ESTER HYDROLASE

Introduction

The work presented in chapters 3 to 6 has been concerned with a study of the cholesterol ester hydrolase found in the 105,000 x g supernatant of the bovine adrenal cortex. Much of the earlier work carried out on adrenal cholesterol ester metabolism however involved the use of whole rat adrenals (Sayers et al. (1946)); (Davies and Garren (1966)); (Sayers et al. (1944)); (Brecher et al., (1973)); (Simpson et a (1972)); (Shima et al. (1972)); (Behrman and Greep (1972)); (Trzeciak and Boyd (1973)).

Investigations on the activation of cholesterol ester hydrolase in the rat adrenal have been reported (Behrman and Greep (1972)); (Trzeciak and Boyd (1973)). It was decided to carry out further investigations into the rat adrenal cholesterol ester hydrolase and compare it with the cholesterol ester hydrolase found in the bovine adrenal cortex. The experiments presented in this chapter were designed to study further the properties and the activation mechanism of cholesterol ester hydrolase in the rat adrenal. Experiments were also carried out to investigate the hydrolysis of lipid droplet cholesterol esters in vitro and to study the specificity of cholesterol ester hydrolase for the cholesterol side chain.

General properties of rat adrenal cholesterol ester hydrolase

Assay for cholesterol ester hydrolase activity was carried out as described in chapter 2. Under these conditions the rate of hydrolysis of cholesterol oleate is linear during an incubation period of up to 60 minutes, and within the range of protein concentration 0.5 mg to 3.0 mg protein per ml (Trzeciak and Boyd (1973)).

a) Subcellular distribution

A homogenate of rat adrenals was prepared and the homogenate was fractioned into subcellular particles by the procedur
shown in Figure 2.1. On assaying each fraction for cholesterol
ester hydrolase activity most of the activity was found in
the 105,000 x g supernatant (Table 7.1) however significant
cholesterol ester hydrolase activity was also found in the
microsomal and lysosomal fractions. All subsequent invest-
igations were therefore carried out on the cholesterol ester
hydrolase situated in the 105,000 x g supernatant.

b) pH optimum

The effect of varying pH on rat adrenal 105,000 x g
supernatant cholesterol ester hydrolase activity is shown
in Figure 7.1.

Potassium phosphate buffer 1.0M or 1.0M Tris-HCl buffer
were added to the 105,000 x g adrenal supernatant at a final
concentration of 50mM and at the pHs indicated in Figure 7.1.
After the addition, the pH of the resulting solution was
checked and the solution assayed for cholesterol hydrolase
activity. Optimal cholesterol ester hydrolase activity was
exhibited in the range pH 7.3 to 7.9.

c) Increasing substrate concentration

Figure 7.2 shows the effect of increasing concentration
of cholesterol oleate on cholesterol ester hydrolase activity.
The Km value for cholesterol oleate determined by the graph-
ical method of Hanes (1932) was found to be 14μ M. This
compares with the Km value found for cholesterol oleate in
the bovine adrenal of 14.5μ M (Chapter 3).

Subcellular Fraction	Cholesterol Ester Hydrolase % of the total activity
Supernatant	67
Microsomes	18
Lysosomes	9
Mitochondria	4
Nuclei	2

Table 7.1 The subcellular distribution of rat
adrenal cholesterol ester hydrolase .
Subcellular fractionation was carried out as described
in Figure 2.1 and cholesterol ester hydrolase activity
was determined as described in chapter 2.
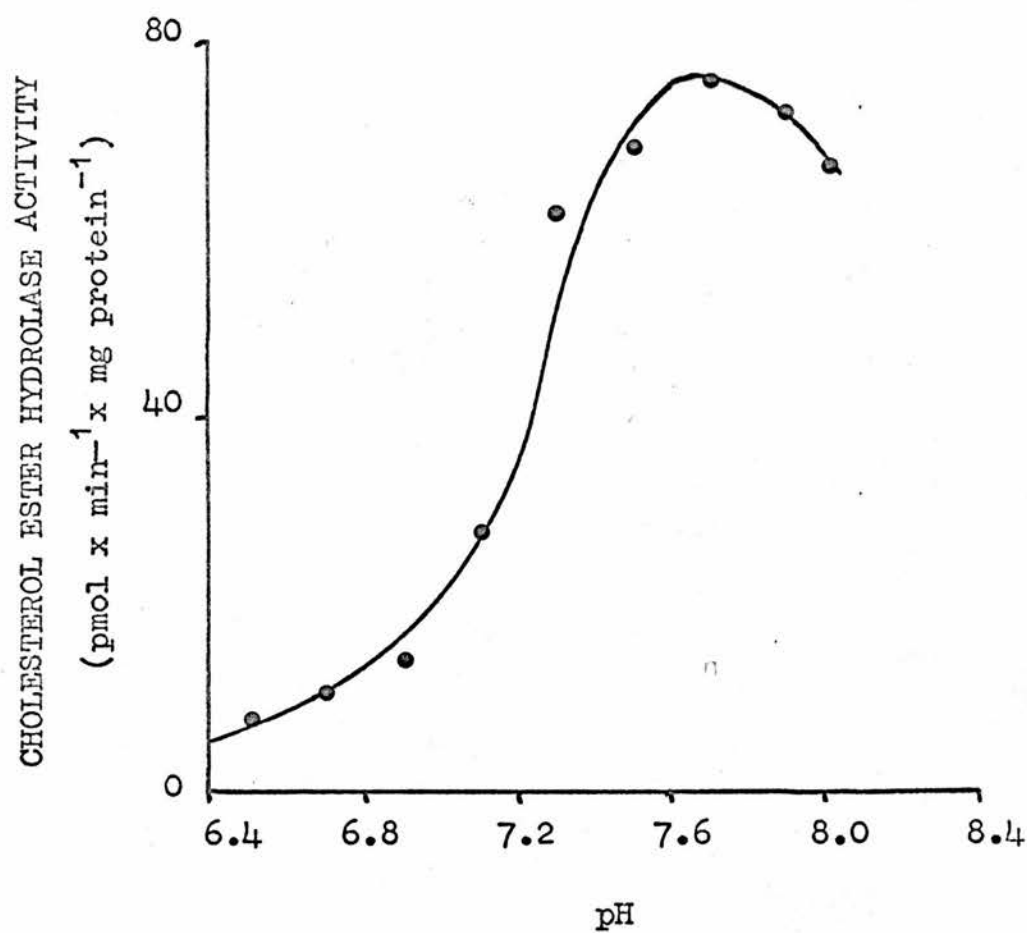
Figure 7.1   The effect of pH on cholesterol ester

hydrolase activity in rat adrenal supernatant.

Experimental procedure as text.

$\frac{S}{V}$ v/s S PLOT FOR RAT ADRENAL CHOLESTEROL ESTER HYDROLASE
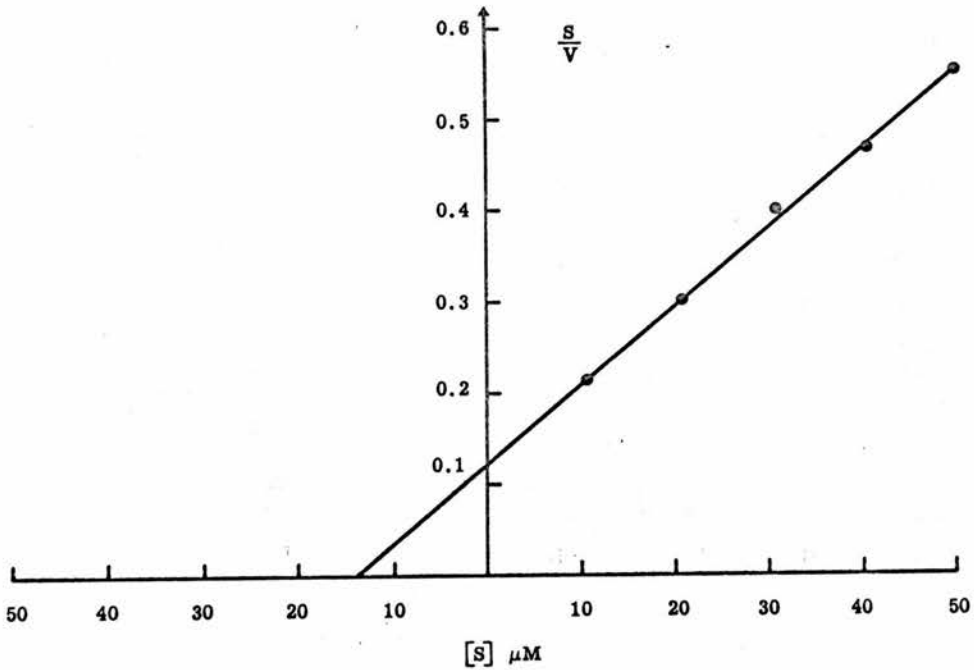
Figure   7.2 The effect of increasing concentration

of cholesterol oleate on cholesterol ester hydrolase

activity in rat adrenal 105,000 x g supernatant.

Cholesterol ester hydrolase activity was determined as

is described in chapter 2 in the presence of increasing

concentrations of cholesterol oleate as indicated above

and in the text.

The rate of hydrolysis of cholesterol ester analogues by cholesterol ester hydrolase

The assay system for cholesterol ester hydrolase activity employed in this thesis is based on the measurement of the rate of hydrolysis of cholesterol oleate. Cholesterol oleate was chosen as the substrate as it is the predominating ester in the adrenal lipid droplets. Another reason for the use of cholesterol oleate is the fact that cholesterol oleate has been shown to be hydrolysed at a faster rate in vitro and in vivo (Dailey et al. (1963)) than any other cholesterol ester with the possible exception of cholesterol arachidonate.

It has been shown that cholesterol ester hydrolase in the adrenal shows a specificity for the fatty acid side chain (Dailey et al. (1963)); (Table 9.5). No studies on the specificity of adrenal cholesterol ester hydrolase for the cholesterol side chain have been reported.

It is known that the rate of side chain cleavage of cholesterol in the bovine adrenal mitochondria (Arthur et al. (197 and the rate of 7α hydroxylation of cholesterol in rat liver (Boyd et al. 1974) is governed by the nature of the cholesterol side chain. These results suggest that the cholesterol side chain cleavage enzyme and the cholesterol 7α hydroxylation enzyme have a specific apolar binding site for the side chain of cholesterol.

It was therefore decided to investigate the rate of hydrolysis of [4-^{14}C] cholesterol oleate, [25-^{3}H] norcholesterol oleate and [20-^{3}H] pregnenol oleate synthesised by the method described in chapter 2.

The 105,000 x g desalted supernatant from the adrenals of 2 groups of 10 rats was prepared in the usual way. One group of rats, the control, were kept in a quiescent state and the other group were subjected to a 10 minute ether anaesthesia stress before killing by decapitation to maximise the activity of the hydrolase.

Cholesterol ester hydrolase activity was then assayed in the supernatants from each group of animals in the presence and absence of the cofactors 5mM ATP-MgCl$_2$ and 10μ M cyclic AMP. The assay was carried out in the usual way with the addition of either 74nmoles of $[$ 25-^{3}H $]$ norcholesterol oleate, 85nmoles of $[$ 20-^{3}H $]$ pregnenol oleate or 60nmoles of $[$ 4-^{14}C$]$cholesterol oleate per assay.

The results are shown in Table 7.2. It can be seen that both norcholesterol oleate and pregnenol oleate were hydrolysed at a faster rate than cholesterol oleate. In each case the rate of hydrolysis of the esters were increased by ether anaesthesia stress or by cofactor addition in vitro. However the different rates of hydrolysis found for the cholesterol ester analogues were small compared to the differences observed when the fatty acid side chain was altered (Dailey et al. (1963)); (Table 9.5).

It seems therefore that cholesterol ester hydrolase shows little specificity for the cholesterol side chain, but it does exhibit a specificity for the fatty acid moiety.

Activation of cholesterol ester hydrolase by ATP and cyclic AMP and ether anaesthesia stress

Behrman and Greep (1972) and Trzeciak and Boyd (1973) have reported that in the rat elevated plasma ACTH levels

Table 7.2 The hydrolysis of cholesterol ester analogues by rat adrenal

cholesterol ester hydrolase.

Substrate	Cholesterol oleate pmol x min x mg		Norcholesterol oleate pmol x min mg		Pregnenol oleate pmol x min x mg	
Animals	Stressed	Quiescent	Stressed	Quiescent	Stressed	Quiescent
No addition	67	64	103	99	98	79
+ Cofactors	76	70	137	108	144	99

Experimental procedure as text.

Cofactors = 5mM ATP + 10 μM cyclic AMP + 5mM $MgCl_2$

Table 7.3 Activation of rat adrenal Cholesterol
ester hydrolase by cyclic AMP and ATP and ether stress

Additions	Exp No.	Cholesterol ester hydrolase	
		Quiescent	Stressed
		pmol x min^{-1} x mg	
None	1	147 ± 5	273 ± 13
+ Cofactors		219 ± 3	274 ± 10
None	2	97 ± 8	136 ± 11
+ Cofactors		227 ± 30	216 ± 12
None	3	119 ± 5	150 ± 10
+ Cofactors		212 ± 6	218 ± 3

Experimental procedure as text

Cofactors = 5mM ATP + 5Mm $MgCl_2$ + 10 µM cyclic AMP

Results are expressed as the mean of duplicate assays

± S.E.M.

stimulate cholesterol ester hydrolase activity in the adrenal.
Trzeciak and Boyd (1973) have also reported that cholesterol .
ester hydrolase activity in the 105,000 x g adrenal supernatant
could be stimulated by the in vitro addition of ATP and
cyclic AMP.

The results shown in Table 7.3 confirm the findings
of Trzeciak and Boyd (1973) that cholesterol ester hydrolase
activity can be stimulated in vivo by ether anaesthesia stress
or in vitro by the addition of 5mM ATP and 10 µM cyclic AMP
cofactors.

The animals were randomly divided into two groups for
the experiment. The animals of one group, the controls,
were kept in a quiescent state, transferred to a separate
room for killing to minimise stress due to handling, these
animals were killed by decapitation. The second group were
subjected to a 10 minute ether anaesthesia stress before
killing. After killing the adrenals were quickly removed
and the 105,000 x g supernatant was prepared from the adrenal
homogenate as described in chapter 2. The 105,000 x g super-
natant was then desalted by passage through a Sephadex G-25
column (30cm x 2cm) equilibrated with 5mM Tris-HCl buffer ·
pH 7.4 and the protein fractions were pooled. The desalted
supernatant from each group of rats was then assayed for
cholesterol ester hydrolase activity in the presence and
absence of 10µ M cyclic AMP and 5mM ATP.

It can be seen that in each of the three experiments
a stimulation of cholesterol ester hydrolase activity was
produced by ether anaesthesia. Cofactor addition in vitro
produced a stimulation of activity up to the same maximal
activity in each of the three experiments.

Evidence for the phosphorylation of cholesterol ester hydrolase in the rat adrenal

Trzeciak and Boyd (1973) have postulated that in the rat adrenal activation of cholesterol ester hydrolase takes place by a phosphorylation of the enzyme involving the transfer of the terminal phosphate from ATP to the protein. It was decided therefore to devise an experiment to investigate this postulate.

Thirty male Wistar rats were killed by decapitation and the adrenals were quickly removed and homogenised in 5mls of sucrose. The 105,000 x g supernatant was then prepared in the normal way (Figure 4.1)and desalted by passage through a Sephadex column (30cm x 2cm) equilibrated with 50mM sodium glycerophosphate buffer pH 6.8. This desalted preparation was incubated for 30 minutes at $30^{o}C$ with 1mM $MgCl_2$, 50mM potassium chloride, 10μ M cyclic AMP, 0.1mM ATP and 50μ Ci of $[\gamma-{}^{32}P]$ ATP. After this preincubation period the mixture was desalted by passage through a Sephadex G-25 column equilibrated with 20mM potassium phosphate buffer pH 7.4. The pooled protein fractions were then applied to a Biogel A 0.5M column equilibrated with the same buffer. Fractions of 2.5mls were collected from the column and each fraction was assayed for cholesterol ester hydrolase activity, protein and $[{}^{32}P]$ radioactivity bound to the protein before and after precipitation with 10% trichloroacetic acid by the methods described in chapter 2.

The results obtained are shown in Figures 7.3 and 7.4. It can be seen that all of the cholesterol ester hydrolase activity was eluted in the void volume of the column and these protein fractions also contained $[{}^{32}P]$ radioactivity before and after precipitation of the protein with 10% trichloro-

Figure 7.3 The elution profile of phosphorlated

105,000 x g rat adrenal supernatant from a Biogel

A 0.5 M column. The protein was firstly preincubated

with [γ ^{32}P] ATP under the conditions described in the

text. The fractions obtained from the column were assayed

for cholesterol ester hydrolase , protein, total and

protein bound[^{32}P] radioactivity as described in chapter

2.

ELUTION PROFILE OF PHOSPHORYLATED RAT ADRENAL 105,000 x g SUPERNATANT FROM
A BIOGEL A-0.5 M COLUMN
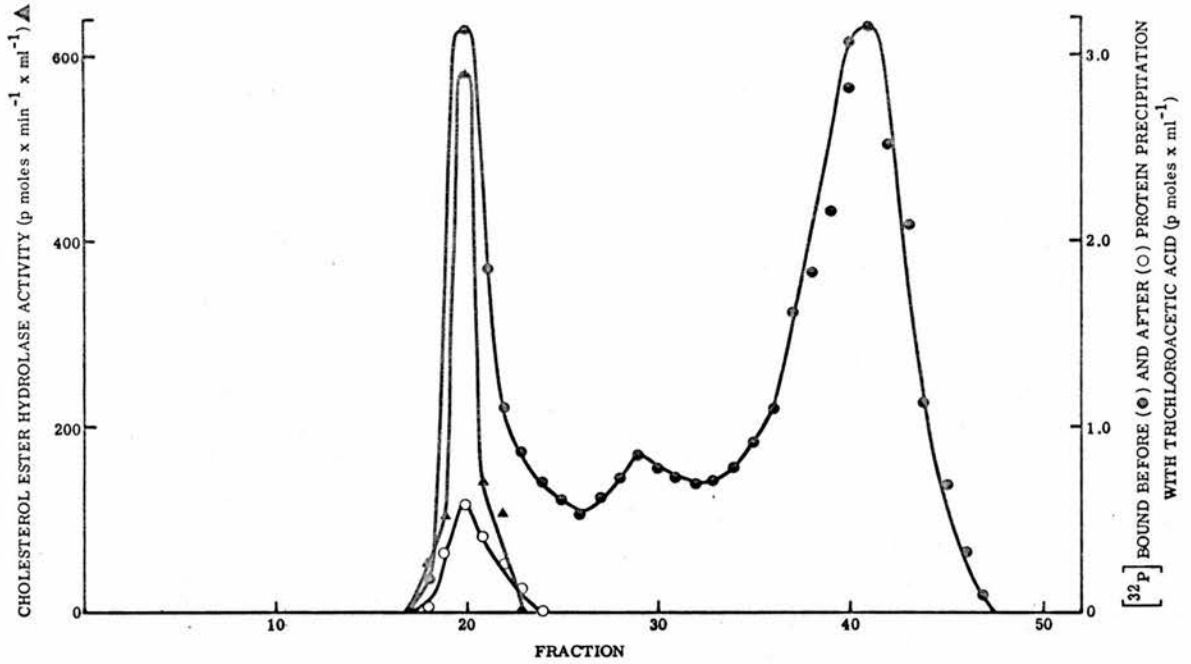
Figure   7.4 The elution profile of phosphorylated

105,000 x g rat adrenal supernatant from a Biogel A 0.5 M

column. Experimental procedure as Figure 7.2

acetic acid. This is similar to the elution profile obtained
from Sephadex G-200 in the bovine adrenal system (Figures
4.4 and 4.5). Unlike the bovine system no second cholesterol
ester hydrolase was obtained eluted from the column after
the void volume enzyme.

Two further peaks of $[^{32}P]$ radioactivity were eluted
from the Biogel column after the void volume fraction. The
final $[^{32}P]$ component eluted was probably $[\gamma-^{32}P]$ - ATP which
had dissociated from the protein during the filtration. The
second of these $[^{32}P]$ radioactivity peaks was associated
with protein but after precipitation of the protein with
trichloroacetic acid no $[^{32}P]$ radioactivity remained bound.

Using gel filtration a 10 fold purification of cholesterol
ester hydrolase was achieved as the specific activity was
increased from 93pmol x min^{-1} x mg protein^{-1} in the 105,000 x g
supernatant to 900pmol x min^{-1} x mg protein^{-1} in the void
volume fractions.

The 105,000 x g supernatant was subjected to polyacrylamide
disc gel electrophoresis on 5% gels as is described in chapter 2.
After running at 2mA per gel until the dye marker had reached
the end of the gel, the gel was stained for esterase activity
using the histochemical method of Gomori (1952). The results
are shown in Figure 7.5. Seven bands of esterase activity
were observed. When the void volume fraction 20 was subjected
to gel electrophoresis and staining for esterase activity
in the same manner three bands of esterase activity were
found (Figure 7.6). When the gels were sliced into 1mm strips
and counted for $[^{32}P]$ radioactivity it was found that the
two esterases with the higher mobilities incorporated $[^{32}P]$
radioactivity and the third did not.

BOTTOM

TOP

ESTERASE ACTIVITY STAIN
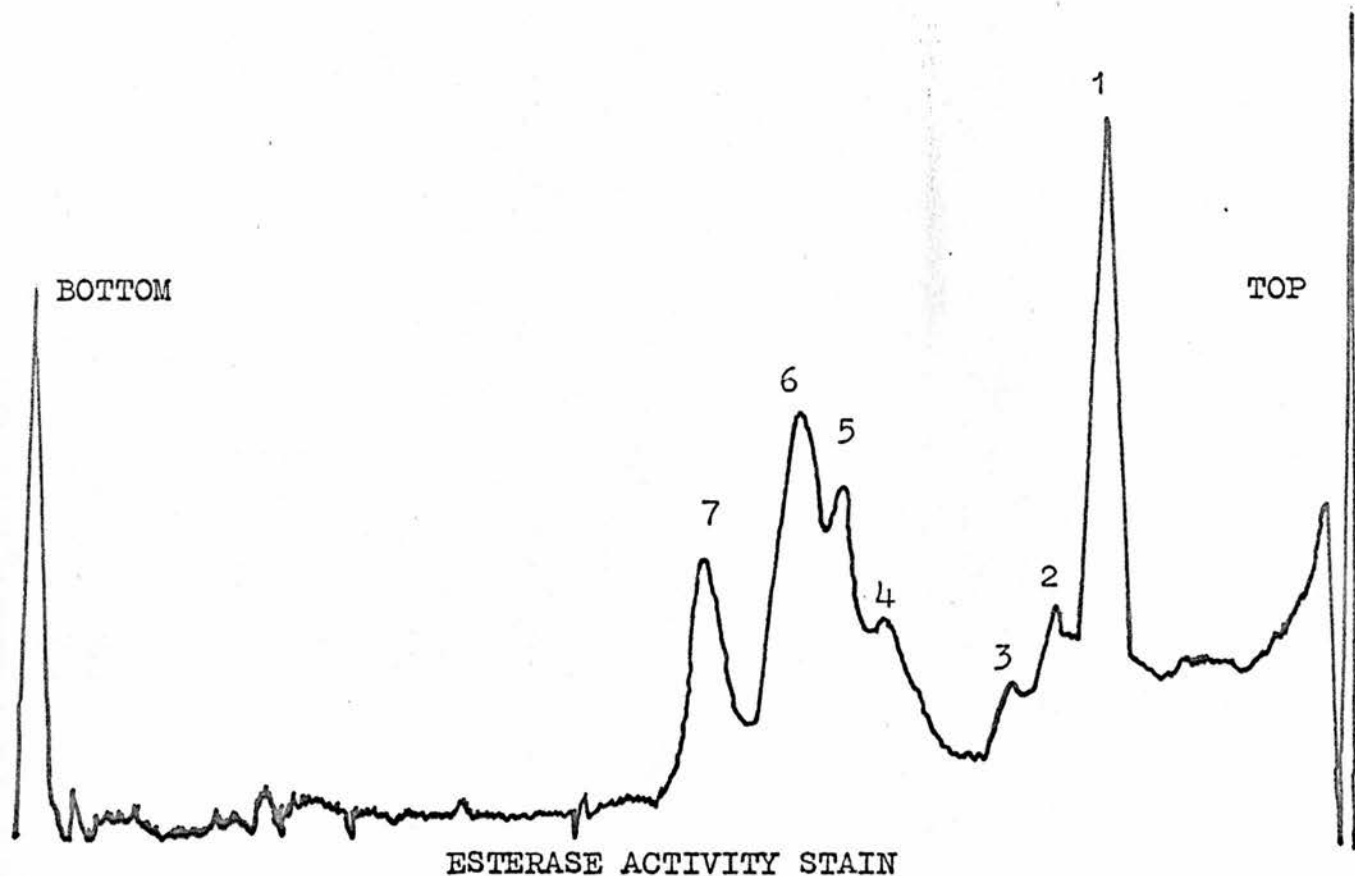
Figure 7.5 Esterase activity stain of rat adrenal
cortical supernatant subjected to polyacrylamide
gel electrophoresis. Electrophoresis and staining was
carried out as described in chapter 2 on 5% gels.
The gels were scanned at 540nm. At least 7 bands of
esterase activity were seen.

BOTTOM   TOP
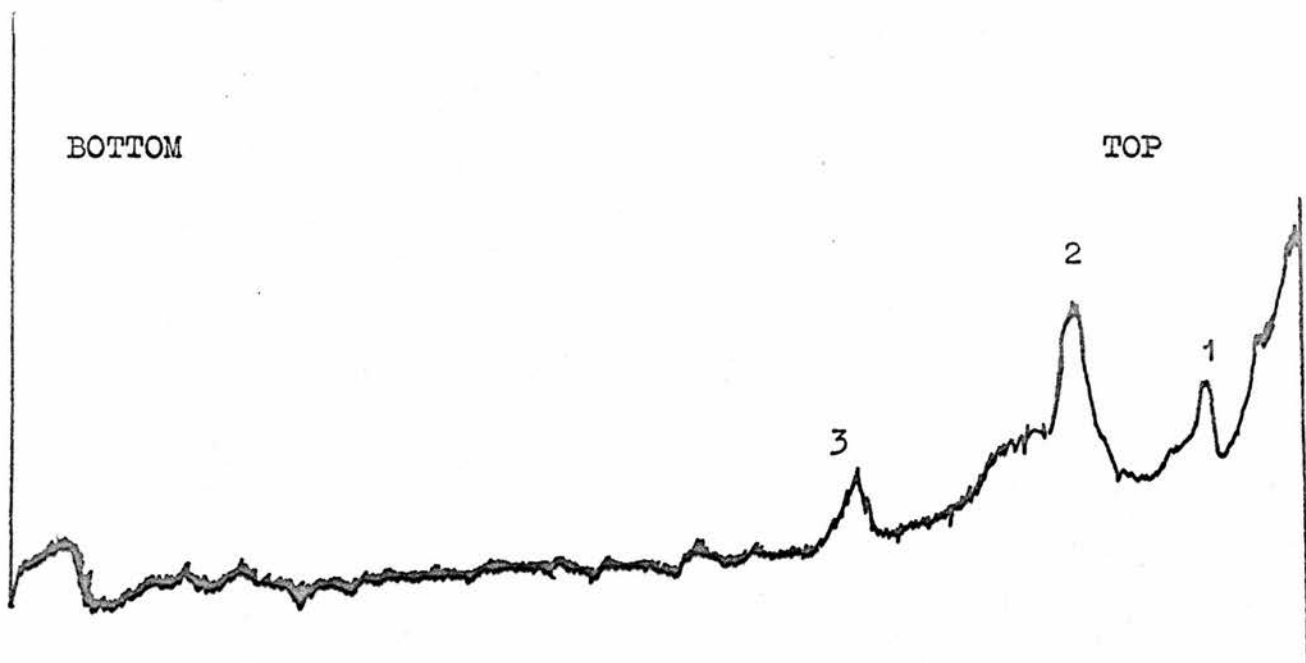
ESTERASE ACTIVITY STAIN

Figure 7.6 Esterase activity stain of a partially

purified preparation of rat adrenal cholesterol

ester hydrolase subjected to polyacrylamide gel

electrophoresis. Experimental procedure as text.

Three bands of esterase activity were seen and

bands 2 and 3 incorporated [^{32}P] radioactivity.

Hydrolysis of lipid droplets in vitro by 105,000 x g supernatant

Garren et al. (1971) and Boyd and Trzeciak (1973) have shown that under conditions where plasma ACTH is elevated the amount of free cholesterol in the cellular lipid droplets increase and cholesterol esters are depleted. This effect was therefore tried to be reproduced in vitro by incubating lipid droplets with 105,000 x g supernatant.

12 male rats were killed by decapitation and the adrenals homogenised in the usual way (chapter 2). After the 105,000 x g centrifugation the floating lipid droplets were removed with a syringe and the clear infranatant decanted from the microsomal pellet. Additions of ATP (5mM), $MgCl_2$ (5mM) and cyclic AMP (10 μM) were made to the infranatant, Tris-HCl buffer 1.0M was also added to a final concentration of 0.1M. After these additions the lipid droplets were resuspended in the supernatant and incubated at $37^{\circ}C$ for three hours. Samples (1ml) were taken from the incubation at the start, 30 minutes and every hour. The samples were centrifuged for 30 minutes at 105,000 x g and after this time the floating lipid layer was removed. Tracer amounts of $[4-^{14}C]$ cholesterol and $[4-^{14}C]$ cholesterol oleate were then added to the lipid droplets and the supernatant. The supernatant and lipid droplets were then extracted with 3mls of methanol and the protein precipitate formed on the extraction was extracted with 3mls of boiling ethyl acetate. The extracts were taken to dryness, redissolved in a small amount of chloroform and applied to a thin layer chromatography plate and run in 75:25:1 petroleum ether:diethyl ether: and acetic acid. The plates were then scanned for the radioactive tracers previously added and the radioactive spots scraped

from the plate. The cholesterol and cholesterol ester fractions were eluted with chloroform and the amounts of cholesterol and cholesterol esters were then determined by gas liquid chromatography as is described in chapter 2. The procedure is summarised in Figure 7.7.

The results are shown in Figures 7.8, 7.9 and 7.10. It can be seen that the amount of free cholesterol present in the supernatant increased significantly over the three hour incubation (Figure 7.8). No significant decrease in the cholesterol ester content of the supernatant however could be detected (Figure 7.9). This is probably due to the high levels of cholesterol ester already present and therefore any small decrease in this large cholesterol ester pool could not be detected by this method. When the ratio of cholesterol/cholesterol esters were measured in the lipid droplet samples (Figure 7.10) an increase in the ratio of cholesterol/cholesterol esters was observed over the three hour incubation period.

The blood volume of a rat is approximately 64mls per Kg (Wang (1959)). The rats employed in this experiment had an average body weight of 170 gms and an average adrenal weight of 25mgs.

From this experiment it can be calculated that:-
The supernatant from 1 adrenal can produce 7.3 μg of cholesterol per hour $\underline{in\ vitro}$. Thus the total output of free cholesterol per rat = 14.6 μg per hour. The rats used in this experiment had an approximate blood volume of 11mls. The maximal corticosteroid production after the first 5 minutes of an applied stressful situation is 30 μg/100mls plasma (Dallman and Jones (1973)). Thus in the rats employed in this experiment maximal corticosteroid production $\underline{in\ vitro}$ could be about 40 μg of corticosteroid produced per hour.

Figure 7.7

Procedure for the in vitro hydrolysis of rat adrenal

cholesterol esters

Adrenals from 12 rats were
homogenised in 0.25M sucrose

↓

Homogenate centrifuged
8,500 x g 10 minutes

↓

Supernatant centrifuged
105,000 x g 60 minutes

Lipid droplets **removed**
with a syringe

Infranatant mixed with
5mM ATP, 10 μM cyclic
AMP, 5mM MgCl$_2$ and 0.1M
Tris-HCl buffer pH 7.4

Mix and incubate
at 37°C.

1ml sample at zero
time.

Further samples (1ml)
taken at 30 mins, 1hr,
2hr and 3hr.

Centrifuge 30 mins ◄
105,000 x g

Lipid droplets
removed.

0.5 mls of supernatant

[4-^{14}C] cholesterol and
cholesterol oleate added

↓

Lipid extracted

↓

Lipid subjected to thin
layer chromatography

↓

Cholesterol and cholesterol
ester determined by GLC.

Figure 7.8   Cholesterol production in the 105,000 x g
rat adrenal supernatant on incubation of the supernatant
with adrenal lipid droplets and cofactors.
Experimental procedure as text. Results are shown as
the average of two determinations.
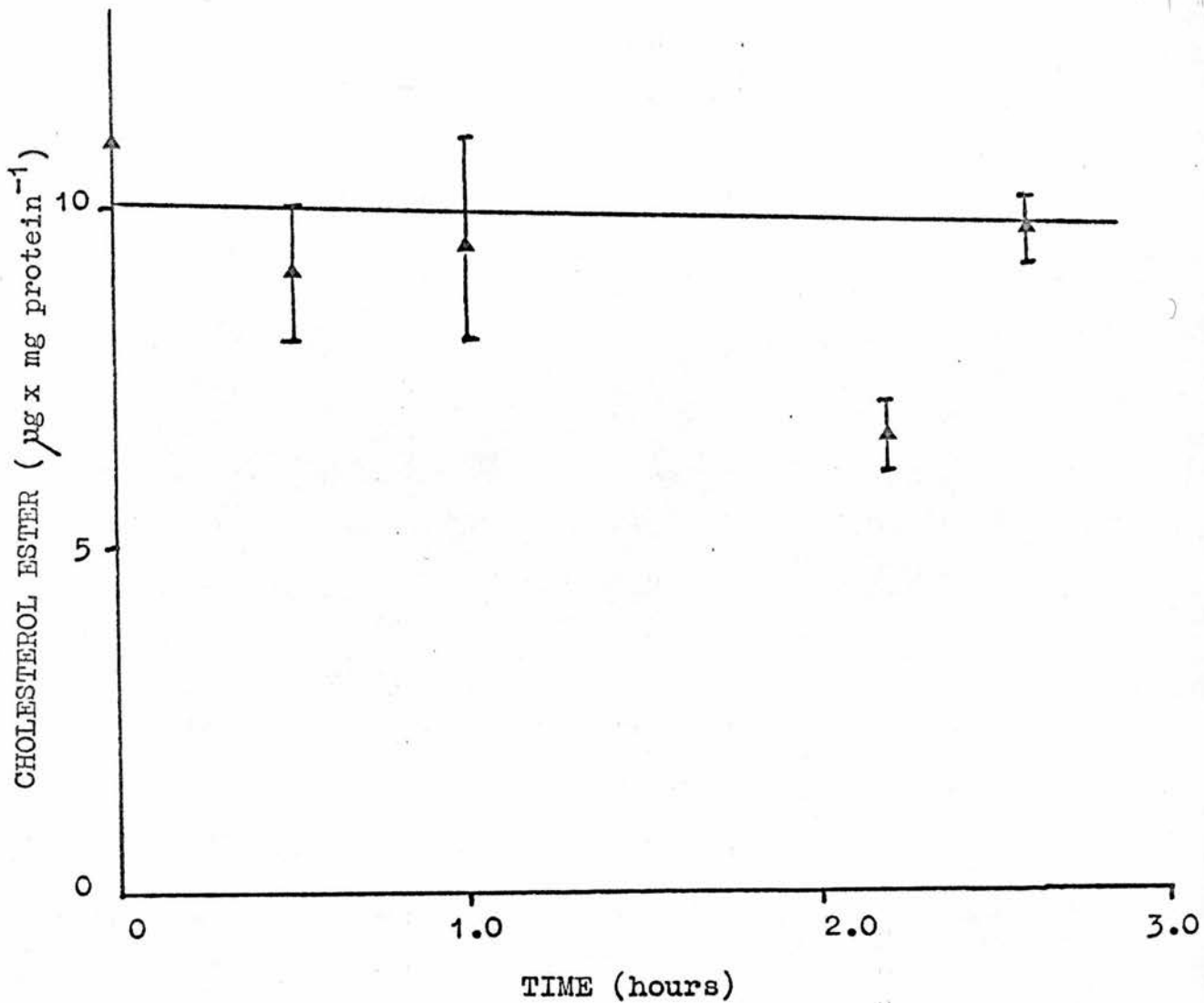
Figure 7.9 Cholesterol ester content of the rat
adrenal 105,000 x g supernatant on incubation of the
supernatant with adrenal lipid droplets.
Experimental procedure as text. Results are shown as
the average of two determinations.
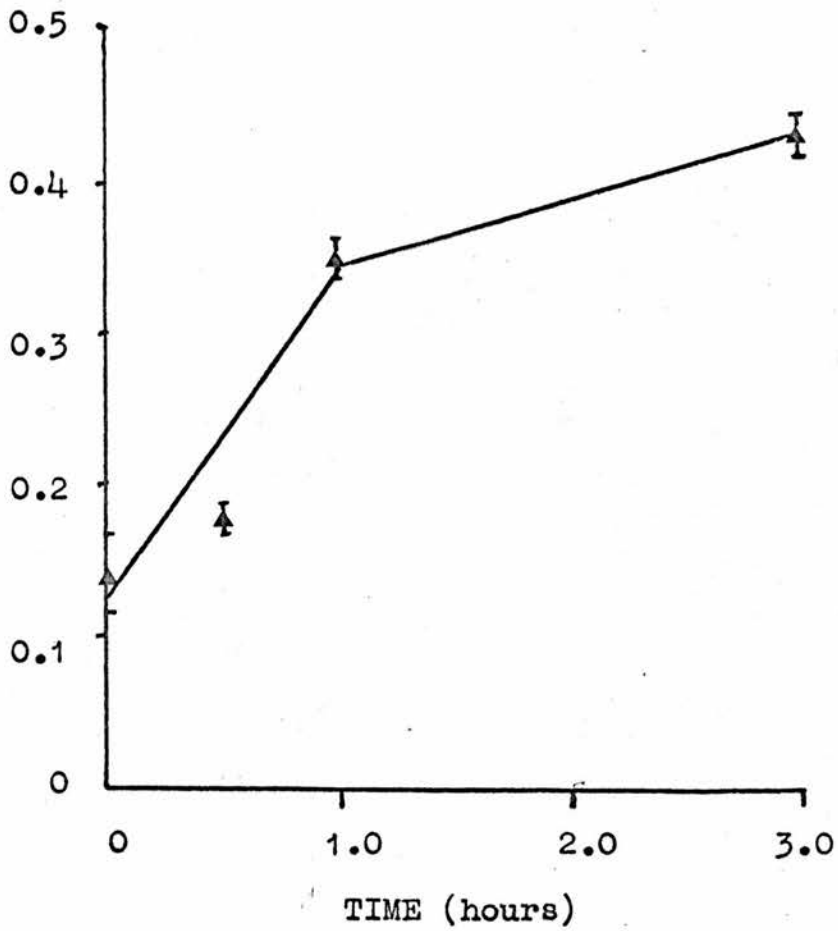
Figure 7.10 The ratio of cholesterol/cholesterol
ester in adrenal lipid droplets after incubation with
rat adrenal cortical supernatant.
Experimental procedure as in text. Results are shown
as the average of two determinations.

It can be seen that the rate of cholesterol production from cholesterol ester _in vitro_ is of the same order as corticosteroid production _in vivo_. As was shown earlier in this chapter some cholesterol ester hydrolase activity exists in other subcellular fractions of the adrenal cell. This would therefore increase the available cholesterol and would increase the calculated rate of cholesterol production to a similar value to the rate of corticosteroid production observed _in vivo_.

From these studies it seems possible that the adrenal lipid droplet cholesterol esters are important precursors of the adrenal steroid hormones.

Summary

a) Cholesterol ester hydrolase was found mainly in the 105,000 x g supernatant fraction.

b) Cholesterol ester hydrolase exhibited maximum activity in the pH range 7.3 to 7.9 and was found to have a Km of 14 μM for cholesterol oleate.

c) Cholesterol ester hydrolase activity was found to be stimulated in vitro by the addition of magnesium, ATP and cyclic AMP and in vivo by ether anaesthesia.

d) Preincubation of the 105,000 x g supernatant with cyclic AMP and $[\gamma -^{32}P]$ ATP followed by purification resulted in a protein fraction which contained cholesterol ester hydrolase activity and protein bound $[^{32}P]$ radioactivity. Electrophoresis of this purified protein fraction resulted in three bands of esterase activity, two of which contained $[^{32}P]$ radioactivity.

e) Adrenal lipid droplet cholesterol esters were found to be hydrolysed in vitro by incubation with 105,000 x g supernatant.

f) Cholesterol ester hydrolase shows little specificity for the cholesterol side chain, but it does exhibit specificity for the esterified fatty acid.

CONTENTS

CHAPTER 8

STUDIES ON RAT LUTEAL CYTOPLASMIC CHOLESTEROL ESTER HYDROLASE

Introduction

The work which has been presented in chapters 3 to 7 has been concerned with the study of the cholesterol ester hydrolases found in the rat and bovine adrenal gland. Coutts and Stansfield (1968) and Behrman and Armstrong (1969) have demonstrated cholesterol ester hydrolase activity in the corpus luteum of superovulated rats.

Two conflicting reports have been published concerning the subcellular distribution of cholesterol ester hydrolase in rat luteal tissue. Coutts and Stansfield (1968) found that cholesterol ester hydrolase activity resided in the 5000 x g pellet of the luteal homogenate. Behrman and Armstrong (1969) however found cholesterol ester hydrolase activity resided mainly in the 100,000 x g supernatant from a rat luteal homogenate. This is similar to the distribution of the enzyme found in the rat adrenal (Treziak and Boyd (1973)). The luteal supernatant cholesterol ester hydrolase in the rat is stimulated by injections of LH administered 1 hour before killing (Behrman and Armstrong (1969); Armstrong and Flint (1973)). Injections of LH antiserum 24 hours before killing has been shown to decrease cholesterol ester hydrolase activity by 90% (Behrman et al. (1972)). Stimulation of cholesterol ester hydrolase activity by cyclic AMP has not been demonstrated in corpus luteum (Behrman and Armstrong (1969); Flint et al. (1973)). No stimulation of cholesterol ester hydrolase in the rat or bovine adrenal by the in vitro addition of cyclic AMP per se

has been demonstrated, however in the presence of ATP, stimul-ation of cholesterol ester hydrolase by cyclic AMP occurs (Trzeciak and Boyd (1973); Trzeciak and Boyd (1974)).

The failure of cyclic AMP to promote activation of cholesterol ester hydrolase in the 105,000 x g luteal superna-tant does not rule out the possibility of activation of cholesterol ester hydrolase by a phosphorylation involving a protein kinase. Menon (1973) has isolated a protein kinase from bovine corpus luteum which is stimulated directly by LH and it is possible that this enzyme may be responsible for the activation of cholesterol ester hydrolase in this tissue.

In the following experiments the effect of hormonal treatment, cycloheximide injections, and in vitro addition of magnesium ions, cyclic AMP and ATP on rat luteal cytoplasmic cholesterol ester hydrolase activity is investigated.

Luteinized ovaries were induced in immature female Wistar rats by pretreatment of the animals with pregnant mare serum gonadotrophin (Parlow (1958)), as described in chapter 2. The 105,000 x g supernatant was obtained from the homogenate of the luteal tissue by the centrifugation procedure outlined in Figure 4.1. The 105,000 x g super-natant was desalted by passage through a Sephadex G-25 column (25cm x 2cm) equilibrated with 20mM Tris buffer pH 7.4. The desalting procedure was employed in all of the experi-ments reported in this chapter.

General properties of luteal cholesterol ester hydrolase

a) Rate of cholesterol oleate hydrolysis

It can be seen from Fig. 8.1 that when cholesterol oleate was incubated with desalted 105,000 x g supernatant, cholest-erol was released at a linear rate over a 40 minute incubation
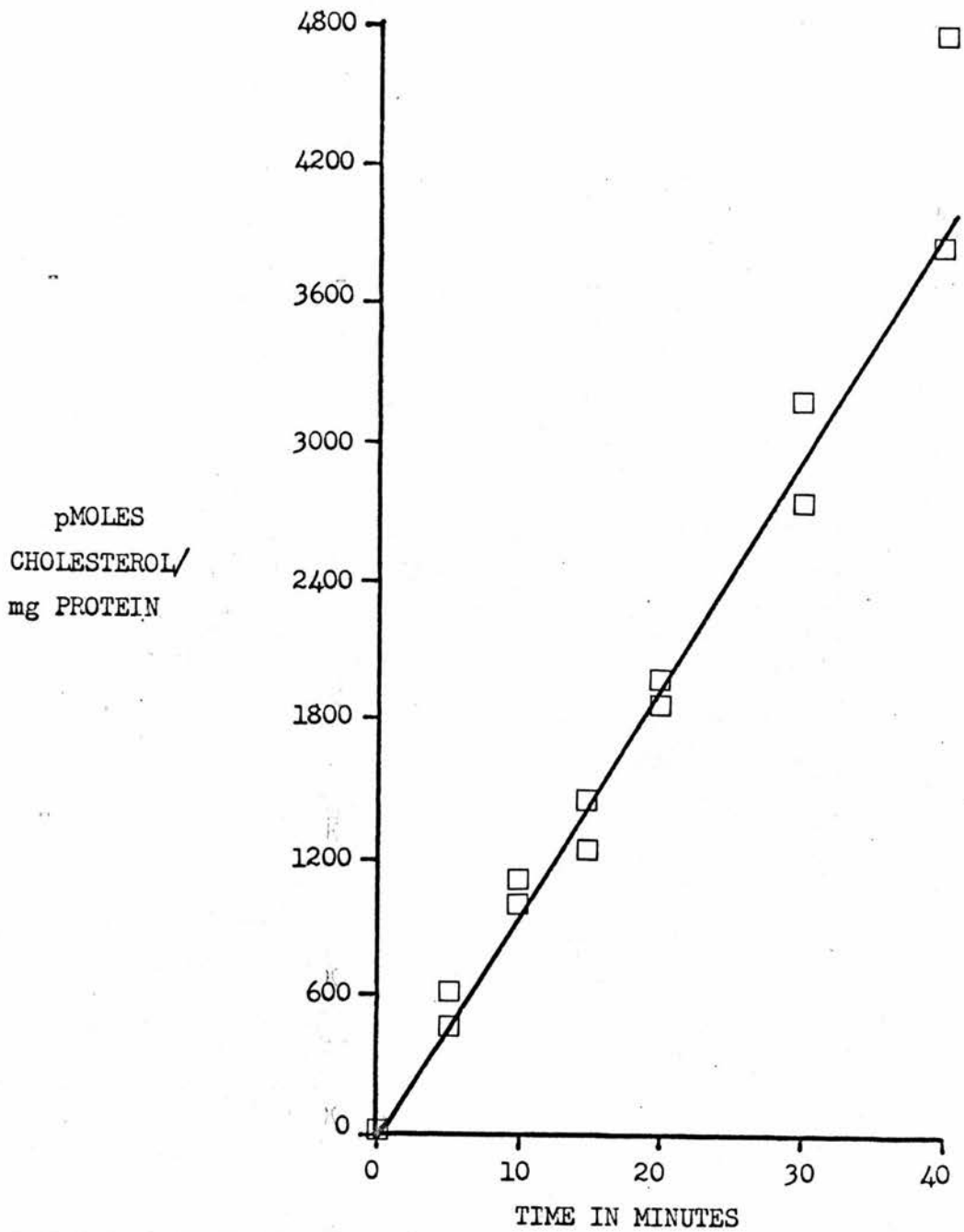
Figure 8.1. The time course of cholesterol appearance from cholesterol oleate catalysed by luteal cholesterol esterase. The assay was carried out as described in chapter 2 except that the time of incubation was varied.

period. The rate of production of cholesterol from cholest-
erol oleate has been reported by Flint and Armstrong (1973)
and Behrman and Armstrong (1969) to be directly proportional
to the amount of protein added to the incubation.

b) The effect of magnesium ions on luteal cholesterol ester
hydrolase activity

Incubations of cholesterol ester hydrolase were carried
out in the presence of $MgCl_2$ concentrations up to 7mM.
Figure 8.2 shows the results obtained from these incubations.
Magnesium chloride was found to stimulate cholesterol ester
hydrolase activity at all the concentrations employed with
a maximal stimulation obtained at 3mM $MgCl_2$. When a $MgCl_2$
concentration of 7mM was reached cholesterol ester hydrolase
activity was returned to the value found in the desalted
preparation therefore 5mM $MgCl_2$ was included in incubations.

c) The effect of ATP and cyclic AMP on luteal cholesterol
ester hydrolase

Various combinations of cofactors were added to the
desalted supernatant and their effect on cholesterol ester
hydrolase activity was determined (Table 8.1). Addition
of 5mM $MgCl_2$ produced a 100% stimulation of cholesterol
ester hydrolase activity while 5mM ATP added in the absence
of $MgCl_2$ produced a 50% stimulation of cholesterol ester
hydrolase activity. Addition of 5mM ATP in the presence
of $MgCl_2$ produced a 150% stimulation of enzymatic activity.
Cyclic AMP 1 μM added together with 5mM ATP produced no further
stimulation of cholesterol ester hydrolase activity in the
presence or absence of 5mM $MgCl_2$.
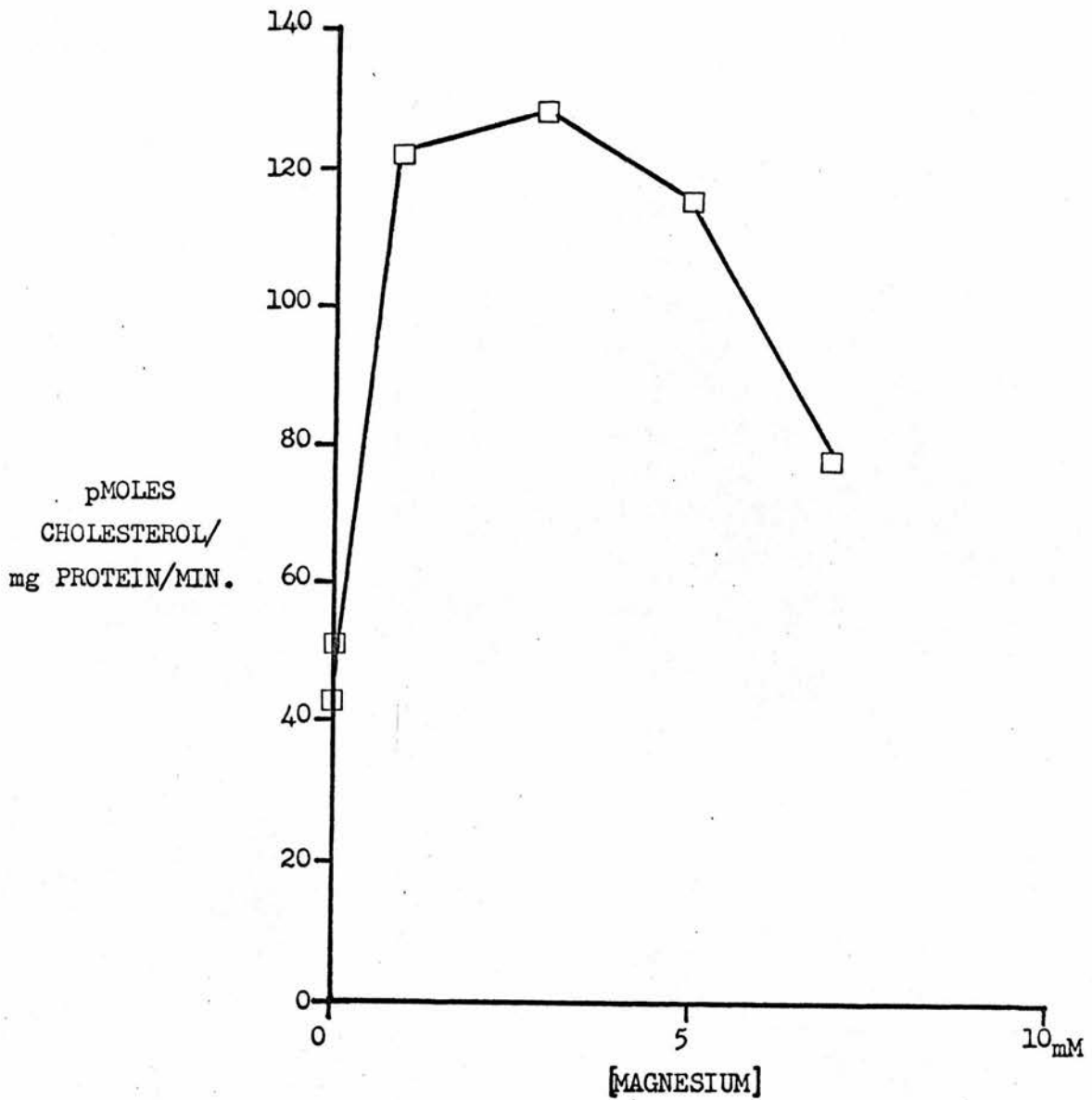
Figure 8.2. The effect of different concentrations of magnesium ions on the activity of rat luteal cholesterol esterase. The assay was carried out as described in chapter 2 except that the magnesium concentration was varied.

Table 8.1

Activators of rat luteal cholesterol esterase

Additions	Activity
	p mol/min/mg protein
None	48
5 mMATP	76
1 μM cAMP, 5 mMATP	75
5 mMMg^{++}	106
5 mMATP/Mg^{++}	136
1 μM cAMP, 5 mMATP/Mg^{++}	116

Cholesterol esterase was assayed as described in chapter 2 except magnesium ions were omitted from the incubation medium; other additions are shown on the table. Incubations were carried out in duplicate.

Table 8.2

The effect of cycloheximide and saline injection twenty minutes before killing on the activity of rat luteal cholesterol esterase

Additions	Activity	
	Cycloheximide	saline
	p mol/min/mg protein	
None	62	54
1 μM cAMP	63	49
5 mMATP	122	115
1 μM cAMP, 5mMATP	134	118

Cholesterol esterase was assayed as described in chapter 2. Incubations were carried out in duplicate. Additions to the incubations are shown on the table.

<u>The influence of cycloheximide administered twenty minutes</u>
<u>before killing on rat luteal cholesterol ester hydrolase activity</u>

Rats received cycloheximide twenty minutes prior to
killing (chapter 2). Cholesterol ester hydrolase activity
in the 105,000 x g desalted supernatant from the luteal
tissue was determined and compared with values obtained from
control rats injected with saline (Table 8.2). The effect
of the <u>in vitro</u> addition of cyclic AMP and ATP was studied.
Cycloheximide has been shown to inhibit luteal mitochondrial
side chain cleavage (Arthur and Boyd (1975)) but appeared
to have no significant effect on cholesterol ester hydrolase
activity. Cyclic AMP 1 μM added <u>in vitro</u> gave no stimulation
of cholesterol ester hydrolase activity in the control or
cycloheximide injected animals. <u>In vitro</u> addition of 5mM
ATP gave a two fold stimulation of cholesterol ester hydro-
lase activity in both groups of rats. Addition of cyclic
AMP with ATP gave no further stimulation of activity above
that observed on ATP addition alone.

Cycloheximide, a known inhibitor of protein synthesis,
does not affect the activity of cholesterol ester hydrolase
when administered <u>in vivo</u> a short time before killing, nor
does it appear to block the activation of cholesterol ester
hydrolase by the <u>in vitro</u> addition of ATP.

<u>Stimulation of luteal cholesterol ester hydrolase activity</u>
<u>by LH injection</u>

LH injections, administered 1 hour before killing as
described in chapter 2, produced a two fold stimulation of
luteal cholesterol ester hydrolase activity in the desalted
105,000 x g supernatant compared to the controls injected

with saline (Table 8.3). This effect has been observed before
(Behrman and Armstrong (1969); Armstrong and Flint (1973)).

In vitro addition of 5mM ATP was able to stimulate
significantly cholesterol ester hydrolase activity in the
105,000 x g supernatant of both groups of animals. Cyclic
AMP added together with ATP to the desalted supernatants
only produced a further significant stimulation of cholest-
erol ester hydrolase activity in the saline injected animals.
It is interesting to note that cofactor addition gave a
stimulation of cholesterol ester hydrolase activity to approxi-
mately the same degree in the desalted supernatants from
both groups of rats.

From these results it can be seen that LH injection
causes a stimulation of cholesterol ester hydrolase. It
was decided to determine whether the stimulation was due
to enzyme synthesis or an activation of an existing inactive
enzyme. An experiment was devised to clarify this point.

The influence of LH/cycloheximide, LH, cycloheximide and
saline injection on cholesterol ester hydrolase activity

The combination of trophic hormone and a protein synthe-
sis inhibitor on luteal cholesterol ester hydrolase was
studied as follows. Injections of LH, cycloheximide, saline
and cycloheximide followed by LH administration to rats
45 minutes before killing produced changes in cholesterol
ester hydrolase activity shown in Table 8.4. As demonstrated
previously injection of LH caused a two fold stimulation
of cholesterol ester hydrolase activity in the desalted
105,000 x g supernatant compared to the saline injected controls.
Cycloheximide treatment did not affect cholesterol ester
hydrolase activity or the two fold stimulation of activity

Table 8.3

The effect of LH injection one hour before killing on the activity of rat luteal cholesterol esterase

Additions	Activity	
	LH	Saline
	p mol/min/mg protein	
None	33	14
1 μM cAMP	32	16
5 mMATP	65	49
1 μM cAMP 5 mMATP	73	65

Cholesterol esterase was assayed as described in chapter 2.   Incubations were carried out in duplicate.   Additions to incubations are shown on the table.

Table 8.4

The effect of injections with LH and cycloheximide, LH, cycloheximide and saline, forty five minutes before killing, on the activity of rat luteal cholesterol esterase

Additions	Activity			
	Cycloheximide	Saline	LH	LH/Cycloheximide
	p mol/min/mg protein			
None	17	15	34	30
5 mMMg^{++}	86	53	53	88
1 μM cAMP	17	9	40	29
5 mMATP/Mg^{++}	95	57	100	95
1 μM cAMP 5 mMATP/Mg^{++}	107	63	95	112

Cholesterol esterase was assayed as described in chapter 2, except magnesium ions were omitted from the incubation medium, other additions are shown on the table. Incubations were carried out in duplicate.

achieved by the injection of LH. This is in contrast to
the effect of cycloheximide on mitochondrial side chain cleav-
age in luteal tissue where cycloheximide abolishes the stimula-
tory effect of the trophic hormone (Arthur and Boyd (1975)).

Again, injection of cycloheximide and LH did not affect
the stimulation of cholesterol ester hydrolase achieved by
the in vitro addition of cofactors to the desalted supernatant.

The effect of LH, cycloheximide and LH/cycloheximide injection
on the cholesterol/cholesterol ester ratio in the luteal
lipid droplets

The effect of LH and cycloheximide injection on cholest-
erol ester hydrolase and cholesterol side chain cleavage
activity is reflected in the composition of the luteal lipid
droplets isolated from this tissue (Table 8.5).

The ratio of cholesterol/cholesterol ester in the luteal
lipid droplets isolated from rats receiving LH 45 minutes
before killing was double that found in the lipid droplets
isolated from animals injected with saline. This increase
in free cholesterol was probably due to the activation of
cholesterol ester hydrolase by LH. As stated previously,
LH stimulates the side chain cleavage of cholesterol in luteal
mitochondria (Arthur and Boyd (1975)). The increased cholest-
erol side chain cleavage activity produced by trophic hormone
must have been less than the extra cholesterol produced by
the hydrolysis of the cholesterol esters.

Cycloheximide also produced an increase in the ratio
of cholesterol/cholesterol esters in the luteal lipid droplet.
The accumulation of cholesterol in this case is probably
due to the inhibition of cholesterol side chain cleavage
(Arthur and Boyd (1975)) as cholesterol ester hydrolase
activity is not altered by cycloheximide administration.

Table 8.5

The effect of injections with LH and cycloheximide, LH, cycloheximide and saline, forty five minutes before killing, on the ratio of cholesterol/ cholesterol esters in the lipid droplets of the luteal cell.

Pretreatment	ratio cholesterol/cholesterol esters
Saline	0.014
LH	0.027
Cycloheximide	0.030
LH/Cycloheximide	0.044

The cholesterol and cholesterol ester content of lipid droplets were determined as described in chapter 2.

The greatest increase in the ratio of cholesterol/cholesterol esters was observed in groups of animals injected with cycloheximide and LH. A three fold increase in the ratio of cholesterol/cholesterol esters was found in the lipid droplets isolated from these animals. This is consistent with the effect of LH and cycloheximide on cholesterol ester hydrolase and on the cholesterol side chain cleavage activity.

Discussion

The experimental results presented in this chapter confirm the findings of Behrman and Armstrong (1969) that LH treatment of rats causes a stimulation of cholesterol ester hydrolase activity in the 105,000 x g luteal supernatant.

In vitro addition of $MgCl_2$ has been shown in this chapter to significantly stimulate cholesterol ester hydrolase activity. This is in contrast to the in vitro addition of $MgCl_2$ to bovine adrenal 105,000 x g supernatant which is inhibitory at the same concentrations. It was postulated in chapter 6 that in the bovine adrenal cortex supernatant there is a magnesium dependent phosphoprotein phosphatase responsible for deactivation of cholesterol ester hydrolase. It is possible that in the luteal supernatant no magnesium ion dependent phosphoprotein phosphatase is present. It is difficult however to explain the stimulatory effect of magnesium ions on the luteal cholesterol ester hydrolase. Magnesium ions could have a direct effect on the cholesterol ester hydrolase or it could activate a protein kinase.

In the adrenal cortex a cyclic AMP dependent protein kinase is thought to activate cholesterol ester hydrolase (chapter 5). No conclusive effect was observed on the in vitro addition of cyclic AMP on luteal cholesterol ester hydrolase activity.

A small (0.1%) contamination of cyclic AMP in the 5mM
ATP however would give a concentration of cyclic AMP in the
incubation great enough to produce maximal stimulation of
protein kinase activity.

Protein kinase activity has been demonstrated in the
bovine corpus luteum. Menon (1973) demonstrated in the
bovine corpus luteum the presence of two protein kinases
KI and KII. Both KI and KII could be stimulated by cyclic
AMP but KII was also stimulated by LH in vitro. Other pituitary
hormones were found to be ineffective. It has been demonstr-
ated that cyclic AMP activates protein kinase by forming
a complex with a regulatory subunit to release a catalytic
subunit in an active state (Gill and Garren (1970)). Azhar
and Menon (1975)have shown that activation of KII by cyclic
AMP follows the same mechanism. Stimulation of KII by the
in vitro addition of LH however does not follow the same
mechanism. It has been postulated that LH could cause a
greater affinity of the histone used in the kinase assay
for KII. Alternatively LH could directly activate the catalytic
subunit without affecting the regulatory-catalytic subunit
complex. It is possible therefore that the protein kinases .
KI and KII are also present in the rat corpus luteum. KII
activated by LH could then be responsible for the phosphor-
ylation and activation of cholesterol ester hydrolase by ATP.
This would explain why no activation of cholesterol ester
hydrolase by cyclic AMP was achieved. KII however activated
by LH will not promote the phosphorylation of ribosomes in
the presence of ATP. KII activated by cyclic AMP will promote
the transfer of the terminal phosphate from ATP to the ribos-
omes (Azhar and Menon (1975)). It cannot be excluded however
that LH activated KII may be able to phosphorylate cholesterol

ester hydrolase. Failure of cyclic AMP to produce activation of cholesterol ester hydrolase in the luteal tissue may also be due to the protein kinase in the 105,000 x g supernatant being already fully activated.

The regulatory subunit is easily dissociated from the catalytic subunit (H. Nimmo personal communication) and it is possible that this occurs in the preparation of the 105,000 x supernatant.

Cycloheximide administration at a dose of 5mg per rat had no effect on the stimulation of cholesterol ester hydrolase activity by LH. This indicates that the cycloheximide sensitive factor involved in the mitochondrial side chain cleavage reaction is not involved in the activation of cholesterol ester hydrolase. The induction of activity in both the side chain cleavage enzyme and the cholesterol ester hydrolase is probably controlled by different mechanisms. The results presented here also indicate that the mechanism of activation and deactivation of cholesterol ester hydrolase in the corpus luteum may be different from that found in the adrenal.

The three fold increase in the ratio of cholesterol/ cholesterol esters produced in the lipid droplets isolated from animals injected with cycloheximide followed by LH compared to the saline injected controls is consistent with the effect of LH and cycloheximide on cholesterol ester hydrolase and cholesterol side chain cleavage activity. LH injection would increase the amount of free cholesterol produced from the hydrolysis of cholesterol esters in the luteal lipid droplets and cycloheximide treatment would prevent the metabolism of the free cholesterol released by inhibiting cholesterol side chain cleavage.

Summary

a) Cholesterol ester hydrolase activity in the 105,000 x g supernatant of rat luteal homogenate is stimulated by _in vitro_ addition of $MgCl_2$ and ATP.

b) Cyclic AMP addition _in vitro_ to the supernatant in the presence or absence of ATP or $MgCl_2$ gave no stimulation.

c) Injection of LH 1 hour before killing resulted in a two fold stimulation of cholesterol ester hydrolase activity.

d) Injections of cycloheximide did not affect cholesterol ester hydrolase activity or inhibit the stimulation of cholesterol ester hydrolase by LH injection.

e) LH treatment of rats caused an increase in the ratio of free cholesterol/cholesterol esters in the luteal lipid droplets.

CONTENTS

CHAPTER 9

THE EFFECTS OF DIETARY RAPE-SEED OIL ON CHOLESTEROL ESTER

METABOLISM AND CHOLESTEROL ESTER HYDROLASE ACTIVITY IN THE

RAT ADRENAL

Introduction

There are circumstances in which adrenal cholesterol
esters accumulate and the ability of the adrenal to remove
these esters seems to be impaired. One such case is when
animals are fed physiological amounts of rape seed oil, a
vegetable oil obtained from the seed of the rape plant.
It has been known since the 1940's that dietary rape seed
oil has many adverse physiological effects on various animals.

It has been reported that feeding rape seed oil to male
rats causes an accumulation of lipid in the heart, skeletal
muscle (Abdellatif and Vles (1970)) and adrenals (Carrol (1951)).
The lipid accumulation in the heart has been shown to be
accompanied by a decrease in the rate of ATP synthesis in
isolated heart mitochondria (Houtsmuller et al. (1970)).
It has been shown that the lipid accumulation in the heart
decreased on prolonged feeding of the oil (Abdellatif and
Vles (1970)) and this decrease is thought to be due to an
increased lipase activity (Jansen et al. (1975)).
Boer et al. (1947) have found that the feeding of rape seed
oil retards the growth of rats and this is has been confirmed
by Thomasson (1955) who identified the substance responsible
for the growth retardation as erucic acid (Thomasson et al. (1955)
Some of the subsequent studies on the nutritional character-
istics of rape seed oil (Alexander and Mattson (1966));
(Middleton and Campbell (1958)); but not all of them (Beare
et al. (1957)); (Salmon (1969)) showed that the growth
retardation could be largely accounted for by the decreased

acceptibility of the rape seed oil diet.

Erucic acid in theory can be β-oxidised to oleic acid
(Craig and Beare (1967)), but in fact erucic acid has been
shown to be oxidised more slowly than oleic or palmitic acid
(Swarttouw (1974)). Erucic acid does not inhibit the oxida-
tion of palmitic or oleic acid.

The accumulation of lipid in rat adrenals on feeding
rape seed oil is due mainly to a 3 to 5-fold increase in
the amount of cholesterol esters (Carrol (1951)), and in
particular cholesterol erucate (Carrol (1962)). Carrol (1953)
showed that erucic acid was the constituent of rape seed
oil responsible for this effect.

When animals are subjected to a stressful situation
a depletion of cholesterol esters in the adrenal occurs
(Trzeciak and Boyd (1974)); (Walker and Carney (1971)).
Walker and Carney (1971) also showed that in rats fed ethyl
erucate plus corn oil and subjected to a 30 minute cold
stress, cholesterol ester depletion was less than in the
control animals fed olive oil. A prominent metabolic feature
of the animals fed ethyl erucate was the lack of utilisation
of cholesterol erucate.

Carrol and Noble (1952) have reported that although
producing an accumulation of adrenal cholesterol esters,
rape seed oil had no marked influence on adrenal function
and such adrenals respond to ACTH and stress. Injection
of large doses of ACTH however failed to reduce the concen-
tration of adrenal cholesterol to low levels in rats fed
rape seed oil. Walker and Carney (1971) showed that plasma
corticosteroids in animals fed corn oil and ethyl erucate
were not elevated to the same level as corticosteroids in
the plasma of animals fed olive oil when both groups of animals

were subjected to a cold stress. Prostaglandin production
which is stimulated by ACTH in adrenal homogenates was also
lower in animals fed rape seed oil compared to animals fed
on corn oil (Carney et al. (1972)).

The aim of the experiments described in this chapter
was to establish whether adrenal cholesterol ester accumul-
ation in rats fed rape seed oil was due to an inhibition
of the adrenal cholesterol ester hydrolase or an inability
of the cholesterol ester hydrolase to metabolise cholesterol
erucate.

The effect of ether anaesthesia stress on cholesterol
ester hydrolase activity in animals fed rape seed oil has
also been studied.

Animals and Animal Treatment

Male Wistar rats (Edinburgh University Small Animal
Breeding Centre, Bush) were maintained for six weeks on a
stock diet of 25% skimmed milk powder, 5% dried yeast and
70% wholemeal flour, supplemented with 25% by weight olive
oil or rape seed oil, and were given water ad libitum.

The animals on each diet were randomly divided into
two groups at the termination of the experiment. The anim-.
als of one group, the controls, were kept in a quiescent
state, transferred individually to a separate room for killing
to minimise stress due to handling and killed by decapitation.
The second group were subjected to an ether anaesthesia stress
for ten minutes before killing.

Results

The fatty acid composition of the oil diets is given
in Table 9.1. Low levels of saturated fatty acids were found
in both diets. A high concentration (60%) of erucic acid
(22:1) was found in the rape seed oil diet with oleic acid

Table 9.1

Fatty Acid Composition of Dietary Fats

Fatty Acid	Percentage* of Total Fatty Acid	
	Olive Oil	Rape Seed Oil
16:0	14	4
18:0	1	-
18:1	61	11
18:2	10	13
20:1	1	11
22:1	6	60

*Minor constituents omitted from table

(18:1) contributing only 11% of the total fatty acid composition in this oil. In the olive oil diet a relatively small amount (6%) of the 22:1 acid was found, 18:1 being the major (61%) fatty acid constituent in this oil.

Effects of feeding olive oil and rape seed oil on adrenal and body weight in the rat

The adrenals of rats kept on the rape seed oil diet for 6 weeks appeared larger than the adrenals of rats fed on the olive oil diet and had a white appearance. There was however no significant difference in adrenal weight, although the body weights of rats fed rape seed oil for six weeks were significantly lower ($p < .001$) than the control rats fed olive oil (Table 9.2). Due to the lower body weight of rats fed rape seed oil, these animals had a higher adrenal weight expressed per gram body weight.

The effects of ether stress on the composition of the cholesterol esters in lipid droplets from the adrenals of rats fed stock diet, olive oil and rape seed oil

Table 9.3 shows the composition of the cholesterol esters in the lipid droplets isolated from quiescent rats and ether stressed rats fed stock diet, rape seed oil diet and olive oil die

It can be seen that in the lipid droplets of rats kept on rape seed oil there was an accumulation of the 22:1 ester whilst little of this ester accumulated in rats kept on the other diets.

Ether stress resulted in a decrease in the concentration of the 14:0, 16:0, 16:1, 18:1, 18:2 and 20:4 esters in lipid droplets of rats receiving rape seed oil, while rats receiving olive oil showed a selected decrease in concentrations of 16:0, 18:1 and 18:2 esters. Stock diet fed animals showed a decrease of 16:1, 18:0, 18:1, 18:2 and 20:4 esters as a

The Effect of Dietary Olive Oil and Rape Seed Oil on
the Body Weight and Adrenal Weight

Treatment	Exp. Number	*Average weight per rat (gms) (12 rats)	Average weight per adrenal (mgs) (12 rats)	Average weight per adrenal per gram body weight (μgrams)
Olive Oil Diet	1	307 ± 7	26.5	86
	2	390 ± 10	31.5	81
	3	249 ± 4	31.0	124
Rape Seed Oil Diet	1	253 ± 7	27.2	107
	2	306 ± 4	32.4	105
	3	221 ± 5	28.6	129

*Table shows Mean Body Weight ± S.E.M.

Rats were fed the olive oil and rape seed oil diets for 5 to 6 weeks

Adrenals from each group were pooled after killing and weighed

TABLE 9.3

Effect of Ether Stress on the Cholesterol Esters from Adrenal Lipid Droplets

of Rats Fed Stock, Olive Oil and Rape Seed Oil Diets

Percentage of Cholesterol Ester

Ester*	Stock Diet		Olive Oil Diet		Rape Seed Oil Diet	
	Quiescent	Stressed	Quiescent	Stressed	Quiescent	Stressed
14:0	4.3	4.3	1.1	1.3	2.2	1.3
16:0	28.6	29.5	16.0	14.3	14.3	12.7
16:1	7.1	5.7	2.7	2.7	4.7	3.1
18:0	7.1	6.1	6.9	7.3	2.3	2.6
18:1	25.5	24.3	41.4	40.1	32.8	31.3
18.2	13.3	11.4	15.4	13.5	10.7	8.0
20:1	2.0	6.1	3.3	3.4	9.2	9.7
20:4	8.8	7.8	7.2	7.6	3.4	1.9
22:1	–	–	1.2	2.7	18.7	27.5
22:4	3.3	4.8	4.8	7.1	1.7	1.9

*Minor constituents omitted from the table.

Ester composition was determined by G.L.C. as described in the Methods section.

consequence of ether anaesthesia.

The lack of utilization of the 22:1 and 20:1 esters
is consistent throughout all the groups of rats fed the
different diets.

The influence of a rape seed oil diet on corticosteroid
production in rats subjected to an ether anaesthesia stress

A rat fed on a rape seed oil diet for 6 weeks was subjected
to an ether stress for 1.5 hours. A 0.5ml sample of plasma
was removed from the tail of the rat every 30 minutes and
assayed for corticosterone by the method described in chapter 2.
A control experiment was also carried out at the same time
on a rat fed on olive oil diet for 6 weeks.

The results are shown in Figure 9.1. It can be seen
that on ether stress plasma corticosterone in the rape seed
fed animal did not increase to the same level as plasma
corticosterone in the animal fed olive oil. These results
are consistent with the findings of Walker and Carney (1971)
who used an ethyl erucate diet and a 30 minute cold stress.

The effects of ether stress, cyclic AMP and ATP addition
in vitro on the activity of adrenal 105,000 x g supernatant
cholesterol ester hydrolase in rats subjected to stock,
olive oil and rape seed oil diets

Table 9.4 shows the effect on adrenal cholesterol ester
hydrolase of feeding rats stock, olive oil and rape seed
oil diets. In the quiescent state only in experiment 2 was
there a significant difference in cholesterol ester hydro-
lase activity between rats on the olive oil diet and rats
fed rape seed oil. In three out of the four experiments,
rats maintained on rape seed oil had a significantly higher
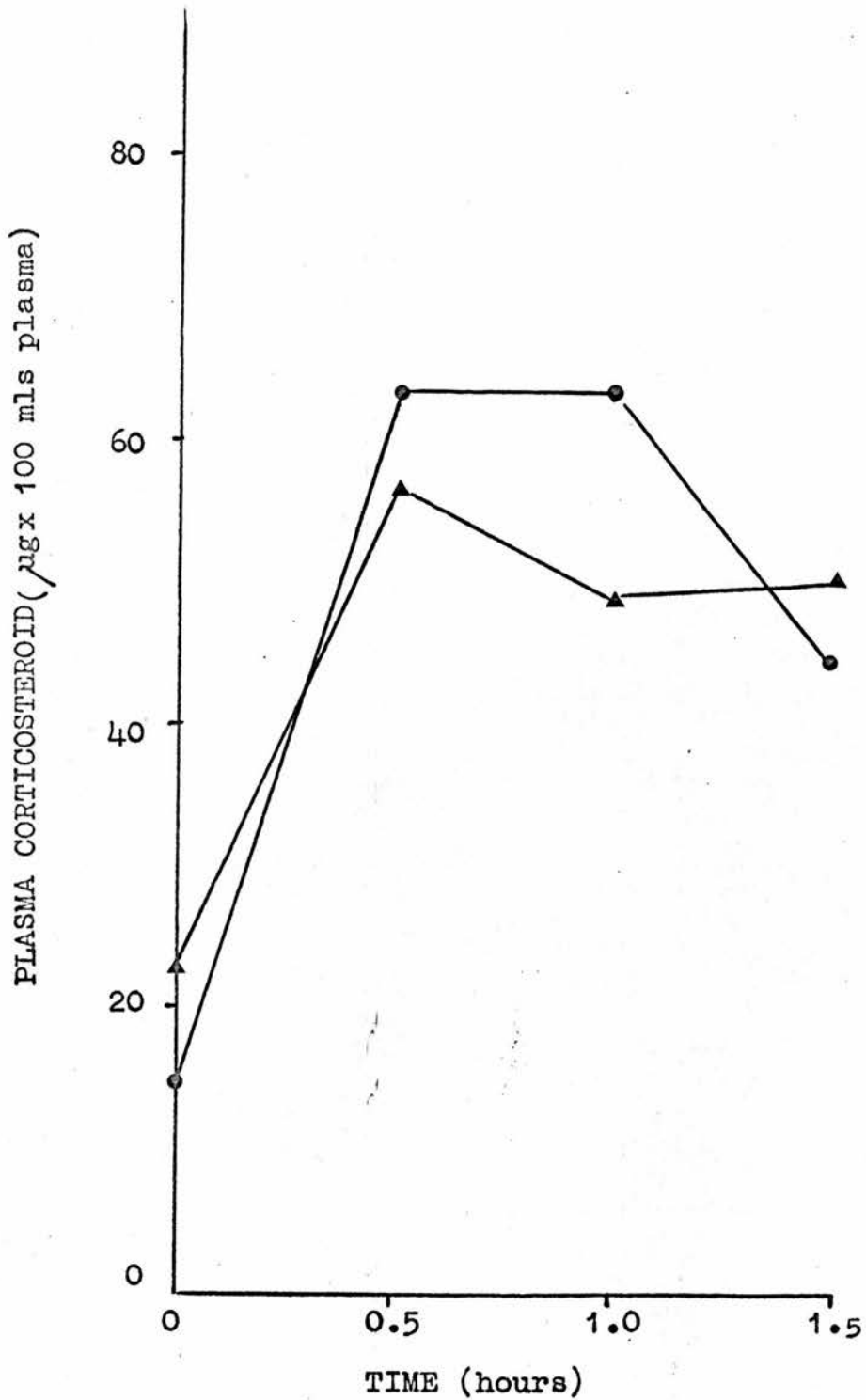
Figure 9.1 The influence of a rape seed oil diet on corticosteroid production in rats subjected to an ether anaesthesia stress.

Experimental procedure as text. Animals fed rape seed oil (▲) and olive oil (●).

TABLE 9.4

The Effect of Ether Stress and Cofactor Addition on Rat Adrenal

Supernatant Cholesterol Ester Hydrolase Activity in Rats fed Stock,

Olive Oil and Rape Seed Oil Diets

Additions	Exp. No.	*Cholesterol Ester Hydrolase Activity p moles $\times$ min^{-1} $\times$ mg^{-1} Protein					
		Stock Diet		Olive Oil Diet		Rape Seed Oil Diet	
		Quiescent	Stressed	Quiescent	Stressed	Quiescent	Stressed
None **+ cofactors	1	147 ± 5 219 ± 3	273 ± 13 274 ± 10	158 ± 25 324 ± 20	319 ± 10 409 ± 5	198 ± 15 210 ± 11	109 ± 5 136 ± 22
None **+ cofactors	2	97 ± 8 227 ± 30	136 ± 11 216 ± 12	99 ± 2 250 ± 20	121 ± 4 230 ± 17	166 ± 11 206 ± 20	149 ± 14 143 ± 19
None **+ cofactors	3	28 ± 2 104 ± 5	43 ± 1 113 ± 13	58 ± 10 154 ± 18	71 ± 4 140 ± 7	77 ± 12 180 ± 32	68 ± 0 82 ± 4
None **+ cofactors	4	119 ± 5 212 ± 6	150 ± 10 218 ± 3	118 ± 6 286 ± 4	269 ± 26 296 ± 20	120 ± 17 168 ± 5	121 ± 10 188 ± 5

*Results are quoted as the mean of duplicate (experiments 3 and 4) or triplicate assays ± S.E.M.

**Cofactors added were 5x10^{-3}M ATP + 5x10^{-3}M MgCl$_2$ + 10^{-5}M cAMP.

Assays were carried out as described in the methods section.

cholesterol ester hydrolase activity ($p<0.05$) than animals maintained on stock diet. When the probabilities found in each of the four experiments were combined (Fisher (1957)) the overall probability that the differences in cholesterol ester hydrolase activity, assayed with cholesterol oleate, between the two groups of rats, is due to chance, is less than 0.01.

Cholesterol ester hydrolase activity was significantly increased ($p<0.05$) in animals maintained on olive oil or stock diet when the animals were subjected to a ten minute ether anaesthesia stress. Animals fed rape seed oil showed no significant increase in cholesterol esterase activity on ether stress and in experiments 1 and 2 there was observed a significant ($p<0.01$) decrease in cholesterol ester hydrolase activity.

Animals maintained on olive oil or stock diet and not subjected to an ether stress gave a significant ($p<0.05$) stimulation of cholesterol ester hydrolase activity when 5mM ATP and 1 μM cyclic AMP were added in vitro. Cholesterol ester hydrolase activity was stimulated in vitro to a higher value with the cofactor additions than was observed when the animals had been subjected to ether stress alone. The addition of 5mM ATP and 1 μM cyclic AMP in vitro to the adrenal supernatant from ether stressed animals fed olive oil or stock diet also stimulated cholesterol ester hydrolase activity to values similar to or greater than those found on adding the cofactors to adrenal supernatant from animals not subjected to an ether stress.

Animals maintained on rape seed oil and not subjected to an ether anaesthesia stress gave no significant stimulation of cholesterol ester hydrolase activity when these cofactors were added in vitro.

Animals fed rape seed oil and subjected to ether stress exhibited a slight increase in cholesterol ester hydrolase activity in vitro by cofactor addition.

Table 9.5 shows the effect of feeding rats diets of rape seed oil, olive oil and stock diets on adrenal cholesterol ester hydrolase activity using 24 μM [4-^{14}C] cholesterol erucate as substrate compared with 24 μM [4-^{14}C] cholesterol oleate. Cholesterol erucate was hydrolysed at a slower rate than cholesterol oleate in enzyme preparations obtained from all groups of animals. Cholesterol ester hydrolase activity could only be stimulated in animals fed olive oil or stock diet by a ten minute ether anaesthesia before killing or by cofactor addition in vitro. Animals fed rape seed oil showed no increase in the rate of cholesterol erucate hydrolysis when subjected to stress but cofactor additions stimulated cholesterol ester hydrolase activity in the supernatant obtained from adrenals of stressed and unstressed animals.

The endogenous cholesterol concentration of the 105,000 g adrenal supernatant obtained from rats fed stock diet, olive oil and rape seed oil diets after passage through a Sephadex G-25 column was 1.8, 6.6 and 6.6 μg per mg protein respectively. Cholesterol ester concentration was 13.6, 21.7 and 19.6 μg per mg^{-1} protein respectively. The endogenous ester concentrations were measured to show that the radioactive assay system employed, was valid to compare cholesterol

TABLE 9.5

The Effect of Ether Stress and Cofactor Addition on Rat Adrenal

Supernatant Cholesterol Ester Hydrolase Activity in Rats Fed Stock,

Olive Oil and Rape Seed Oil Diets using $[4-^{14}C]$ Cholesterol Oleate and

Erucate as Substrate

Substrate	Additions	*Cholesterol Ester Hydrolase Activity p moles x min^{-1} x mg^{-1} Protein					
		Stock Diet		Olive Oil Diet		Rape Seed Oil Diet	
		Quiescent	Stressed	Quiescent	Stressed	Quiescent	Stressed
Oleate	None	119 ± 5	150 ± 10	118 ± 6	269 ± 26	120 ± 17	124 ± 10
	**+ cofactors	212 ± 4	218 ± 3	286 ± 4	296 ± 20	168 ± 5	188 ± 5
Erucate	None	50 ± 5	61 ± 1	41 ± 3	95 ± 8	31 ± 1	31 ± 7
	**+ cofactors	96 ± 10	72 ± 3	106 ± 31	104 ± 1	61 ± 4	103 ± 2

*Results are quoted as the mean of duplicate assays ± S.E.M.

**Cofactors added were 5x10^{-3}M ATP + 5x10^{-3}M MgCl$_2$ + 10^{-5}M cAMP.

Assays were carried out as described in the methods section.

ester hydrolase activity between the groups of rats fed different diets.

The effects of erucic acid, oleic acid, 5mM ATP and 10 μM Cyclic AMP on cholesterol ester hydrolase activity using $[4-^{14}C]$ cholesterol oleate as substrate

Figure 9.2 shows the effect of varying the concentration of free oleic and erucic acids in vitro on the activity of cholesterol ester hydrolase from rats fed stock diet.

Oleic acid gave a significant inhibition of cholesterol ester hydrolase activity at a concentration of 2 μM. The inhibition increased as the concentration of oleic acid was increased until at 160 μM cholesterol ester hydrolase activity had fallen from 70 pmol per min per mg protein to 20 pmol per min per mg protein.

Erucic acid gave a significant activation of cholesterol ester hydrolase activity over the same concentration range as oleic acid with a maximum stimulation to 128 pmol per min per mg protein found at a concentration of 160 μM.

When 5mM ATP and 10 μM cyclic AMP were added, cholesterol ester hydrolase activity was stimulated from 70 pmol per min per mg protein to 154 pmol per min per mg protein. Oleic acid when added in the presence of cofactors again gave a strong inhibition until at a concentration of 160 μM the cholesterol ester hydrolase activity had again fallen to 22 pmol per min per mg protein Figure 9.3.

Erucic acid exhibited no further activation when added in the presence of ATP and cyclic AMP but unlike oleic acid it did not exhibit any inhibition at a concentration of 33 μM and even at a concentration of 160 μM cholesterol ester
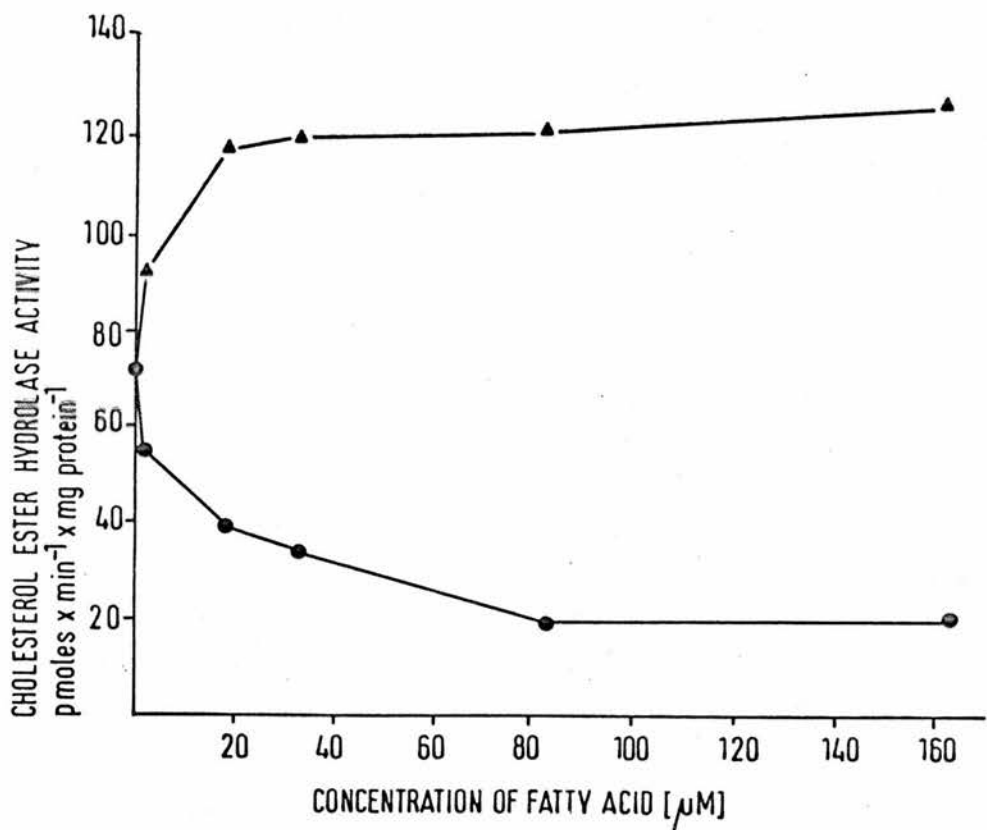
Figure 9.2 The effect of oleic acid (●) and erucic
acid (▲) added in vitro on cholesterol ester hydrolase
activity in the 105,000 x g supernatant from adrenals
of rats fed stock diet.

Incubations were carried out as described in the text.
The acids were added in 10 μl of acetone
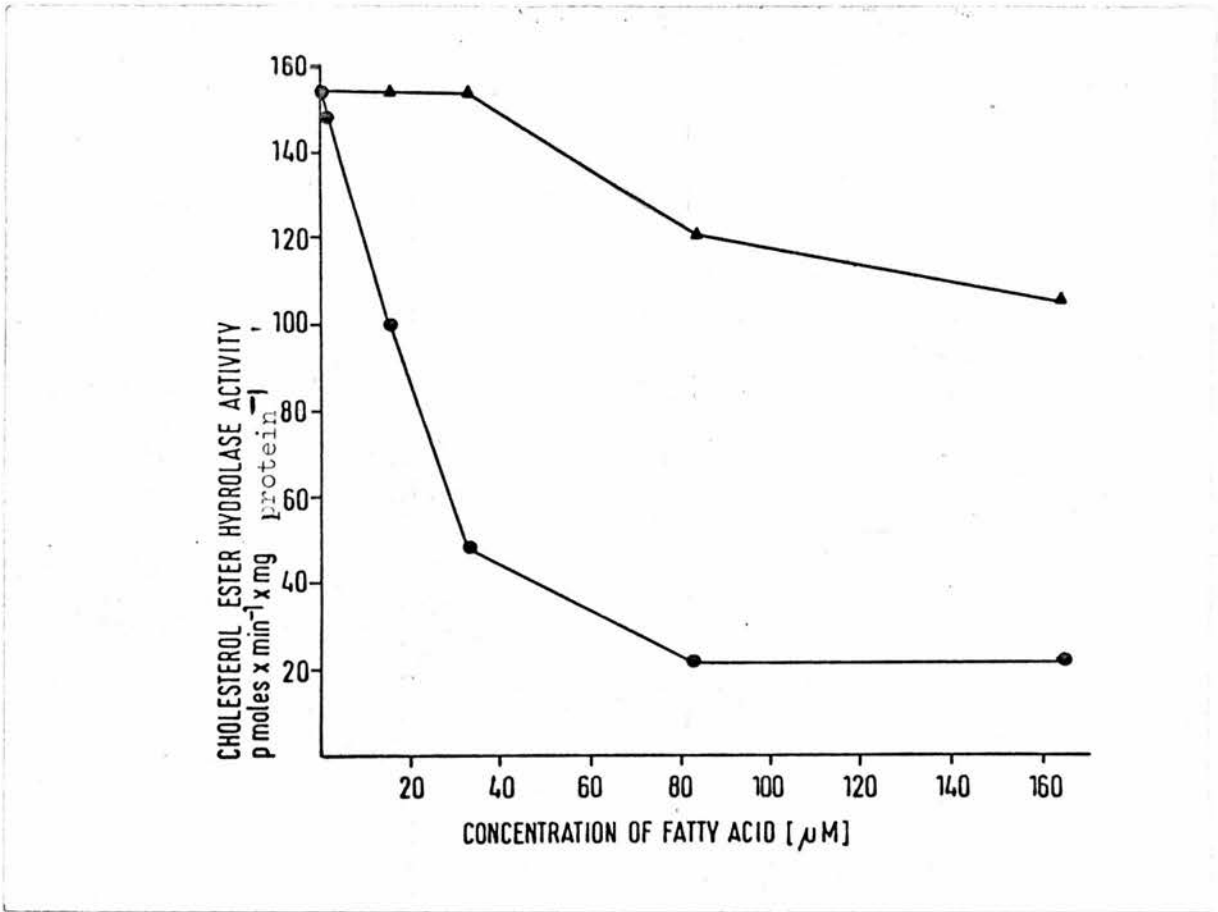
Figure 9.3 The effect of oleic acid (●) and erucic acid (▲) added in vitro on cholesterol ester hydrolase activity of 105,000 x g supernatant in the presence of cofactors from the adrenals of rats fed stock diet. Incubations were carried out as described in the text.
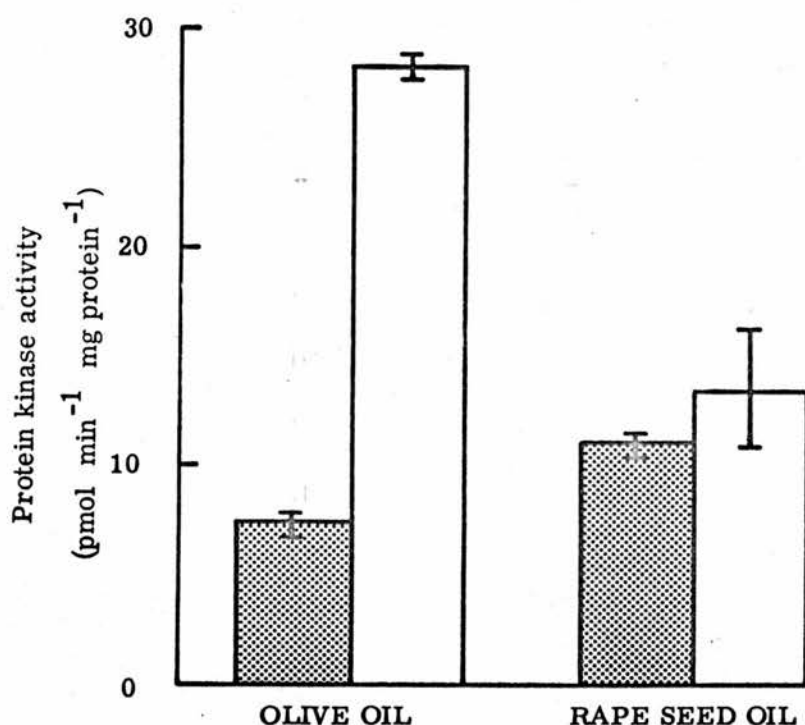
Figure 9.4 The effect of ether stress on protein
kinase activity of the 105,000 x g supernatant from
adrenals of rats fed olive oil and rape seed oil diets.
Incubations were carried out as described in the text.
12 rats were used in each group. Unshaded bars represent
protein kinase activity in animals subjected to a
ten minute ether anaesthesia stress. Shaded bars
represent protein kinase activity in control animals.

hydrolase activity had not fallen below the unstimulated
value of 70 pmol per min per mg protein.

The activity of adrenal 105,000 x g supernatant protein
kinase in rats subjected to olive and rape seed oil diets

Figure 9.4 shows the effect of a ten minute ether anaesth-
esia stress on rat adrenal supernatant protein kinase acti-
vity in animals fed olive oil and rape seed oil diets.
Protein kinase activity was significantly increased ($p < 0.05$)
in rats fed olive oil when these animals were subjected
to a ten minute ether anaesthesia stress. Rats fed the
rape seed oil diet did not exhibit any significant increase
in protein kinase activity.

Discussion

Carrol (1951) has shown that feeding Sprague Dawley
rats a diet of 45% by weight rape seed oil for four weeks
produced an increase in adrenal size and an increase in
the absolute amount of adrenal cholesterol compared with
a control where the oil was absent. The oil produced nearly
the same effect when fed as 25% of the diet and the adrenals
had the same white appearance which was also noted when the
whole seed was fed. The growth rate of rats fed 25% rape
seed oil was lower than the control animals although the
growth rate was much better than when 45% rape seed oil was given

Beare et al. (1959) using Wistar rats found no differ-
ence in adrenal weights after animals had been fed rape seed
oil at 20% for four weeks. Animals fed 20% rape seed oil
failed to gain weight at the same rate as a group of anim-
als fed 20% corn oil for four weeks. Beare et al. (1959)
and Craig et al. (1963) have attributed this slow weight
gain to a lower food consumption of the rape seed diet and

possibly to the low level of saturated acids particularly palmitic acid.

The results described in this chapter confirms those of Beare et al. (1959) in that although the adrenals of animals fed 25% by weight rape seed oil appeared larger and had a white appearance there was no increase in total adrenal weight compared with animals fed 25% by weight olive oil. Animals fed 25% rape seed oil also failed to gain weight at the same rate as the controls.

Walker and Carney (1971) have reported that when rats are subjected to a cold stress for 30 min. total adrenal cholesterol esters in animals receiving an olive oil diet decrease by 34% of the control value. In contrast a cold stress applied to animals receiving ethyl erucate resulted in an insignificant decrease in cholesterol esters. Animals fed ethyl erucate showed only a significant decrease in the concentration of the 18:1 ester when subjected to a 30 min. cold stress, while animals fed olive oil showed decreases in 16:1, 18:2, 20:1, 20:4 and 22:4 cholesterol esters.

From the results it can be seen that when animals were subjected to a 10 min ether stress, decreases in the concentration of 14:0, 16:0, 16:1, 18:1, 18:2 and 20:4 cholesterol esters were observed in animals fed rape seed oil, while animals fed olive oil showed decreases in 16:0, 18:1 and 18:2 esters. As reported by Carrol (1951) a high accumulation of cholesterol erucate in the adrenals of rats fed rape seed oil for four weeks was found but little of this ester accumulated in rats fed olive oil or stock diet for the same period of time.

Ether stress, which increases plasma ACTH concentrations (Matsayuma et al. (1971)), caused an increase in the activity of cholesterol ester hydrolase in both animals fed stock diet or olive oil but in animals fed rape seed oil no increase in activity was found, and in fact in two experiments a significant decrease in activity was observed. These results explain Carney and Walker's (1971) findings that animals fed ethyl erucate show little decrease in the cholesterol ester content of the adrenal when the animals are subjected to a cold stress and also explain the findings of Carrol and Noble (1952) that large doses of ACTH failed to reduce the concentration of total adrenal cholesterol to low levels in animals fed rape seed oil.

Cofactor addition in vitro of cyclic AMP and ATP stimulated cholesterol ester hydrolase activity in stressed and unstressed animals fed stock diet or olive oil. In only one case was a significant (p<0.05) stimulation of cholesterol ester hydrolase activity achieved in vitro in animals fed rape seed oil.

It has been inferred that activation of cholesterol ester hydrolase in the bovine adrenal cortex is achieved by phosphorylation of an inactive cholesterol ester hydrolase by a cyclic AMP dependent protein kinase in the presence of ATP (Trzeciak and Boyd (1974)) and it has been suggested that a similar system occurs in the rat (Trzeciak and Boyd (1973)).

Protein kinase activity in the rat adrenal 105,000 g supernatant from animals fed stock or olive oil diet was elevated as reported previously (Trzeciak and Boyd (1973)) when animals were subjected to ether anaesthesia. Animals

fed rape seed oil, however did not exhibit the usual stimul-
ation of protein kinase activity on stress.

It has been reported (Houtsmuller et al. (1970)) that
ATP production in heart mitochondria isolated from rats fed
rape seed oil is inhibited and there was an inverse linear
relationship between the amount of erucic acid injected and
ATP production. Swarttouw (1974) has also shown that erucic
acid is oxidised more slowly by rat heart mitochondria than
palmitic and oleic acid. If it is true that ATP is necess-
ary in the activation of cholesterol ester hydrolase, then
a reason for the enzyme not being activated when the animal
is subjected to an ether stress could be that ATP product-
ion from adrenal mitochondria is impaired and thus ATP
levels are not sufficiently high to activate the enzyme.
We found a significant stimulation of cholesterol ester
hydrolase on addition of cyclic AMP and ATP in only one
experiment, so it seems unlikely that the failure of cholest-
erol ester hydrolase to respond normally in rats fed rape
seed oil is due to a lower concentration of ATP in the
adrenals of these animals.

It is possible that feeding rape seed oil may impair .
the ability of the protein kinase to phosphorylate the cholest-
erol ester hydrolase or may even cause an inability of the
pituitary to release ACTH. Griesback (1941) showed that
pronounced changes in pituitary cell structure occurred
when rats were fed rape seed oil for seven days.

Free erucic acid as shown by the results does not exhibit
any inhibitary properties on cholesterol ester hydrolase
unlike oleic acid which strongly inhibits at μM concentrations.

Erucic acid in fact activated cholesterol ester hydrolase
in vitro and it is possibly this activating property of the
free acid which caused the quiescent animals fed rape seed
oil to have a higher basal level of cholesterol ester hydro-
lase activity than animals fed stock diet. Electron micro-
scopy showed no differences in the structure of the adrenals
from animals fed rape seed oil or olive oil (A. Wyllie
personal communication).

As the results have shown, cholesterol erucate is hydro-
lysed in vitro at only 25% of the rate of cholesterol oleate
in animals fed rape seed oil and this together with the
inability of cholesterol ester hydrolase to respond normally
to a stress situation explains why cholesterol erucate
accumulates in adrenals of animals fed rape seed oil.

Rats fed olive oil diet, which respond normally to a
stressfull situation to give an increase in cholesterol
ester hydrolase activity, hydrolyse cholesterol erucate in vitro
at only 30% of the rate of cholesterol oleate. It would
seem therefore that in rats fed rape seed oil diet the init-
ial cause of the accumulation of cholesterol erucate is an
inability of the cholesterol ester hydrolase to hydrolase
the cholesterol erucate at a sufficient rate to stop its
accumulation. On a prolonged diet of rape seed oil the adren-
al cholesterol ester hydrolase system seems to loose its
ability to respond to a stressfull situation. No increase
in cholesterol ester hydrolase activity can be observed in vitro
and this would favour further accumulation of cholesterol oleate.

These studies on the effect of dietary rape seed oil
on cholesterol ester hydrolase activity and corticosteroid

production lend support to the view that cholesterol ester
hydrolase plays a key role in the control of steroidogenesis.
Corticosteroid production in animals fed rape seed oil diet
and subjected to an ether stress is lower than in the control.
As cholesterol side chain cleavage activity is not altered
by dietary rape seed oil (J. Arthur personal communication)
it is possible that corticosteroid production in these
animals subjected to an ether anaeshtesia stress is lower
due to a diminished supply of cholesterol from the lipid
droplet cholesterol esters to the mitochondrial side chain
cleavage enzyme. This will be discussed in chapter 10.

Summary

The effects of stock diet and stock diet supplemented by olive oil and rape seed oil on rat adrenal cholesterol ester metabolism has been studied.

a) Rats fed rape seed oil failed to gain weight at the same rate as rats fed olive oil.

b) A prominent feature of the rats fed rape seed oil was on accumulation of high concentrations of cholesterol erucate in the adrenal lipid droplets. No percentage decrease in the amount of cholesterol erucate was observed in these rats when they were subjected to an ether stress.

c) Plasma corticosteroid levels were not elevated to the same degree on ether stress in animals fed rape seed oil compared to the control animals fed on an olive oil diet.

d) Adrenal cholesterol ester hydrolase activity was higher in rats fed the olive oil and rape seed oil diets than rats fed the stock diet.

e) A ten minute ether anaesthesia stress resulted in a two fold increase in adrenal cholesterol ester hydrolase activity in rats fed stock or olive oil diets. Cofactor addition of ATP, cyclic AMP and $MgCl_2$ in vitro resulted in a stimulation of cholesterol ester hydrolase to a similar activity in both quiescent and ether stressed rats.

f) Rats fed the rape seed oil diet gave no significant stimulation of cholesterol ester hydrolase activity when given an ether stress or when cofactors were added.

g) Cholesterol erucate was hydrolysed at only 25% to 30% of the rate of cholesterol oleate in vitro in all groups of animals.

h) Oleic acid added _in vitro_ gave an inhibition of cholesterol ester hydrolase activity in rats fed stock diet while erucic acid activated the enzyme.

It is postulated that the initial cause for the accumulation of cholesterol erucate in the adrenal when rats are fed rape seed oil is the inability of cholesterol ester hydrolase to hydrolyse the ester at a sufficient rate. Prolonged feeding of rape seed oil however causes a inability of the enzyme to respond to a stressfull situation and increase the rate of hydrolysis of the esters thus enhancing the accumulation of the cholesterol oleate.

CHAPTER 10

GENERAL DISCUSSION

The object of these studies was to investigate the mechanism of control of a cholesterol ester hydrolase in adrenal cortical and luteal tissue. The role of cyclic AMP, ATP, cyclic AMP dependent protein kinase and phosphoprotein phosphatase in the control of this cholesterol ester hydrolase has been investigated. From these studies a role for cholesterol ester hydrolase in the control of steroidogenesis has been postulated.

Most of the studies described in this thesis have been concerned with the cholesterol ester hydrolase found in the bovine adrenal cortex cytosol. This tissue was chosen as it was easy to obtain it in the quantities required for a purification procedure. In some cases however, it was necessary to treat the animals with hormones and various diets prior to killing. This procedure would have been impractical with large animals such as sheep, pigs and cows and for this reason the rat was employed as an experimental animal.

It was essential to determine if the cholesterol ester hydrolase found in the bovine adrenal cortex was under a similar hormonal control to the adrenal cholesterol ester hydrolase (Garren et al. (1971)); Boyd and Trzeciak (1973); Trzeciak and Boyd (1973); Brecher et al. (1973).

Trzeciak and Boyd (1973) have shown that in vitro addition of cyclic AMP and ATP to the rat adrenal 105,000 x g cortical supernatant could activate cholesterol ester hydrolase activity to values equal to or greater than that obtained by ether anaesthesia stress. In a similar manner cholesterol ester hydrolase in the bovine adrenal cortical

105,000 x g supernatant can be activated _in vitro_ by the
addition of ATP and cyclic AMP (Trzeciak and Boyd (1974);
Figure 3.5). The ability of bovine adrenal cholesterol
ester hydrolase to be activated _in vitro_ in a similar manner
to the rat adrenal enzyme is taken as evidence for hormone
sensitivity of the bovine adrenal cortical cholesterol ester
hydrolase. The work presented in chapters 3, 4, and 7 show
that the cholesterol ester hydrolases found in the rat and
bovine adrenal cortex also exhibit other similar properties.

Studies on the sub-cellular distribution of cholesterol
ester hydrolase in the adrenals of the rat and the bovine
have shown that most of the activity resides in the 105,000 x g
supernatant (Table 3.1 and Table 7.1). This is not surpris-
ing as the lipid droplets which are the store of the cholest-
erol esters, the substrate for the cholesterol ester hydrolase,
are dispersed in the cell cytosol (Figure 1.2).

The effect of pH on bovine adrenal cholesterol ester
hydrolase activity was shown in Figure 3.2. The pH activity
curve did not appear to be symmetrical. Cholesterol ester
hydrolase activity decreased more rapidly at high pH values
than at low pH values; the reason for this is unclear.
It is possible that sulphydryl groups are involved in the
activity of the cholesterol ester hydrolase. If this is
the case, then at high pH, deprotonation of the sulphydryl
groups may take place rendering the enzyme inactive. Para-chloro-
mercuri-benzoate has been shown to inhibit cholesterol ester
hydrolase activity in the bovine adrenal cortical 105,000 x g
supernatant (W.H. Trzeciak personal communication) and this
again lends support to the view that sulphydryl groups are

involved in the enzymatic reaction. It was also shown in
Table 3.3 that reducing agents promoted the activation of
cholesterol ester hydrolase and no further stimulation of
enzymatic activity could be produced by the addition of
cyclic AMP and ATP when these reducing agents were present.
It is possible but unlikely that the reducing agents inhibit
the phosphorylation of cholesterol ester hydrolase by the
protein kinase as phosphorylation of phosphorylase kinase
by cyclic AMP dependent protein kinase has been demonstrated
in the presence of mercaptoethanol (Cohen (1973)) and this
protein kinase will also phosphorylate and activate cholest-
erol ester hydrolase (Figure 5.1). It is possible that
reducing agents may produce a similar conformational change
in cholesterol ester hydrolase as is produced by phosphory-
lation and this would explain why reducing agents stimulate
cholesterol ester hydrolase activity but inhibit further
activation on the addition of ATP and cyclic AMP.

It was demonstrated in Figure 5.2 that complete activation
of cholesterol ester hydrolase on preincubation with ATP
and cyclic AMP occurred after 15 minutes and after only
4 minutes 90% activation of cholesterol ester hydrolase
had taken place. This rate of activation is comparable
with the rate of activation of phosphorylase kinase (Cohen (1973))
in rabbit skeletal muscle. Naghshineh et al. (1974) have
reported that the complete activation of bovine cortical
cholesterol ester hydrolase occurred after 30 minutes preincub-
ation of the tissue homogenate with ATP and dibutyryl cyclic AMP.
The time required for the complete activation of the bovine
cortical cholesterol ester hydrolase found by Naghshineh et al.
(1974) seems too long when one considers that plasma
corticosteroid levels in the rat increase in a matter of

minutes after an applied stressfull situation (Dallman and
Jones (1973)).

The rate of activation of cholesterol ester hydrolase
shown in Figure 5.2 is consistent with the time of the
steroidogenic response to ACTH. Naghshineh et al. (1974)
used a 2 hour preincubation of the crude homogenate before
the addition of ATP and dibutyryl cyclic AMP. As was shown
in Table 6.1 preincubation of the bovine 105,000 x g cortical
supernatant for greater than 60 minutes resulted in a decreased
ability of the enzyme to be activated by cyclic AMP and
ATP and this could account for the time required for the
activation of cholesterol ester hydrolase in the preparation
of Naghshineh et al. (1974). The findings of Naghshineh et al.
(1974) that in many cases stimulation of cholesterol ester
hydrolase in the bovine adrenal cortical homogenate could
not be produced in vitro by the addition of dibutyryl cyclic
AMP and ATP, unless the homogenate was preincubated for
2 hours, is difficult to explain. By contrast in the experi-
ments reported in chapters 3 and 4 the addition of ATP and
cyclic AMP to the bovine cortical supernatant resulted in
a marked activation of cholesterol ester hydrolase. It is
possible however that the conflicting results of Naghshineh et al.
(1974) and those in this thesis are due to the use by the
former of a homogenate rather than the delipidated 105,000 x g
cortical supernatant used in the experiments in this thesis.
The use of a homogenate would confuse the results due to
the high levels of endogenous cholesterol esters in the
homogenate. These esters are normally removed as a floating
lipid layer from the 105,000 x g supernatant in the experiments
described in this thesis. These high levels of cholesterol
esters in the homogenate would dilute the radioactive label

of the $[^{14}C]$cholesterol oleate employed by Naghshineh et al. (1974)
to different degrees in different preparations. It has
been inferred however (Dailey et al. (1963)) that little
mixing of the two pools of cholesterol esters, exogenous
and endogenous, occurs. The study of cholesterol ester
hydrolase activity in an adrenal cortex homogenate as used
by Naghshineh et al. (1974) would involve the study of
possibly more than one cholesterol ester hydrolase as cholest-
erol ester hydrolase activity is present in other subcellular
fractions as well as the 105,000 x g supernatant. This
again would confuse the results obtained on the study of
cholesterol ester hydrolase by Naghshineh et al. (1974).

The work presented in chapters 4 and 5 has indicated
that activation of cholesterol ester hydrolase occurs by
phosphorylation. It was shown in Figure 4.12 that on electrophor-
esis two protein bands were produced from the purified
preparation of cholesterol ester hydrolase. Only one of
these bands exhibited esterase activity using α napthol propionate
as substrate and this same protein band also incorporated
$[^{32}P]$ radioactivity when the crude protein preparation had
been preincubated with $[α-^{32}P]$ ATP prior to purification.
It is possible that the other protein band which is not
phosphorylated is cholesterol ester hydrolase and this cholest-
erol ester hydrolase cannot hydrolyse α napthol propionate
to give an activity stain.

It was shown in Figure 5.1 that a variety of cyclic
AMP dependent protein kinases could activate cholesterol
ester hydrolase in vitro and each of these kinases gave a
similar degree of activation. As the cyclic AMP dependent
protein kinase inhibitor stopped the activation of cholesterol
ester hydrolase by the in vitro addition of ATP and cyclic

AMP in a crude preparation it is reasonable to assume that the cyclic AMP dependent protein kinase, which is known to occur in bovine and rat adrenal supernatant (Trzeciak and Boyd (1974); Garren et al. (1971)) is responsible for the activation and phosphorylation of cholesterol ester hydrolase.

The reservation of Trzeciak and Boyd (1974) that the cholesterol ester hydrolase in their semi purified preparation may not be phosphorylated but rather another protein which can stimulate or participate in the hydrolysis of cholesterol esters can be excluded by the work presented in chapter 4. Another phosphorylated protein certainly could be involved in steroidogenesis but it is certainly not required for the in vitro hydrolysis of cholesterol esters.

In testes and adrenal tissue stimulation of steroido-genesis with very low doses of trophic hormone can occur without any detectable changes in the concentration of cyclic AMP in the cell (Rommerts et al. (1973); Beall and Sayers (1972); Moyle and Ramachandran (1973); Rao and Saxena (1973)). This may indicate that cyclic AMP does not have a direct role in the stimulation of steroidogenesis or alternat-ively that the changes in the cyclic AMP concentration necessary to stimulate steroidogenesis can not be detected using the conventional techniques. However administration of cyclic AMP or dibutyryl cyclic AMP to the animals or to tissue slices can mimic the effects of the trophic hormone stimulation of steroidogenesis. (Hermier and Jutisz (1969); Mahaffee et al. (1974)). From the experiments carried out in chapters 3, 4 and 5 it can be deduced that cyclic AMP has a role in the activation of cholesterol ester hydrolase in the adrenal gland.

From the results presented in this thesis it has been concluded that activation of cholesterol ester hydrolase takes place by a phosphorylation of the enzyme. This phosphorylation is catalysed by a cyclic AMP dependent protein kinase found in the cell cytosol. The activation of the cyclic AMP dependent protein kinase involves the removal of the regulatory subunit from the catalytic subunit by the increased levels of cyclic AMP produced in the cell by the trophic hormone stimulation of adenyl cyclase (Garren et al. (1971)). This activated cholesterol ester hydrolase can then act upon the cholesterol esters in the lipid droplets of the cell to produce free cholesterol. The free cholesterol produced is then available for passage into the mitochondria where cleavage of the cholesterol side chain occurs to produce pregnenolone for corticosteroid production (Figure 10.1).

While cyclic AMP has been implied as being involved in the activation of cholesterol ester hydrolase, in some experiments it was found that ATP and magnesium addition in vitro to rat or bovine adrenal cortical supernatant in the absence of cyclic AMP also gave a marked stimulation of the cholesterol ester hydrolase activity. Cyclic AMP added to these preparations with ATP and magnesium could not further stimulate cholesterol ester hydrolase activity. There are a number of explanations for the failure of cyclic AMP to stimulate cholesterol ester hydrolase activity in these preparations.

When animals are stressed plasma ACTH and intracellular cyclic AMP concentrations in the adrenal increase (Matsayuma et al. (1971); Boyd and Trzeciak (1973)). It is possible that the stressful situation induced in the animal during its slaughter causes a rise in the intracellular cyclic AMP

levels and hence an activation of the cyclic AMP dependent
protein kinase. The amount of adrenal active protein kinase
produced by the stress would then be sufficient to activate
cholesterol ester hydrolase in the presence of ATP and
magnesium ions alone. The activation of the protein kinase
may also occur during the preparation of the 105,000 x g
desalted supernatant as the regulatory subunit is easily
dissociated from the catalytic subunit during the isolation
of rabbit skeletal muscle cyclic AMP dependent protein kinase
(H. Nimmo personal communication). As ATP is a required
substrate for the protein kinase a small (0.1%) contamination
of cyclic AMP in the 5mM ATP used in the experiments would
produce a concentration of cyclic AMP great enough to produce
a stimulation of protein kinase activity. This explanation
is unlikely however as using the same preparation of ATP
it was found that the activation of cholesterol ester hydro-
lase by cyclic AMP in the presence of ATP was variable.
There has been found in the corpus luteum a protein kinase
which is activated directly by LH (page 101). It is possible
that in the adrenal a protein kinase exists which is activa-
ted directly by ACTH and this activated protein kinase may
be responsible for the activation of cholesterol ester
hydrolase. However no such ACTH sensitive protein kinase
has as yet been isolated. The results presented in this
thesis do indicate that it is a cyclic AMP dependent protein
kinase which is responsible for the activation of cholesterol
ester hydrolase in the adrenal cortex. The role of cyclic
AMP in the activation of luteal cholesterol ester hydrolase
has been discussed in chapter 8.

Trzeciak and Boyd (1974) have shown that magnesium ions
are required for the inactivation of cholesterol ester
hydrolase. More extensive studies on the deactivation of.
cholesterol ester hydrolase have been described in this
thesis. It was shown that magnesium and calcium ions inhib-
ited cholesterol ester hydrolase activity in the crude
105,000 x g supernatant (Figure 3.4) but these additions
were not inhibitory in the purified preparation (Figure 4.24).
The findings of Trzeciak and Boyd (1974),have been confirmed
that deactivation of cholesterol ester hydrolase on preincub-
ation of a semi purified phosphorylated adrenal cortical
supernatant protein fraction with magnesium ions was accompan-
ied by a loss of $[{}^{32}P]$ radioactivity from the protein (Figure 6.2)
Alkaline phosphatase has also been shown to increase the
rate of deactivation of cholesterol ester hydrolase in a
semi purified preparation (Figure 6.3). α and β phosphorylase
kinase phosphatase have also been shown to inactivate and
dephosphorylate cholesterol ester hydrolase in a purified
phosphorylated preparation (Figure 6.5). It is proposed
(Figure 10.1) that cholesterol ester hydrolase in the adrenal
cortex is deactivated by a dephosphorylation carried out
by a phosphoprotein phosphatase. Merlevede and Riley (1966),
have shown that there is in the adrenal cortex an ATP-magnes-
ium dependent phosphorylase phosphatase which is responsible
for the dephosphorylation and inactivation of phosphorylase a.
Cyclic AMP added together with ATP and magnesium ions was
shown to inactivate the phosphorylase phosphatase and it
is possible that this enzyme or another phosphoprotein phosph-
atase is involved in the inactivation of cholesterol ester
hydrolase. Tsai et al. (1973) have shown that inactivation
of hormone sensitive lipase, which is thought to be activated

by a phosphorylation (Tsai et al. (1970); Huttunen et al. (1970a); Huttunen and Steinberg (1971); Huttunen et al. (1970b)), is inactivated by ATP, magnesium ions and ascorbic acid. It has been proposed that inactivation is accomplished by dephosphorylation of the enzyme by a phosphatase (Tsai and Vaughn (1972)). It is possible that cholesterol ester hydrolase is inactivated in a similar manner.

Figure 10.1 portrays activation of cholesterol ester hydrolase by a single site phosphorylation mechanism, however control of cholesterol ester hydrolase activity may involve a second site phosphorylation mechanism. Control of phosphorylase kinase (Cohen and Antoniw (1973); Yeaman and Cohen (1975)) and glycogen synthetase activity (Nimmo and Cohen (1974)) are thought to occur by a second site phosphorylation of the enzyme.

Rabbit skeletal muscle phosphorylase kinase is composed of three types of polypeptide chain (Cohen (1973)) and has the structure $(\alpha\beta\gamma)_4$. The cyclic AMP dependent activation of the enzyme has been found to correlate with the phosphorylation of a unique serine site on the β subunit. A second site on the α subunit has however been found to be rapidly labelled after a short lag period (Cohen (1973); Cohen et al. (1975) Yeaman and Cohen (1975)). After phosphorylation of phosphorylase kinase in vitro Cohen and Antoniw (1973) showed that the preparation contained traces of endogenous phosphorylase kinase phosphatase activity which was capable of dephosphorylating both the α and β subunits. It was also shown that phosphorylation of the α subunit enhanced the dephosphorylation of the β subunit and this was accompanied by the loss of activity. Intravenous injection of adrenalin also resulted in the phosphorylation of the same α and β sites in vivo
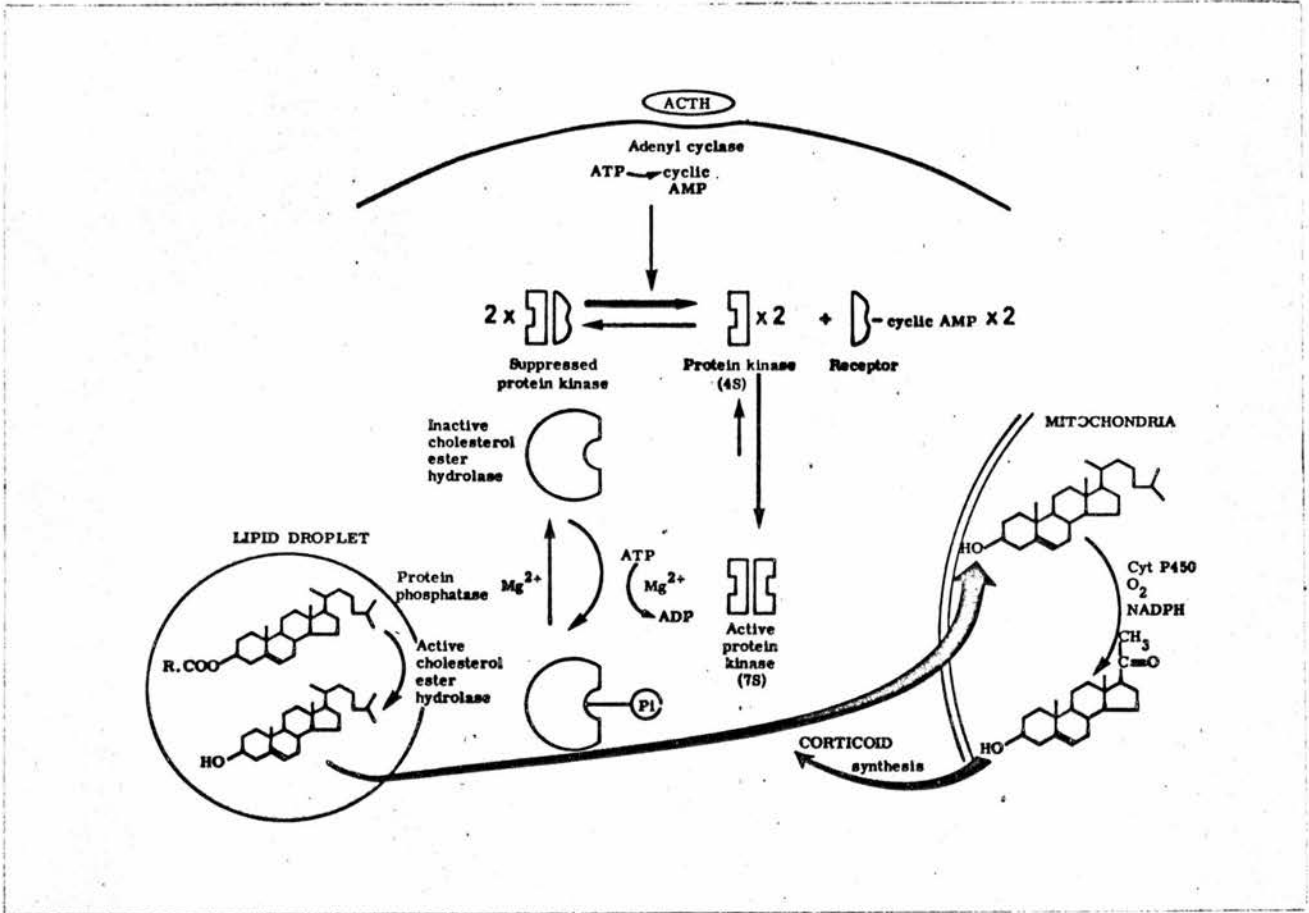
Figure 10.1 Hypothetical mechanism of cholesterol ester hydrolase activation and deactivation in the adrenal cortex.

as were labelled in vitro (Yeaman and Cohen (1975)). The
phosphorylation has been shown to occur on the α and β
subunits at specific serine residues (Cohen et al. (1975)).
The presence of two distinct phosphorylase kinase phosphat-
ases in rabbit skeletal muscle have been demonstrated (Antoniw
and Cohen (1975)), one of these is specific for the α subunit
and the other specific for the β subunit of phosphorylase
kinase. The results have led to the concept that phosphory-
lase kinase is controlled by a second site phosphorylation
mechanism (Figure 10.2 taken from Yeaman and Cohen (1975)).

Control of glycogen synthetase has also been implicated
as involving a second site phosphorylation and Nimmo and
Cohen (1974) have isolated a protein kinase GSKII which is
distinct from cyclic AMP dependent protein kinase and may
be involved in the control of glycogen synthetase activity.

It is possible that enzyme regulation by second site
phosphorylation may occur in other enzyme systems including
cholesterol ester hydrolase. Pyruvate dehydrogenase has
also been reported as being phosphorylated at more than
one site (Linn et al. (1972)).

In Figure 5.2 it was shown that while cholesterol ester
hydrolase activation had plateaued after 15 minutes a slow
incorporation of $[^{32}P]$ radioactivity into the protein still
continued. This is consistent with the observations of
Cohen (1973) concerning the phosphorylation of phosphorylase
kinase. This could be indicative of a second site phosphory-
lation similar to that of phosphorylase kinase, or merely
an exchange of an unlabelled phosphate group on the enzyme
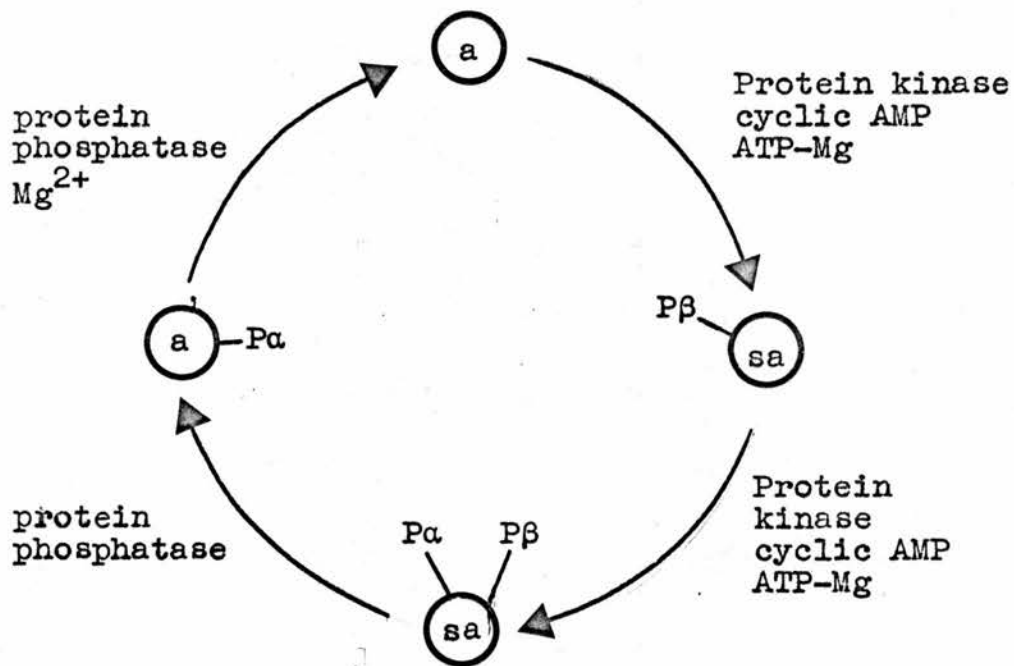for a $[^{32}P]$ radioactive group.

Figure 10.2 Mechanism of the hormonal control
of phosphorylase kinase activity.

a = phosphorylase kinase a, sa = phosphorylase kinase
sa, Pβ = phosphorylated β subunit, Pα = phosphorylated
α subunit.

(After Yeaman and Cohen (1975))

Further evidence for a second site phosphorylation of cholesterol ester hydrolase is shown in Figure 6.6. It was shown that at zero cholesterol ester hydrolase activity 50% of the $[^{32}P]$ radioactive label remained bound to the protein. It was also shown in Figure 6.1 that even in the presence of ATP and cyclic AMP a rapid inactivation of cholesterol ester hydrolase took place after a period of 60 minutes and this again is consistent with a second site phosphorylation enzyme control system.

It was shown in Figure 4.4 that a second cholesterol ester hydrolase was isolated from the 105,000 x g cortical supernatant which was not phosphorylated. Teale et al. (1972) isolated two cholesterol ester hydrolases from the pancreas but concluded that both were the same enzyme, one being aggregated with more lipid than the other. Eto and Suzuki (1971; 1973) have isolated three different cholesterol ester hydrolases from the rat brain, one being located in the mitochondrial fraction and the other two in the myelin sheath and microsomes. Pittman et al. (1972) have isolated two forms of hormone sensitive lipase both of which are activated by a protein kinase. The behaviour on agarose gel chromatography of these two enzymes were similar to the behaviour of the two cholesterol ester hydrolase found on Sephadex G200 gel filtration (Figure 4.4). One of the hormone sensitive lipases was eluted in the void volume fraction of a 6% agarose column and the other was retained and had a molecular weight of 100,000 - 200,000. This is similar to the elution pattern of the two cholesterol ester hydrolases (Figure 4.4). It is however possible from the findings of Pittman et al. (1972) that the two hormone sensitive lipases isolated by them are the same enzyme in different degrees of aggregation.

The origin and nature of the second cholesterol ester hydrolase eluted from the Sephadex G200 column after the void volume enzyme is unknown. It was proposed (page 49) that this cholesterol ester hydrolase may be a disaggregated form of the enzyme eluted in the void volume. If this were so it must consist of cholesterol ester hydrolase phosphorylated and activated in vivo as preincubation of a crude cortical protein fraction with $[\gamma^{-32}P]$ ATP prior to purification did not render the protein fraction from the Sephadex G200 column containing the second cholesterol ester hydrolase radioactive.

The other possibility is that the second cholesterol ester hydrolase which is not phosphorylated is a completely different enzyme. This may be a non-specific esterase which also originates from the cell cytosol, or it may be a contaminant from another subcellular fraction, as cholesterol ester hydrolase has been shown to exist in other subcellular fractions. The fact that the cholesterol ester hydrolases in different preparations appeared in different ratios may indicate that the second cholesterol ester hydrolase is a subcellular contaminant, the amount of the second cholesterol ester hydrolase depending on the degree of homogenisation. It is possible however that the second cholesterol ester hydrolase originates from the cell cytosol and is inducable by several different external stimuli. No second cholesterol ester hydrolase was found on the purification of the rat adrenal 105,000 x g supernatant (Figure 7.3). This supports the view that the second cholesterol ester hydrolase in the bovine adrenal cortex were a disaggregated form of the void volume enzyme as it might have been expected that a similar disaggregation would have also occurred in the rat system to give two cholesterol ester hydrolase on gel filtration.

The specificity of rat adrenal cholesterol ester hydro-
lase has been studied in vitro and in vivo. Walker and Carney
(1971) have shown that cold stress applied to a rat fed
olive oil resulted in decreases of the 16:1, 18:2, 20:1,
20:4 and 22:4 cholesterol esters in the adrenal. In contrast
Muraoka (1965) has shown that only cholesterol arachidonate
decreased significantly in rat adrenals in vivo after the
animals had received an injection of ACTH.

Studies on cholesterol ester hydrolysis in vitro (Dailey
et al. (1963)) have shown that the rate of cholesterol ester
hydrolysis follows the order arachidonate>oleate>linoleate>
palmitate. However Dailey et al. (1963) have suggested
that added labelled cholesterol ester may not mix with the
metabolic pool of cholesterol esters in the adrenal. This
would indicate that these added cholesterol esters may not
be presented to cholesterol ester hydrolase in the same
manner as it is presented in vivo. This may affect the
order of preference of cholesterol ester hydrolase for the
substrate in vivo compared to the addition of the cholesterol
esters in vitro.

It was shown in Table 9.3 that ether anaesthesia stress
resulted in selected decreases in the percentage of the
14:0, 16:0, 18:0, 18:1, 18:2, and 20:4 esters. A lack of
utilisation of the 22:1 and 20:1 esters was consistent through-
out all the groups of animals. It was also shown in Table 9.5
that cholesterol oleate was hydrolysed in vitro at a much
greater rate than cholesterol erucate. Alteration of the
cholesterol side chain using oleic acid as the esterified
fatty acid in each case did not significantly alter the
rate of hydrolysis of the steroid esters (Table 7.2).

These results indicate that cholesterol ester hydrolase
shows a specificity for the fatty acid of the cholesterol
esters and not for the sterol side chain, the size and
configuration of which has been shown to be important for
7αhydroxylation and side chain cleavage of cholesterol Boyd
et al. (1974); Arthur et al. (1975)).

The results presented in the preceeding chapters have
indicated a role for cholesterol ester hydrolase in the
control of steroidogenesis. The importance of the cholest-
erol side chain cleavage reaction in the control of steroido-
genesis is now well established (Stone and Hechter (1954);
Hall and Eik Wes (1964); Hall and Koritz (1965): Karaboyas
and Koritz (1965); Channing and Villee (1966); Hall and
Young (1968)). Bell et al. (1973) and Bell and Harding (1974)
have concluded that the supply of cholesterol to the cholest-
erol side chain cleavage enzyme is the rate limiting step
in adrenal steroidogenesis but the origin of the cholesterol
supply was not deduced. Arthur and Boyd (1974) have shown
that in vitro the cholesterol supply to the luteal mitochon-
dria is important in controlling pregnenolone production,
Arthur (1975) has also shown that LH injection causes an
apparent decrease in the amount of pregnenolone produced
from the endogenous cholesterol in luteal mitochondria but
on adding more cholesterol in vitro to the isolated mitochon-
dria from the LH treated rats it was found that pregnenolone
production was substantially higher than in the controls.
ACTH injection of rats produces a similar effect on adrenal
mitochondrial cholesterol side chain cleavage (J.I. Mason
and J.R. Arthur personal communication). It seems therefore
that the LH effect on the corpus luteum or the ACTH effect
on the adrenal in vivo causes an activation of the cholesterol

side chain cleavage enzymes as subsequently assayed in isolated mitochondria. This activation can only be expressed on the provision of the mitochondria with adequate supplies of cholesterol.

There is much documented evidence to suggest that cholesterol for corticosteroid production originates from the cholesterol esters stored in the lipid droplets (Page 3). It was also shown (Page 79) that the rate of production of cholesterol *in* *vitro* from the cholesterol esters in the lipid droplets by the action of cholesterol ester hydrolase was of the same order as corticosterone production *in* *vivo* in the rat.

Further evidence for the involvement of cholesterol ester hydrolase in the control of steroidogenesis was shown in chapter 9. Cholesterol side chain cleavage activity is not affected by dietary rape seed oil whilst activation of cholesterol ester hydrolase was abolished. Corticosterone production in animals fed rape seed oil was not stimulated to the same levels as the controls when the animals were subjected to ether anaesthesia stress. One explanation for these observations is that if the supply of cholesterol to the mitochondria is important in the control of steroidogenesis, then while the mitochondrial cholesterol side chain cleavage enzymes will have the potential to produce more pregnenolone for corticosterone production; on stress this is not possible due to the inadequate supplies of cholesterol from the hydrolysis of cholesterol esters, due to an inhibition of cholesterol ester hydrolase. Another possibility which must be considered is that dietary rape seed oil could inhibit other enzymes in the pathway from pregnenolone to corticosterone.

In conclusion it can be stated that cholesterol esters have many important functions and are actively metabolised and abundantly present in tissues where cholesterol is converted to other metabolically important compounds. Adrenals, placenta and gonadal tissue all have high levels of cholesterol ester and all synthesise steroid hormones (Goodman (1965)). Except for these tissues it is possible that the cholesterol esters found in most tissues are the reservoir for free cholesterol that can be utilised during membrane turnover.

Cholesterol ester hydrolase activity has been shown in all tissues which produce steroid hormones (Goodman (1965)) and the work presented in this thesis has shown that the adrenal and corpus luteum cholesterol ester hydrolase is under hormonal control. It is possible that cholesterol ester hydrolase plays a role in the control of steroid hormone production in all steroid hormone producing tissues. Certainly when rat adrenal mitochondria are subjected to a 30 minute incubation free cholesterol is significantly diminished (Boyd and Trzeciak (1973)). This cholesterol must be replenished _in vivo_ when rats are subjected to a stressfull situation and it is possible that adrenal cholesterol ester hydrolase being under hormonal control can produce a release of cholesterol from cholesterol esters which then replenishes the mitochondria with cholesterol. The supply of cholesterol from cholesterol esters in steroidogenic tissue could be efficient as the hydrolysis of the cholesterol esters to produce free cholesterol also produces free fatty acids. These acids could be available to the mitochondria for oxidation to support cholesterol side chain cleavage.

The free fatty acid could also exert a direct effect on
cholesterol ester hydrolase and hence control the supply
of cholesterol to the cholesterol side chain cleavage system.
It was shown in Figure 9.2 that oleic acid accumulation
in vitro inhibits cholesterol ester hydrolase activity.
It is also known that the cholesterol ester preferentially
hydrolysed by cholesterol ester hydrolase is cholesterol
arachidonate and it is known that arachidonic acid is the
precursor for prostaglandins (Hamberg et al. (1974)). Prostaglan-
dins have been implicated in the control of steroidogenesis (page 7).

Other factors may be involved in the control of steroido-
genesis apart from the activity of the cholesterol side chain
cleavage mixed function oxidase and cholesterol ester hydro-
lase enzymes. A rapidly turning over protein has been propo-
sed to control steroidogenesis by mediating the supply of
cholesterol to the sterol side chain cleavage enzymes (Garren
et al. (1965)), and to be involved in the binding of the
cholesterol to the side chain cleavage cytochrome P 450
(Simpson et al. (1972)). Walton et al. (1971) have shown
that ACTH stimulation of protein kinase in the adrenal cortex
cell can catalyse the phosphorylation of ribosomes. It was
postulated that this may be important in the steroidogenic
response of the adrenal cortex to ACTH. Caron et al. (1974)
have reported that phosphorylation of the cytochrome P 450
fraction of a reconstituted bovine corpus luteum mitochondrial
side chain cleavage system caused a stimulation of steroido-
genesis, however the mechanism of the stimulation is not
yet known.

It seems therefore that the acute stimulatory effect of LH and ACTH on steroidogenesis in steroid hormone producing tissues depends upon at least two processes. Cholesterol ester hydrolase activity is increased and the cholesterol side chain cleavage is stimulated in the mitochondria. The activation of cholesterol ester hydrolase is effected by a phosphorylation involving a cyclic AMP dependent protein kinase and does not involve a rapidly turning over protein. The activation of the mitochondrial side chain cleavage system depends on a rapidly turning over protein of extra mitochondrial origin, which enhances the binding of cholesterol to the mitochondrial side chain cleavage P 450. The production of the rapidly turning over protein and the binding of the cholesterol to the side chain cleavage P 450 may also involve a phosphorylation involving a cyclic AMP dependent protein kinase.

Whether it is the extra supply of cholesterol from the cholesterol esters which is important controlling corticosteroid production or whether this cholesterol supply is only important in supporting corticosteroid production when the system has been activated is however unclear and further experimentation must be carried out to clarify this point.

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THE ROLE OF CHOLESTEROL AND CYTOCHROME P-450 IN THE CHOLESTEROL SIDE CHAIN CLEAVAGE REACTION IN ADRENAL CORTEX AND CORPORA LUTEA

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SUMMARY

1. Rat adrenal mitochondria, and rat corpora luteal mitochondria *in vitro* convert endogenous cholesterol into pregnenolone by a cholesterol side chain cleavage mixed function oxidase.

2. These mitochondria contain a relatively small pool of cholesterol so that the decline in the observed rate of pregnenolone production over a few minutes at 37°C is due to substrate depletion.

3. It is possible to maintain the initial rate of pregnenolone formation by the inclusion of cholesterol in the incubation medium added in a small quantity of acetone or cholesterol offered in the form of a lecithin-cholesterol micelle.

4. The cytosol of the rat adrenal cortex and the cytosol of the rat corpora lutea both contain cholesterol ester hydrolases which are activated by a protein kinase in the presence of 3'5'-cyclic AMP and ATP. This activation of the cholesterol ester hydrolase is independent of protein synthesis blockers such as cycloheximide.

5. It is suggested that one method by which the trophic hormones ACTH and LH may activate their appropriate target tissues is by a 3'5' cyclic AMP dependent activation of a protein kinase resulting in a phosphorylation and activation of the cholesterol esterase.

6. This esterase can attack the cholesterol esters in the lipid droplets bringing about a release of unesterified cholesterol.

7. The cholesterol which is in the cytoplasm moves to the mitochondria by a mechanism which is not yet clear. After the initial rapid uptake by the mitochondria, the cholesterol has to move throughout the cristae to the active site of the cholesterol side chain cleavage enzyme system.

8. The evidence suggests that the translocation or the transportation of the sterol molecule within the mitochondrion may be the rate limiting event in the overall cholesterol side chain cleavage oxygenase reaction. Labile protein(s) may be involved in this event and also in other aspects of the cholesterol side chain cleavage reaction.

9. From studies on intact mitochondria and on the isolated cytochrome P-450 involved in the cholesterol side chain cleavage reaction it seems clear that the micro-environment of the cholesterol (?phospholipid) cytochrome P-450 in the cristae may be a significant factor in the ultimate rate of the cholesterol side chain cleavage reaction.

INTRODUCTION

It has been shown that the conversion of cholesterol to pregnenolone occurs in the mitochondria of steroid hormone producing tissues [1, 2] and is catalysed by a mixed function oxidase involving cytochrome P-450 [3, 4]. Regulation of the rate of production of steroid hormones by tissues such as the adrenal cortex, corpora lutea and testes is often assumed to be through a rate limiting step in the mitochondria of these tissues namely, the cholesterol side-chain cleavage reaction [5]. It is known that oxygen interacts with the side chain of cholesterol and the suggestion has been made that the hydroxylations follow the pattern 20α, −20α, 22 and then by a desmolase event produce pregnenolone and isocaproic aldehyde [6]. Alternative proposals have been put forward that in fact the oxygen interacts with the 20 position of the sterol molecule to produce a hydroperoxide and then by a radical mechanism the side chain is cleaved [8]. There are of course further possibilities including the suggestion that a 20, 22 epoxide or radical could be

involved [7]. Whatever the mechanism of this cholesterol side chain cleavage reaction, it appears that the haemoprotein cytochrome P-450 is involved and consequently, oxygen and suitable electron donors are obligatory components of this multi-enzyme reaction. One possible mechanism is shown in Fig. 1.

There is evidence that the stimulation of steroidogenesis in the adrenal cortex and the gonads is mediated by the appropriate trophic hormone and the secondary messenger 3'5' cyclic AMP [9, 10]. In many events involving 3'5' cyclic AMP, there appears to be a protein kinase component in this response [11, 12]. The issue is then to establish how this phosphorylation event is related to the control process under study.

In this paper we discuss some studies which have been performed on the control of steroidogenesis in rat adrenal cortex and in rat corpora lutea. This report deals with studies on the events occurring in mitochondria and the exploration of the optimal conditions for the conversion of cholesterol to pregneno-

Suggested scheme for the side chain cleavage of cholesterol
occurring in the mitochondria of the adrenal cortex, testes
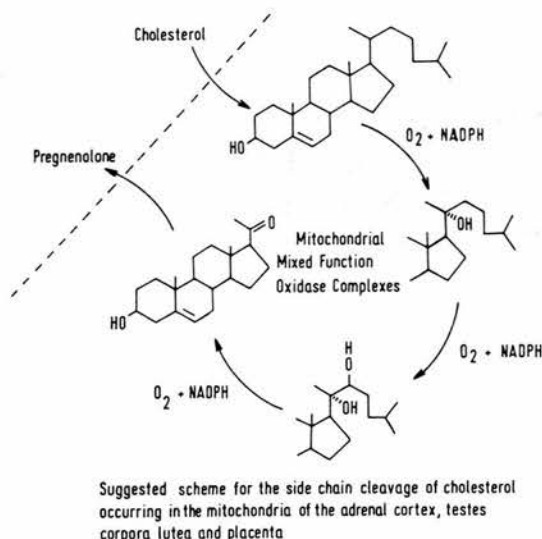corpora lutea and placenta

Fig. 1. Suggested scheme for the side chain cleavage of
cholesterol occurring in the mitochondria of the adrenal
cortex, testes, corpora lutea and placenta.

lone under *in vitro* conditions. The source of choles-
terol for this mitochondrial side chain cleavage reac-
tion has been investigated. The mechanisms which
may operate in the cytosol for the delivery of this
relatively hydrophobic molecule to the cristae of the
mitochondrion will be considered as a factor in the
overall control of steroidogenesis.

EXPERIMENTAL

Chemicals and radiochemicals

The sources and purity of the chemicals and radio-
chemicals used in these studies have been stated else-
where [13–15].

Sterol and steroid assays

Cholesterol and pregnenolone were determined by
gas–liquid chromatography and by enzymic-fluori-
metric methods as previously published [13].

Adrenals

Adrenal mitochondria were prepared as previously
described [13] from male and female rats in the
weight range 150–250 g. The adrenals were trimmed
and homogenized in 250 mM sucrose. The mitochon-
dria were suspended in 250 mM sucrose to give a
final protein concentration of about 3 mg/ml.

Corpora Lutea

Female rats of the Wistar strain were used in these
experiments. Luteinized ovaries were obtained from
animals using the pretreatment procedure devised by
Parlow[16, 17]. Rats 21–24 days old were injected
subcutaneously with pregnant mare serum gonado-
trophin (Gestyl-Organon-50 I.U.) followed 3 days
later by an injection of human chorionic gonadotro-
phin (pregnyl-Organon-25 I.U.) or a second injection
of pregnant mare serum gonadotrophin (50 I.U.). The

hormones were dissolved in 0·5 ml of 0·9% saline for
injection. The rats were used 5–7 days after the
second injection of hormone. The ovarian weight
varied from 120–150 mg.

The ovaries were trimmed free of fat and placed
in ice-cold 250 mM sucrose. The tissue was homo-
genized in 5 volumes of 250 mM sucrose/g tissue as
described previously [18]. The homogenate was cen-
trifuged at 650 *g* for 10 min to remove nuclei, red
blood cells and intact cells. The supernatant was cen-
trifuged at 8500 *g* for 10 min to give the mitochon-
drial pellet. This pellet was resuspended in one half
of the original homogenate volume of 250 mM suc-
rose and centrifuged again at 8500 *g* for 10 min. This
washing procedure was repeated and the final mito-
chondrial pellet suspended in 250 mM sucrose to give
a protein concentration of 6–8 mg/ml.

Enzymic conversion of cholesterol to pregnenolone

Studies on the rate of conversion of cholesterol to
pregnenolone in rat adrenal or corpora luteal mito-
chondrial preparations and studies on the respiration
of these mitochondira were conducted in a medium
consisting of 250 mM sucrose, 20 mM KCl, 15 mM
triethanolamine hydrochloride, 10 mM potassium
phosphate, 5 mM magnesium chloride and 0·1 mM
EDTA. The buffer solution also contained 0·1%
bovine serum albumin and the pH was adjusted to
7·4. The final mitochondrial protein concentration
was 1–2 mg/ml. Radioactive cholesterol was added
to the mitochondria in acetone.

Respiratory studies on these mitochondrial prep-
arations were performed using a modified Clark elec-
trode in a cell fitted with a pH electrode and a
thermistor. It is possible to obtain an estimate of the
cholesterol side-chain cleavage reaction in rat adrenal
cortical mitochondria or in rat luteal mitochondria
by the incubation of these mitochondrial preparations
in the appropriate buffer in the presence of oxygen,
an electron donor, and radioactive cholesterol [13].

Inhibition of the steroid 3β-hydroxy dehydrogenase

The cholesterol side chain cleavage reaction is com-
plicated in that the product of the reaction, pregneno-
lone, may undergo oxidation in the presence of the
steroid 3β-hydroxy dehydrogenase, present in mito-
chondria, resulting in the conversion of pregnenolone
to progesterone. However, if the dehydrogenase in-
hibitor cyanoketone is added to the incubation mix-
ture, the only radioactive products which can be
detected are the substrate cholesterol and the choles-
terol cleavage product, pregnenolone [13]. It has been
possible to devise a simple radioactive assay for the
cholesterol side chain cleavage activity and this type
of assay has been applied to mitochondria from rat
adrenal cortex, and rat corpora lutea.

RESULTS

When radioactive cholesterol in acetone is added
to mitochondrial preparations from steroidogenic tis-
sues the rate of uptake of this tracer dose of radioac-

Table 1. Uptake of radioactive cholesterol by bovine adrenal cortex mitochondria

Time (min)	1	2	3	5	15	20
4-^{14}C cholesterol taken up by mitochondria as a percentage of the final value	81	90	97	98	99	100

tive cholesterol into the mitochondrial preparations is extremely rapid. Even at temperatures as low as 5°C a mitochondrial preparation adsorbs most of the added cholesterol in 2–3 mins.; this is shown in Table 1. It is for this reason that in certain studies the [4-^{14}C]-cholesterol is pre-incubated with the mitochondria at a low temperature prior to initiating the enzymic oxygenase event.

By the application of standard incubation procedures, it is interesting to compare and contrast the cholesterol side chain cleavage reaction in three tissues obtained from the same species. We have compared the pregnenolone production rate in mitochondria isolated from different steroidogenic tissues in Table 2. It will be noted that in these different mitochondria, the ratio of total cholesterol in the mitochondrion to total cytochrome P-450 varies as does the calculated V_{max} for pregnenolone production.

In the radioactive assay of cholesterol side chain cleavage activity, the mitochondria are isolated, suspended in buffer solution and a tracer quantity of radioactive cholesterol is added. It is assumed that the amount of cholesterol added is insignificant in comparison with the endogenous cholesterol present in the preparation. The radioactive cholesterol is given an adequate period to equilibrate with the endogenous cholesterol. After equilibration is complete the electron donor is added to trigger the reaction. As shown in Table 1, the rate of uptake of cholesterol by rat corpora luteal mitochondria at 4°C from the aqueous environment occurs within a period of about 2 min. This initial uptake event need not be equated with complete equilibration of cholesterol within the various sub-mitochondrial cholesterol pools.

Cholesterol is distributed about all organelles, body fluids, and cell membranes and it is of importance to consider the distribution of the sterol within the cells involved in steroidogenesis. In the rat adrenal cortical cell, we find about 3 nmol cholesterol/mg protein in the cell cytosol, 20 nmol cholesterol/mg protein in the isolated washed mitochondria and about 50 nmol cholesterol/mg protein in the isolated endoplasmic reticulum or microsomal fraction. There is of course a vast reservoir of cholesterol as cholesterol esters in the lipid droplets floating in the cytosol.

In considering the events which occur in association with the addition of cholesterol to mitochondrial preparations it is important to take into account the phospholipid content of these organelles. The phospholipid content of rat adrenal mitochondria exceeds the protein content of these organelles and the analytical data suggest that there may be 3 μmol phospholipid/mg mitochondrial protein[19]. Subfractionation of the phospholipids isolated from rat adrenal mitochondria suggests that the predominant phospholipid is phosphatidyl choline or lecithin with lesser amounts of phosphatidyl ethanolamine or cephalin and then minor quantities of phosphatidyl serine, cardiolipin and some other as yet unidentified polar lipids. Thus if mitochondria from the adrenal cortex are suspended in an aqueous buffer solution and cholesterol in acetone is added to this suspension, the phospholipids undoubtedly play an important part in the uptake and subsequent organisation of this sterol within the membrane structures of the mitochondria. In considering the attack by oxygen, under the influence of the haemoprotein cytochrome P-450, on the cholesterol molecule, the unusual environment of the enzyme and the substrate must be considered. There are many examples of cytochrome P-450 dependent reactions being sensitive to the phospholipid environment [20–22] and in this particular case of the cholesterol side chain cleavage reaction in mitochondria the enormous excess of phospholipids undoubtedly influences the reaction. In these mitochondria there is about 1 nmol cytochrome P-450 per 20 nmol of cholesterol associated with about 3000 nmol of phospholipid per mg mitochondrial protein. Not all the phospholipid is phosphatidyl choline but from the analytical data there may

Table 2. The content of cholesterol and cytochrome P-450 in certain endocrine mitochondria

Species		per mg mitochondrial protein			V_{max} nmol pregnenolone nmol cytochrome P-450^{-1} min^{-1}
		cholesterol nmol A	cytochrome P-450 nmol B	Ratio $^A/_B$	
Human	Adrenal cortex		0·8		
	Placenta	50	0·1	500	0·08
Bovine	Adrenal cortex	30	1·0	30	0·25
	Corpora lutea	60	0·2	300	0·35
Rat	Adrenal cortex	20	0·9	22	1
	Corpora lutea	12	0·4	30	1
	Testes	70	0·01	7000	2
Pig	Testes	20	0·5	40	1

be at least 1000 nmol of phosphatidyl choline/mg of mitochondrial protein.

Although progress has been made in the resolution of the cytochrome P-450 from adrenal cortex and the other tissues [23–29] we do not have a pure sample of this haemoprotein from steroid hormone producing tissues. The inability to resolve the cytochrome P-450s present in the mitochondria from steroidogenic tissues has proved a considerable handicap to the analysis of many of the enzymic events of interest to endocrinologists. In mitochondria from tissues in which steroidogenesis is stimulated by the appropriate trophic hormone there is evidence for an increased production of pregnenolone from cholesterol.

With regard to the cholesterol side chain cleavage reaction being a rate-limiting event in steroidogenesis, one problem which arises in the interpretation of the kinetics of pregnenolone production in these mitochondria is the rate at which the mitochondrial cholesterol is depleted. It will be noted from Table 2 that the reserves of cholesterol in mitochondria from different steroid hormone producing tissues vary considerably. In the case of mitochondria isolated from the bovine adrenal cortex, it is possible to add a tracer amount of cholesterol to these mitochondria and obtain a linear production of pregnenolone over a period of 10–20 min [4]. This is because the radioactive tracer is discharged into a reactive pool of cholesterol in the mitochondrion such that subsequent enzymic events associated with the conversion of cholesterol to pregnenolone occur at a rate which does not materially affect the magnitude of the pool of cholesterol in the mitochondria. On the other hand, in the case of mitochondria isolated from the rat adrenal cortex or mitochondria isolated from rat corpora lutea, the cholesterol content of these organelles in the presence of O_2 and an electron donor at 37°C would be markedly depleted in a few minutes.

In the mitochondrion there is only a limited amount of cytochrome P-450 and if we assume that there will be a minimum of 1 molecule of substrate associated with 1 molecule of cytochrome P-450 at the appropriate reaction centre, there must be some mechanism in the mitochondrion for the movement of this non-polar material-cholesterol through the protein and phospholipid membrane structure of the cristae to the enzyme reaction centre. Spectral studies have been employed to attempt to quantitate the interaction of cholesterol with a specific cytochrome P-450. The data suggest that trophic hormone stimulation of the adrenal cortex results in an increase in the cholesterol complex of cytochrome P-450 [13, 14]. It is well known that cholesterol and phospholipids also interact to form micelles [30, 31] and that the polar end of the sterol molecule is often associated in these structures with the polar end of the phospholipid molecule [32].

Pre-treatment of rats with protein synthesis blockers, such as cycloheximide, results in a decrease in the cholesterol side chain cleavage activity in mito-
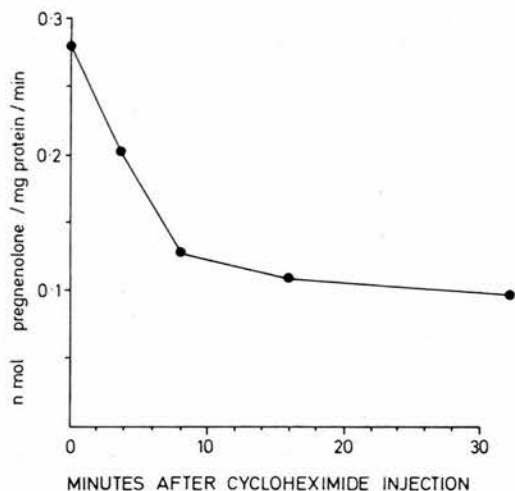

Fig. 2. Groups of immature female rats were injected with cycloheximide at various intervals before killing the animals. The cholesterol side chain cleavage reaction was measured on the mitochondria isolated from corpora lutea.

chondria from steroidogenic tissues such as adrenal cortex, corpora lutea and testes. Figure 2 shows the cholesterol side chain cleavage activity in mitochondria isolated from rat corpora lutea. Groups of animals were pretreated with cycloheximide for varying periods before killing. The corpora lutea were removed, homogenized and processed in an identical way and the cholesterol side chain cleavage activity of these mitochondria measured in the usual way. By operating at 29°C and by using a short observation period, an approximation can be obtained to the maximal rate of cholesterol side chain cleavage activity. It will be noted that the rate of this reaction diminishes rapidly after the administration of cycloheximide and this is an agreement with observations previously made on the rat adrenal cortex system in which the prior administration of cycloheximide resulted in a diminution of the observed cholesterol side chain cleavage activity [13]. The administration of cycloheximide to these animals does not produce any significant change in the total mitochondrial cytochrome P-450 content expressed per mg of mitochondrial protein. It has been suggested that there must be some rapidly turning over "labile protein" which is involved in the overall kinetics of the cholesterol to pregnenolone reaction and this rapidly turning over protein must be of extramitochondrial origin because as shown in Table 3 the administration of chloramphenicol to these animals has no effect on the cholesterol side chain cleavage activity. On the basis of the usual assumption that chloramphenicol is an inhibitor of mitochondrial protein synthesis, this would imply that the labile protein involved in this mitochondrial cholesterol side chain cleavage event must be of extra-mitochondrial origin.

One possible explanation of these results is that in the endoplasmic reticulum of this tissue there is an active synthesis of a protein of peptide which may be involved in the translocation of the cholesterol

Table 3. The effect of chloramphenicol injection on choles-
terol side chain cleavage activity in rat luteal mitochondria

Treatment		% conversion			
		0 min	5 min	10 min	15 min
Saline	(a)	0	17·5	24·4	29·0
	(b)	0	16·0	22·3	26·5
Chloramphenicol	(a)	0	18·0	26·2	29·5
	(b)	0	15·0	22·5	27·0

The table shows the percentage conversion of $[4\text{-}^{14}C]$-
cholesterol into $[4\text{-}^{14}C]$-pregnenolone in luteal mitochon-
dria from rats injected with saline or chloramphenicol (10
mg i.m.).
(a) Injections 3 hours before killing.
(b) Injections 3 and 10 hours before killing.
Cholesterol side chain cleavage assays were carried out
as described in methods. Total mitochondrial cholesterol
was unchanged by chloramphenicol treatment.

Fig. 3. The effect of stress and cycloheximide injection on
the activity of rat adrenal cholesteryl esterase and protein
kinase. □ esterase, ■ kinase.

molecule through the apolar core of the mitochon-
drial membrane to the cytochrome P-450 reaction
centre for pregnenolone formation.

Since there is only a limited amount of cholesterol
in these mitochondria and the sterol is apparently
in more than one pool the kinetics of the side chain
cleavage reaction utilising $[^{14}C]$-labelled cholesterol
require careful interpretation. Alterations in the pool
sizes of cholesterol in the mitochondrion could
greatly influence the apparent outcome of the conver-
sion of the $[^{14}C]$-cholesterol into $[^{14}C]$-pregneno-
lone.

In the rat adrenal cortex, the reservoir of choles-
terol appears to be in the cholesterol ester rich lipid
droplets in the cytosol of the cells. The immediate
precursor of the cholesterol side chain cleavage reac-
tion appears to be non-esterified (free) cholesterol and
not esterified cholesterol. Consequently, if the reser-
voir of cholesterol in the cells as ester cholesterol is
to be utilised in the production of pregnenolone by
the mitochondria the cholesterol esters must be hyd-
rolysed to make the cholesterol available to the mito-
chondrion. We have studied the cytoplasmic choles-
terol ester hydrolase and attempted to establish some
of the characteristics of this particular sterol ester-
ase [33].

It has been shown that $3'5'$ cyclic AMP and ATP
are involved in the activation of this supernatant cho-
lesterol ester hydrolase [15]. The cholesterol ester hy-
drolase activity and a protein kinase activity in rat
adrenal cortex 105,000 g supernatant fraction are sig-
nificantly higher in animals subjected to stressful sit-
uations [33]. Also the injection of cycloheximide did
not prevent the stress induced enhancement of the
activities of either enzyme. The results of these studies
are shown in Figs. 3 and 4. On the basis of these
observations we conclude that in the cytosol of the
cells of the adrenal cortex [33] and corpora lutea [34]
in the rat and in the bovine adrenal cortex [15] there
is present a cholesterol ester hydrolase which appears
to be in a dephosphorylated inactive form. This inac-
tive cholesterol ester hydrolase in the presence of
ATP and an active protein kinase is phosphorylated
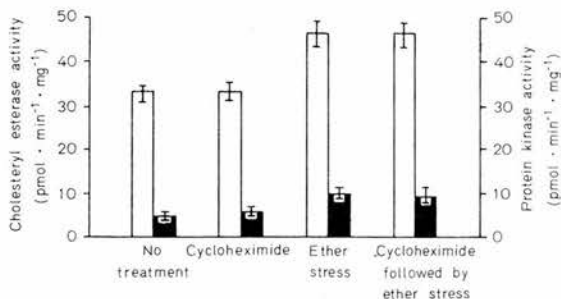to an active form of cholesterol ester hydrolase con-

taining a phosphate grouping. It seems likely that in
the bovine adrenal cortex and in the rat adrenal cor-
tex the protein kinase activity is reduced in the basal,
quiescent or resting state by the presence of an inhibi-
tory protein which has also the characteristics of a
$3'5'$ cyclic AMP binding protein [11]. Accordingly,
when the appropriate trophic signal is administered
to the plasma membrane of the cell, resulting in acti-
vation of the adenyl cyclase system, the rise in the
intracellular concentration of $3'5'$ cyclic AMP results
in this cyclic nucleotide binding to the appropriate
protein and consequently releasing the protein kinase
from this inhibitor. These reactions result in a marked
activation of the protein kinase with a subsequent
phosphorylation of the inactive cholesterol ester hy-
drolase to the active form of this enzyme. Our obser-
vations so far suggest that in the rat adrenal cortex,
in the bovine adrenal cortex, and in rat corpora lutea,
the sequence of events outlined above appears to
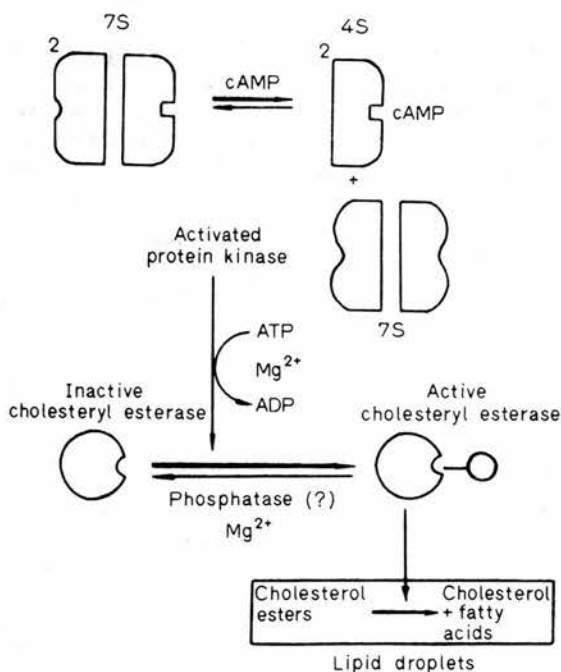operate resulting in an increase of two fold in the

Fig. 4. Possible mechanism for the activation of choles-
terol ester hydrolase enzyme system in bovine adrenal cor-
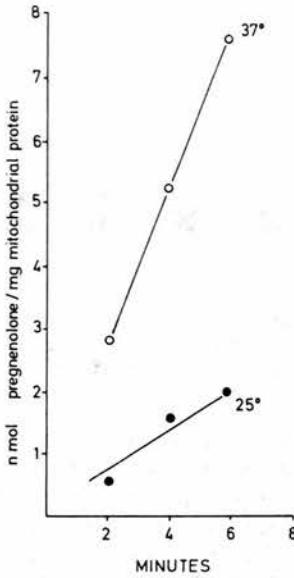tex or in rat adrenal cortex.

Fig. 5. The pregnenolone production rate in rat adrenal cortical mitochondria, in buffer solution as described in the text, in the presence of 10 mM isocitrate, 1 mM NADP and 0·1% BSA.

cholesterol ester hydrolase enzyme present in the cytosol of the cells [33].

This is shown diagrammatically in Fig. 4. In assays of cholesterol ester hydrolase activity, we use [4-^{14}C]-cholesterol oleate as the substrate because in lipid droplets in these cells, the predominant cholesterol ester tends to be cholesterol oleate [35]. In the cell the lipid droplets are present as spherical particles of diameter one micron or less. Lipid droplets contain varying amounts of phospholipid and in addition the droplets contain proteins.

Lipid droplets have been isolated from the adrenal cortex of rats and the corpora lutea from female rats. These lipid droplets (lipoproteins) have been washed by differential centrifugation and delipidated by methods used previously on other lipoprotein pro-

blems [36, 37]. The proteins isolated have been chromatographed by gel chromatography and electrophoresis, and there is evidence that these lipoproteins contain various proteins which appear to be disaggregated in SDS gel electrophoresis. These proteins present in the lipid droplets may have a structural role within the lipid droplet, to achieve effective interaction of the phospholipids and cholesterol esters or they may have a different role in the subsequent translocation of the sterol from the lipid droplets to the mitochondrion or to the plasma membrane of the cell.

Using methods previously described [13] a study has been made of some of the factors which influence the cholesterol side chain cleavage reaction in mitochondria obtained from rat adrenal glands and rat corpora lutea. Figure 5 shows the rate of production of pregnenolone expressed per mg mitochondrial protein in rat adrenal cortex mitochondria where the experiment has been performed at two temperatures. It will be noted that at 37°C about 8 nmol of pregnenolone per mg mitochondrial protein have been generated in 6 mins. This rate of pregnenolone production is such that a sizeable fraction of the total cholesterol present in the mitochondria has been metabolised at 37°C. Figure 5 also shows the rate of pregnenolone production at 25°C.

As a result of these experiments attempts were made to study the respiration of adrenal cortical mitochondria under certain specified conditions. As shown in Fig. 6 when adrenal mitochondria were suspended in the appropriate incubation mixture and isocitrate was added, the respiration rate increased. These mitochondria showed respiratory control. The addition of cyanide to the mitochondria blocks the respiratory chain without affecting the hydroxylating chain.

In Figure 6 these mitochondria have been respiring at 37°C for 12 min. It could be predicted from the previous results that much of the endogenous cholesterol in the mitochondria will have been oxidised to
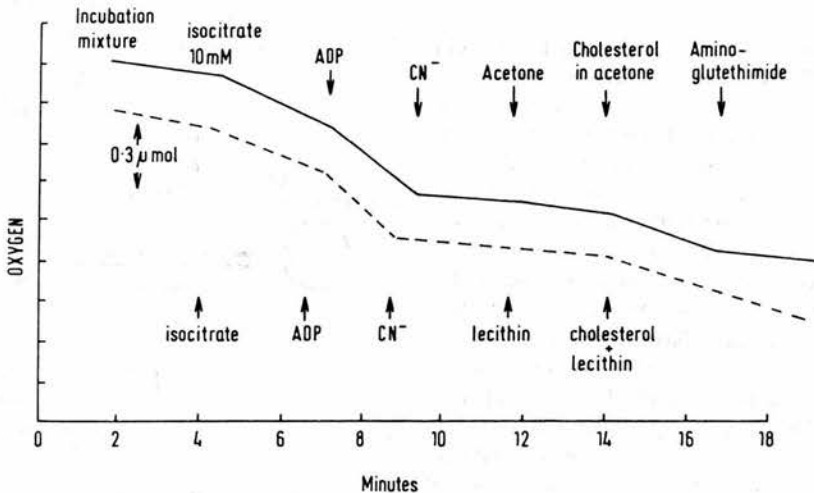

Fig. 6. Respiration of rat adrenal mitochondria in buffer solution as in Fig. 5, the experiment was conducted at 37°C. Note the increase in the rate of respiration upon the addition of cholesterol.
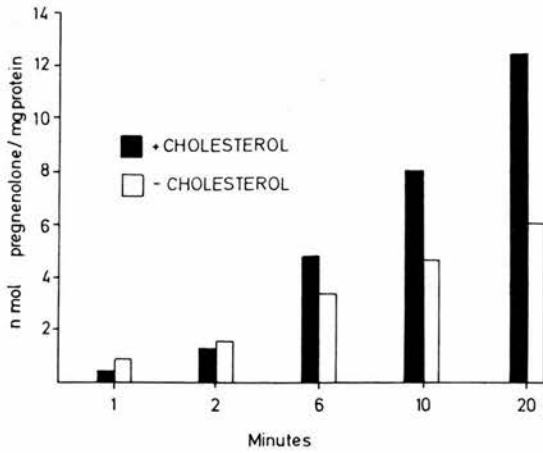
Fig. 7. Pregnenolone production in rat corpora luteal mitochondria in buffer solution in the presence of an electron donor. The solid histograms are incubations in the presence of cholesterol while the open histograms are results obtained in the absence of cholesterol [18].

pregnenolone. To test this acetone is added to the preparation and as shown in Figure 6 there is no change in the rate of respiration but when cholesterol in acetone is added there is an increase in the respiratory rate. That this increase in respiration is due to the cholesterol side chain cleavage reaction is shown by the inhibition of respiration achieved by 1 μM aminoglutethimide.

It could be argued that it is unphysiological to add cholesterol in acetone to these mitochondrial particles. Accordingly as shown in Fig. 6 the experiment was repeated with the variant that after blocking respiration with cyanide, lecithin was added producing no change in the respiratory rate but when lecithin-cholesterol micelles were added, again the rate of respiration increased. These observations suggest that during incubation in the presence of electron donors, rat adrenal mitochondria are quickly depleted of their

available cholesterol. The addition of cholesterol in acetone or as lecithin-cholesterol micelles restores the respiratory rate and restores the rate of production of pregnenolone from cholesterol.

In order to test this hypothesis, mitochondria from rat corpora lutea, which behave in these respiratory experiments in a fashion comparable to mitochondria from the adrenal cortex, were incubated with an electron donor for periods from 1 to 20 min. Similar mitochondria were incubated with excess cholesterol present in the incubation medium. The results of the experiment are shown in Fig. 7 where it can be seen that the pregnenolone production by rat corpora luteal mitochondria is greatly increased in the presence of cholesterol. These observations taken collectively imply that rat adrenal and rat corpora luteal mitochondrial particles are quickly depleted of cholesterol; consequently pregnenolone production is a function of the supply of cholesterol to the mitochondria.

It is well known that all mitochondria and these mitochondria from steroid hormone producing tissues in particular are very rich in phospholipids. From evidence collected in various model systems especially from lecithin-cholesterol liposome models [38, 39] there is a suggestion that cholesterol and lecithin interact in a specific fashion to produce in these artificial membranes a situation similar to that which may prevail in the inner cristae of mitochondria.

If cholesterol and phospholipids do interact in mitochondria in this way it is likely that the interaction will be dependent upon the ratio of cholesterol to phospholipid in the membrane and also dependent upon the temperature. Since these mitochondria rapidly metabolise cholesterol we decided to test the integrity of the mitochondria, through assessment of the respiratory control of the mitochondria at 29°C and then block the respiration by cyanide at this stage. The temperature was raised to 37°C followed by the addition of cholesterol to the mitochondria
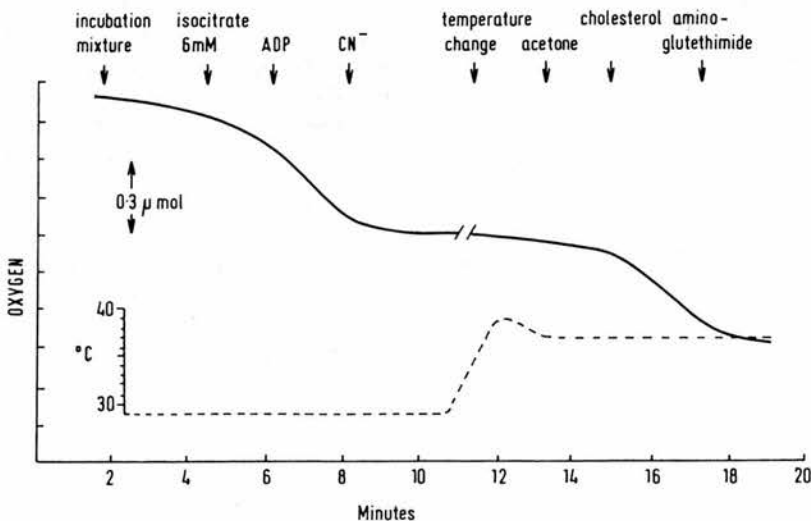

Fig. 8. Respiration of rat adrenal mitochondria at 29°C showing respiratory control. The change in temperature showed that the addition of cholesterol in acetone produced a rapid rise in the respiratory rate.

at this higher temperature. Such an experiment is shown in Fig. 8. The advantage of this approach is that it allowed assessment of the integrity of the mitochondria at a reduced temperature where the rate of cholesterol depletion would be reduced followed by a rapid transition to the physiological temperature.

It is established that calcium ions stimulate mitochondrial hydroxylation reactions *in vitro* [40–43] and it has been suggested that some of these effects could be due to changes in the permeability of the mitochondria induced by these divalent ions. It is well known that calcium ions interact with the polar heads of phospholipids in micelles and it has been shown that the permeability of cholesterol-phospholipid liposomes is affected by calcium ions [44]. It has been shown that calcium ions influence the cholesterol side chain cleavage reaction in adrenal mitochondria *in vitro* [41, 42] and it has been deduced that this effect of calcium ions is due to an increased formation of a cytochrome P-450 cholesterol complex attributed to the formation of a high spin complex [43].

The effect of calcium ions on the cholesterol side chain cleavage reaction revealed that these ions at concentrations as low as 0·1 mM produced a marked increase in the pregnenolone production rate [41, 42]. The effect of calcium ions on the respiration of rat adrenal mitochondria was studied as described previously. As shown in Fig. 9 when calcium ions at 0·1 mM concentration was applied to these mitochondrial preparations there was an immediate increase in the respiration rate which could be confirmed as due to an elevated conversion of cholesterol to pregnenolone. This effect was transient and upon the addition of cholesterol the rate of respiration was markedly elevated. It would be possible to interpret these results in the light of the other observations as due to the displacement of substrate cholesterol from some phospholipid binding site to another site—the cytochrome P-450 reaction centre [43]. Furthermore 0·1 mM calcium ions also alter the permeability of

Fig. 10. Oxygen consumption and pregnenolone production rate of rat adrenal mitochondria respiring in buffer (as previously described) at 37°C in the presence of cyanide.

the mitochondria and so facilitate ingress of sterol to the cytochrome P-450 oxygenase. It will be noted in Fig. 9 that these alterations in the rate of respiration of the mitochondria due to low concentrations of calcium ions is accomplished without substantial change in the pH of the environment so that the change in the cholesterol side chain cleavage reaction cannot be due to alterations in the cholesterol-cytochrome P-450 spin state as a consequence of an alteration in the hydrogen ion concentration of the medium [14].

Employing these *in vitro* respiration studies on rat adrenal cortical mitochondria coupled to pregnenolone assays it has been possible to explore the stoichiometry of the cholesterol side chain cleavage reaction in these organelles. As shown in Fig. 10 the oxygen uptake of rat adrenal cortical mitochondria exceeds the pregnenolone production rate by a factor of about nine. If the cholesterol side chain cleavage event followed the reaction sequence shown in Figure 1 the stoichiometry would be three molecules oxygen consumed for each cholesterol molecule converted to pregnenolone. This three to one molar ratio for the cholesterol side chain cleavage mixed function oxidase has been confirmed in a cytochrome P-450 preparation from bovine adrenal cortex [45]. It appears therefore that in the intact mitochondrial preparation in which the respiratory chain is inhibited by cyanide that there must be other hydroxylation events or peroxidation events occurring or there must be certain "non-productive" cytochrome P-450 cycles which result in the consumption of oxygen without the production of a hydroxylated intermediate—other than perhaps H_2O_2. This situation would be comparable to the uncoupling of hydroxylation events seen in
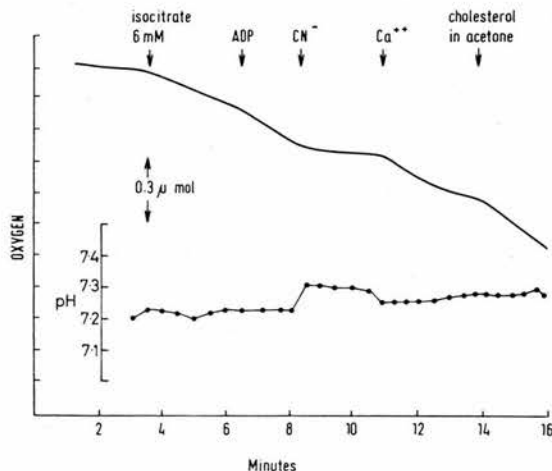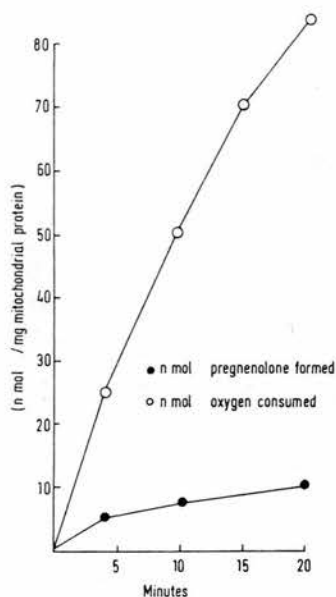

Fig. 9. Respiration of rat adrenal mitochondria at 37°C in buffer solution as previously described. Trace shows the increase in respiration as a result of the addition of 0·1 mM calcium followed by a further increase upon the addition of cholesterol in acetone.

liver microsomal preparations under certain circumstances [47].

During maximum pregnenolone production in mitochondria from rat corpora lutea in the presence of exogenous cholesterol, EPR spectroscopy has shown that the iron sulphur protein in mitochondria is about 70% reduced. By contrast in the absence of exogenous cholesterol, where the cholesterol content of these mitochondria is declining, the iron sulphur protein is about 90% reduced. These observations support the concept that the supply of cholesterol to these mitochondria may influence the electron flux through the hydroxylation chain [48].

DISCUSSION

The work reported in this paper on cholesterol metabolism in mitochondria is in general agreement with recent studies of several other laboratories [42, 46, 50] in that one of the key events in the adrenal cortex, under stimulation with ACTH, or in the corpus luteum under stimulation with LH, is the transportation of cholesterol to the cytochrome P-450 reaction centre located in the inner cristae of the mitochondria. While the overall production of pregnenolone remains the rate limiting event in steroidogenesis in these tissues, the translocation, or trans-

portation component in intact mitochondria appears to impart the actual slow step to this process. Once the cholesterol has been delivered to the cytochrome P-450 enzyme concerned with the side chain cleavage of this sterol the subsequent oxygenase reaction is rapid.

In these mitochondria, the available cholesterol is quite limited (Table 2) and hence there must be a mechanism for the supply of sterol to the oxygenase system as and when required. Some of the results reviewed in this paper show that there is present in the cytosol of the adrenal cortex and corpora lutea a cholesterol ester hydrolase whose activity may be modified under the influence of $3'5'$ cyclic AMP and a protein kinase. This hydrolase is capable of attacking the cholesterol ester stores in the lipid droplet storage granules in the cytoplasm to release unesterified or free cholesterol. The lipid droplets are also rich in phospholipids and it is possible that cholesterol and the phospholipids could form micelles of a liposome type for transportation of the sterol to the mitochondria, as shown in Fig. 11.

The mitochondria of these steroidogenic tissues have been shown to be capable of utilising for pregnenolone formation, exogenous cholesterol whether supplied in an acetone solution or in phospholipid micelles [18, 46]. The interaction of a relatively small amount of cholesterol with an excess of phospholipids in the cristae of the native mitochondria is obviously an area for exploration in the future. The cholesterol side chain cleavage reaction occurring in this essentially hydrophobic environment presents a challenge to enzymologists. The substrate cholesterol may be delivered to the mitochondrial cristae in the form of a micelle, and presumably this micelle may fuse with the micelle like, or liposome like, cristae and achieve a translocation of the cholesterol contents to the cristae. It is known that even in artificial liposomes of cholesterol and phospholipids the structure is not uniform and there are areas of solidity and areas of fluidity in the membrane [30]. As the ratio of cholesterol molecules to cytochrome P-450 molecules in mitochondria is quite low the problem is to discover how the cholesterol molecules get to the cytochrome P-450 enzyme involved in the cholesterol side chain cleavage reaction. There is therefore a requirement for the cholesterol molecule to be removed through the hydrophobic core of the cristae. On the basis of other evidence on the interaction of substrates with cytochrome P-450 it is deduced that the cholesterol-cytochrome P-450 ligand forms a high spin complex. Acetone extracted bovine adrenal mitochondria which have been depleted of cholesterol and phospholipids do in fact produce optical and EPR spectral evidence of the formation of a cytochrome P-450 high spin complex on treatment with lecithin-cholesterol micelles [49]. It is interesting that intact mitochondria which have been depleted of cholesterol by the addition of an electron donor in the presence of oxygen, followed by gentle washing, do not produce a type I characteristic spectrum when treated with choles-
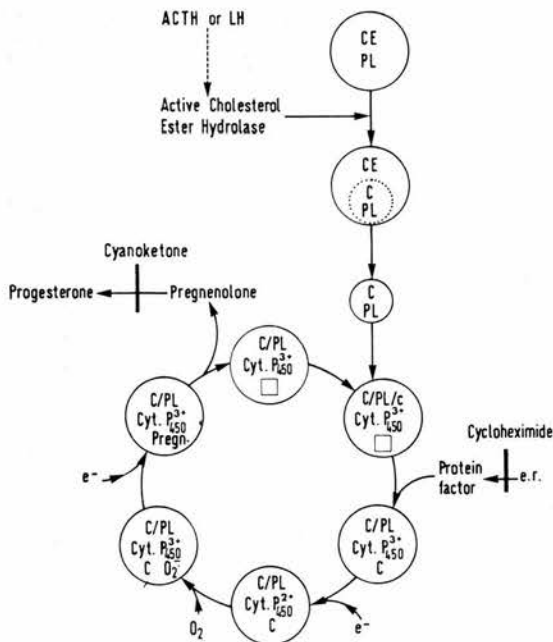
Fig. 11. Diagrammatic representation of the events which may operate in the adrenal cortex and in corpora lutea. It is suggested that the cholesterol esterase is activated by a protein kinase reaction followed by the release of cholesterol to the cytosol. The cholesterol is transferred to the mitochondria where it is taken up into a reserve of available cholesterol. This cholesterol can then move through the cristae of the mitochondrion to the cytochrome P-450 reaction centre. The cholesterol movement may be controlled by a protein or peptide of extramitochondrial origin. The cytochrome P-450-cholesterol complex may be a high spin species which is then subjected to the usual events resulting finally in the production of pregnenolone and a change in the spin state of the cytochrome P-450.

terol. Presumably there is some barrier to the access of the substrate to the haemoprotein in these intact cholesterol depleted mitochondria.

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The Effect of Dietary Rape-Seed Oil
on Cholesterol-Ester Metabolism
and Cholesterol-Ester-Hydrolase Activity in the Rat Adrenal

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The effects of stock diet and stock diet supplemented by olive oil and rape seed oil on rat adrenal cholesterol ester metabolism have been studied. Rats fed rape seed oil failed to gain weight at the same rate as rats fed olive oil.

A prominent feature of the rats fed rape seed oil was an accumulation of high concentrations of cholesterol erucate in the adrenal lipid droplets. When these rats were subjected to an ether stress no percentage decrease in the amount of cholesterol erucate was observed.

Adrenal cholesterol ester hydrolase activity was higher in rats fed the olive oil and rape seed oil diets than rats fed the stock diet.

In rats fed stock or olive oil diets, a ten-minute ether anaesthesia stress resulted in a two-fold increase in activity of adrenal cholesterol ester hydrolase. Cofactor addition of ATP, cyclic AMP and $MgCl_2$ *in vitro* resulted in a stimulation of cholesterol ester hydrolase to a similar activity in both quiescent and ether-stressed rats. By contrast rats fed the rape seed oil diet gave no significant stimulation of cholesterol ester hydrolase activity when given an ether stress or when cofactors were added *in vitro*.

Cholesterol erucate was hydrolysed at only 25% to 30% of the rate of cholesterol oleate *in vitro* in all groups of animals.

Oleic acid added *in vitro* gave an inhibition of cholesterol ester hydrolase activity in rats fed stock diet while erucic acid activated the enzyme.

The accumulation of cholesterol erucate in the adrenal when rats are fed rape seed oil could be due to the reduced ability of cholesterol ester hydrolase to hydrolyse this ester.

It has been reported that feeding rape seed oil to male rats causes an accumulation of lipid in heart, skeletal muscle [1] and adrenals [2]. The lipid accumulation in heart has been shown to be accompanied by a decrease in the rate of ATP synthesis in isolated heart mitochondria [3].

Boer *et al.* [4] have found that feeding rape seed oil retards the growth of rats and this has been confirmed by Thomasson [5] who identified the substance responsible for the growth retardation as erucic acid [6].

The accumulation of lipid in adrenals is due mainly to a 3 to 5-fold increase in the amount of cholesterol esters [2], and in particular cholesterol erucate [7]. Carrol [8] showed erucic acid was the constituent of rape seed oil responsible for this effect.

When animals are subjected to a stressful situation a depletion of cholesterol esters in the adrenal occurs [9,10]. Walker and Carney [10] showed that in rats fed ethyl erucate plus corn oil and subjected to a 30-min cold stress, cholesterol ester depletion was less than in animals fed olive oil. A prominent metabolic feature of the animals fed ethyl erucate was the lack of utilisation of cholesterol erucate.

Carrol and Noble [11] have reported that although producing an accumulation of adrenal cholesterol esters, rape seed oil has no marked influence on adrenal function and such adrenals respond to adrenocortico-

Abbreviations. Cyclic AMP, adenosine 3′:5′-monophosphate; for fatty acids, the number before the colon indicates the number of carbon atoms, and the number after the colon the number of double bonds.

Enzymes. Cholesterol ester hydrolase or sterol ester hydrolase (EC 3.1.1.13); protein kinase or ATP:protein phosphotransferase (EC 2.7.1.37).

Trivial name. Cholesterol, 5-cholesten-3β-ol.

tropin and stress. Injections of large doses of adreno-corticotropin, however, failed to reduce the concentration of adrenal cholesterol to low levels in rats fed rape seed oil. Walker and Carney [10] showed that plasma corticosteroids in animals fed corn oil plus ethyl erucate were not elevated to the same level as corticosteroids in the plasma of animals fed olive oil when both groups of animals were subjected to cold stress. Prostaglandin production which is stimulated by adrenocorticotropin in adrenal homogenates is also lower in animals fed rape seed oil compared with animals on a corn oil diet [12].

Sayers *et al.* [13] have demonstrated that the depletion of adrenal cholesterol which follows adreno-corticotropin treatment involves the cholesterol ester fraction and Shima *et al.* [14] have shown that adreno-corticotropin injections cause a significant increase in cholesterol ester hydrolase activity in the adrenal. Garren [15] has postulated that the adrenocortico-tropin-induced depletion in cholesterol ester concentration in rat adrenals resulted from the activation of cholesterol ester hydrolase and that a cyclic-AMP-dependent protein kinase was involved in the process. Recently work in this laboratory [16] has suggested that in bovine adrenal cortex an active cyclic-AMP-dependent protein kinase phosphorylates an inactive form of cholesterol ester hydrolase to an active phos-phorylated form in the presence of ATP. Work in this laboratory has also shown that ether anaesthesia stress, which is known to increase plasma adreno-corticotropin concentration [17], causes an increase in protein kinase and cholesterol ester hydrolase activity in the rat adrenal [9].

The aim of the present study was to establish whether adrenal cholesterol ester accumulation in rats fed rape seed oil was due to an inhibition of the adrenal cholesterol ester hydrolase or an inability of the cholesterol ester hydrolase to metabolise choles-terol erucate.

The effect of ether stress on cholesterol ester hydrolase in animals fed on rape seed oil has also been studied.

MATERIALS AND METHODS

Chemicals and Radiochemicals

ATP, disodium salt; cyclic AMP; oleic acid (99% pure); erucic acid (99% pure); cholesterol oleate (99% pure) and histone type II, from calf thymus; were purchased from Sigma Chemical Co. (St. Louis). Other chemicals and solvents were obtained from British Drug Houses Ltd (Poole) and were of analytical grade.

The sodium salt of adenosine $5'$-[γ-^{32}P]triphos-phate (specific activity $500-3000$ mCi/mmol), [4-^{14}C]-

cholesterol (specific activity 55.6 mCi/mmol) and [7α-^{3}H]cholesterol (specific activity 9.4 Ci/mmol) were purchased from the Radiochemical Centre (Amersham). [4-^{14}C]cholesterol oleate and erucate were prepared by refluxing equimolar concentrations of [4-^{14}C]cholesterol, fatty acid and dicyclohexyl carbodiimide in dry benzene. All radioactive steroids were purified by thin layer chromatography on silica gel G (Merck, E., Darmstadt).

Olive oil (Pacchini brand) was purchased locally and rape seed oil was generously donated by Dr A. Vergroessen (Unilever Research, Vlaardingen, Holland).

Animals and Animal Treatment

Male Wistar rats (Edinburgh University Small Animal Breeding Centre) were maintained for six weeks on a stock diet of 25% skimmed milk powder, 5% dried yeast and 70% wholemeal flour, supple-mented with 25% by weight olive oil or rape seed oil, and were given water *ad libitum*.

The animals on each diet were randomly divided into two groups at the termination of the experiment. The animals of one group, the controls, were kept in a quiescent state, transferred individually to a separate room for killing to minimise stress due to handling and killed by decapitation. The second group were sub-jected to an ether anaesthesia stress for ten minutes before killing.

Preparation of Enzyme Extract and Lipid Droplets

The adrenals from the animals were quickly removed after killing, trimmed free of adherent fat, pooled and homogenised in ice-cold 0.25 M sucrose in a teflon-pestle Potter-Elvehjem homogeniser.

The $105000 \times g$ supernatant fraction and lipid droplets were prepared as described previously [9]. The $105000 \times g$ delipidated supernatant was passed through a Sephadex G-25 column (30×2 cm) which was equilibrated with 5 mM Tris-HCl buffer, pH 7.4, to desalt the supernatant and the protein fractions pooled. The Sephadex G-25 filtration was carried out at 3 °C and the desalting procedure took approximately 30 min. It is unlikely that significant dephosphoryla-tion to the inactive cholesterol ester hydrolase could take place over this time at 3 °C.

Determination of Cholesterol Ester Hydrolase Activity

The assay based upon the method of Chen and Morin [18] was carried out as described previously [9].

Assays were carried out in a final volume of 1 ml with 1—2 mg of protein per assay. The substrate employed was 24 µM [4-^{14}C]cholesterol oleate (95000 counts/min) added in 10 µl of acetone. At the concentration of cholesterol oleate employed in the incubation mixture the reaction rate was linear over the 30-min incubation [9].

Determination of Protein Kinase Activity

Protein kinase activity was determined by the method of Krebs and co-workers as modified by Gill and Garren [19]. The assay system consisted of 50 mM sodium glycerol-3-phosphate pH 6.5, 10 mM $MgCl_2$, 4 mM sodium fluoride, 2 mM theophylline, histone 0.5 mg/ml and 25 µM [γ-^{32}P]ATP sodium salt (approx. 10^7 counts/assay). The final volume of the reaction mixture was 0.2 ml. The incubation was conducted for 20 min at 30 °C along with a control which contained no histone. The phosphorylated protein was isolated [20] dissolved in 23 M formic acid and an aliquot was taken for counting in a Packard Tri-Carb-liquid scintillation spectrometer in a dioxane-based scintillation fluid [21].

Determination of Cholesterol and Cholesterol Ester Concentration in Delipidated $105000 \times g$ Supernatant

Cholesterol and cholesterol ester were extracted from the adrenal supernatant as described previously [9]. Esterified and free cholesterol were measured by gas liquid chromatography [22]. Results were corrected for losses during the extraction procedure by the addition of tracer amounts of [7-^{3}H]cholesterol and [4-^{14}C]cholesterol oleate prior to extraction by the lipid solvent.

Determination of Fatty Acid Composition of Cholesterol Esters in Lipid Droplets

Steroids were extracted from the lipid droplets as described previously [9]. The cholesterol esters were transesterified with boron trifluoride/methanol. The fatty acid composition of the adrenal cholesterol esters was determined by gas liquid chromatography on 12.5% diethylene glycol adipate on a chromosorb W support at 180 °C, using a Pye-104 gas liquid chromatograph and a flame ionisation detector.

Protein Estimations

Protein concentrations were determined by the method of Lowry *et al.* [23] with bovine serum albumin as a standard.

Table 1. *Fatty acid composition of dietary fats*
Minor constituents omitted from the table. Fatty acids were extracted from the oils and determined by gas-liquid chromatography as described in the Methods section

Fatty acid	Amount in	
	olive oil	rape seed oil
	% total	
16:0	14	4
18:0	1	–
18:1	61	11
18:2	10	13
20:1	1	11
22:1	6	60

RESULTS

The fatty acid composition of the oil diets is given in Table 1. Low levels of saturated fatty acids were found in both diets. A high concentration (60%) of erucic acid (22:1) was found in the rape seed oil diet with oleic acid (18:1) contributing only 11% of the total fatty acid composition in this oil. In the olive oil diet a relatively small amount (6%) of the 22:1 acid was found, 18:1 being the major (61%) fatty acid constituent in this oil.

Effect of Feeding Olive Oil and Rape Seed Oil on Adrenal and Body Weight in the Rat

The adrenals of rats kept on the rape seed oil diet for 6 weeks appeared larger than the adrenals of rats fed on the olive oil diet and had a white appearance. There was however no significant difference in adrenal weight, although the body weights of rats fed rape seed oil for six weeks were significantly lower ($P < 0.001$) than the control rats fed olive oil (Table 2). Due to the lower body weight of rats fed rape seed oil, these animals had a higher adrenal weight expressed per gram body weight.

The Effect of Ether Stress on the Composition of the Cholesterol Esters in Lipid Droplets from the Adrenals of Rats Fed Stock Diet, Olive Oil and Rape Seed Oil Diet

Table 3 shows the composition of the cholesterol esters in the lipid droplets isolated from quiescent rats and ether-stressed rats fed stock diet, rape seed oil diet and olive oil diet.

It can be seen that in the adrenal lipid droplets of rats kept on rape seed oil there was an accumulation of the 22:1 ester whilst little of this ester accumulated in rats kept on the other diets.

Ether stress resulted in a decrease in the concentration of the 14:0, 16:0, 16:1, 18:1, 18:2 and 20:4 esters in lipid droplets of rats receiving rape seed oil, while rats receiving olive oil showed a selected decrease in the concentrations of 16:0, 18:1 and 18:2 esters. Stock diet fed animals showed a decrease of 16:1, 18:0, 18:1, 18:2 and 20:4 esters as a consequence of ether anaesthesia.

The lack of utilization of the 22:1 and 20:1 esters is consistent throughout all the groups of rats fed the different diets.

The Activity of Adrenal 105000 × g Supernatant Cholesterol Ester Hydrolase in Rats Subjected to Stock, Olive Oil and Rape Seed Oil Diets

Table 4 shows the effect on adrenal cholesterol ester hydrolase of feeding rats stock, olive oil and rape seed oil diets. In the quiescent state only in Expt 2 was there a significant difference in cholesterol ester hydrolase activity between rats on the olive oil diet and rats fed rape seed oil. In three out of the four experiments, rats maintained on rape seed oil had a significantly higher cholesterol ester hydrolase activity ($P < 0.05$) than animals maintained on stock diet. When the probabilities found in each of the four experiments were combined [24] the overall probability that the differences in cholesterol ester hydrolase activity, assayed with cholesterol oleate, between the two groups of rats, is due to chance, is less than 0.01.

Cholesterol ester hydrolase activity was significantly increased ($P < 0.05$) in animals maintained on olive oil or stock diet when the animals were subjected to a ten-minute ether anaesthesia stress. Animals fed rape seed oil showed no significant increase in cholesterol esterase activity on ether stress and in Expts 1 and 2 there was observed a significant ($P < 0.01$) decrease in cholesterol ester hydrolase activity.

Animals maintained on olive oil or stock diet and not subjected to an ether stress gave a significant ($P < 0.05$) stimulation of cholesterol ester hydrolase activity when 5 mM ATP and 1 μM cyclic AMP were added in vitro. Cholesterol ester hydrolase activity was stimulated in vitro to a higher value with the cofactor additions than was observed when the animals had been subjected to ether stress alone. 5 mM ATP and 1 μM cyclic AMP added in vitro to the adrenal supernatant from ether-stressed animals also stimulated cholesterol ester hydrolase activity to values similar to or greater than those found on adding the cofactors to adrenal supernatant from animals not subjected to an ether stress.

Animals maintained on rape seed oil and not subjected to an ether stress gave no significant stimu-

Table 2. *The effect of dietary olive oil and rape seed oil on the body weight and adrenal weight*
Rats were fed the olive oil and rape seed oil diets for 5 to 6 weeks. Adrenals from each group were pooled after killing and weighed. For body weight, mean ± S.E.M. is given

Treatment	Experiment number	Average weight of rat (12 rats)	Average weight of adrenal (12 rats)	Average weight of adrenal per gram body weight
		g	mg	μg/g
Olive oil diet	1	307 ± 7	26.5	86
	2	390 ± 10	31.5	81
	3	249 ± 4	31.0	124
Rape seed oil diet	1	253 ± 7	27.2	107
	2	306 ± 4	32.4	105
	3	221 ± 5	28.6	129

Table 3. *Effect of ether stress on the cholesterol esters from adrenal lipid droplets of rats fed stock, olive oil and rape seed oil diets.*
Minor constituents are omitted from the table. Ester composition was determined by gas-liquid chromatography as described in Methods

Ester	Stock diet		Olive oil diet		Rape seed oil diet	
	quiescent	stressed	quiescent	stressed	quiescent	stressed
	%					
14:0	4.3	4.3	1.1	1.3	2.2	1.3
16:0	28.6	29.5	16.0	14.3	14.3	12.7
16:1	7.1	5.7	2.7	2.7	4.7	3.1
18:0	7.1	6.1	6.9	7.3	2.3	2.6
18:1	25.5	24.3	41.4	40.1	32.8	31.3
18:2	13.3	11.4	15.4	13.5	10.7	8.0
20:1	2.0	6.1	3.3	3.4	9.2	9.7
20:4	8.8	7.8	7.2	7.6	3.4	1.9
22:1	–	–	1.2	2.7	18.7	27.5
22:4	3.3	4.8	4.8	7.1	1.7	1.9

lation of cholesterol ester hydrolase activity when these cofactors were added in vitro.

Animals fed rape seed oil and subjected to ether stress exhibited a slight increase in cholesterol ester hydrolase activity in vitro by cofactor addition.

Table 5 shows the effect of feeding rats diets of rape seed oil, olive oil and stock diets on adrenal cholesterol ester hydrolase activity using 24 μM [4-^{14}C]cholesterol erucate as substrate compared with 24 μM [4-^{14}C]cholesterol oleate. Cholesterol erucate was hydrolysed at a slower rate than cholesterol oleate in enzyme preparations obtained from all groups of animals. Cholesterol ester hydrolase activity could only be stimulated in animals fed olive oil or stock

Table 4. *The effect of ether stress and cofactor addition on rat adrenal supernatant cholesterol ester hydrolase activity in rats fed stock, olive oil and rape seed oil diets*
Results are quoted as the mean of duplicate (Expts 3 and 4) or triplicate assays ± S.E.M. 6 rats were used in each group. Cofactors added were 5 mM ATP + 5 mM $MgCl_2$ + 10 µM cyclic AMP. Assays were carried out as described in Methods

Additions	Experiment number	Cholesterol ester hydrolase activity					
		stock diet		olive oil diet		rape seed oil diet	
		quiescent	stressed	quiescent	sressed	quiescent	stressed
		$pmol \times min^{-1} \times mg\ protein^{-1}$					
None	1	147 ± 5	273 ± 13	158 ± 25	319 ± 10	198 ± 15	109 ± 5
+ Cofactors		219 ± 3	274 ± 10	324 ± 20	409 ± 5	210 ± 11	136 ± 22
None	2	97 ± 8	136 ± 11	99 ± 2	121 ± 4	166 ± 11	49 ± 4
+ Cofactors		227 ± 30	216 ± 12	250 ± 20	230 ± 17	206 ± 20	143 ± 19
None	3	28 ± 2	43 ± 1	58 ± 10	71 ± 4	77 ± 12	68 ± 0
+ Cofactors		104 ± 5	113 ± 13	154 ± 18	140 ± 7	180 ± 32	82 ± 4
None	4	119 ± 5	150 ± 10	118 ± 6	269 ± 26	120 ± 17	121 ± 10
+ Cofactors		212 ± 6	218 ± 3	286 ± 4	296 ± 20	168 ± 5	188 ± 5

Table 5. *The effect of ether stress and cofactor addition on rat adrenal supernatant cholesterol ester hydrolase activity in rats fed stock, olive oil and rape seed oil diets using [4-^{14}C]cholesterol oleate and erucate as substrate*
Results are quoted as the mean of duplicate assays ± S.E.M. 6 rats were used in each group. Cofactors added were 5 mM ATP + 5 mM $MgCl_2$ + 10 µM cyclic AMP. Assays were carried out as described in the Methods section

Additions	Substrate	Cholesterol ester hydrolase activity					
		stock diet		olive oil diet		rape seed oil diet	
		quiescent	stressed	quiescent	stressed	quiescent	stressed
		$pmol \times min^{-1} \times mg\ protein^{-1}$					
None	Oleate	119 ± 5	150 ± 10	118 ± 6	269 ± 26	120 ± 17	124 ± 10
+ Cofactors		212 ± 4	218 ± 3	286 ± 4	296 ± 20	168 ± 5	188 ± 5
None	Erucate	50 ± 5	61 ± 1	41 ± 3	95 ± 8	31 ± 1	31 ± 7
+ Cofactors		96 ± 10	72 ± 3	106 ± 31	104 ± 1	61 ± 4	103 ± 2

diet by a 10-min ether anaesthesia before killing or by cofactor addition *in vitro*. Animals fed rape seed oil showed no increase in the rate of cholesterol erucate hydrolysis when subjected to stress but cofactor additions stimulated cholesterol ester hydrolase activity assayed against cholesterol erucate in the supernatant obtained from adrenals of stressed and unstressed animals.

The endogenous cholesterol concentration of the $105000 \times g$ adrenal supernatant obtained from rats fed stock diet, olive oil and rape seed oil diets after passage through a Sephadex G25 column was 1.8, 6.6 and 6.6 µg/mg protein respectively. Cholesterol ester concentration was 13.6, 21.7 and 19.6 µg/mg protein respectively. The endogenous ester concentrations were measured to show that the radioactive

assay system employed, was valid to compare cholesterol ester hydrolase activity between the groups of rats fed different diets.

The Effects of Erucic Acid, Oleic Acid, 5 mM ATP and 10 µM Cyclic AMP on Cholesterol Ester Hydrolase Activity Using [4-^{14}C]Cholesterol Oleate as Substrate

Fig.1 shows the effect of varying the concentration of free oleic and erucic acids on the activity of cholesterol ester hydrolase from rats fed stock diet.

Oleic acid gave a significant inhibition of cholesterol ester hydrolase activity at a concentration of 2 µM. The inhibition increased as the concentration of oleic acid was increased until at 160 µM cholesterol
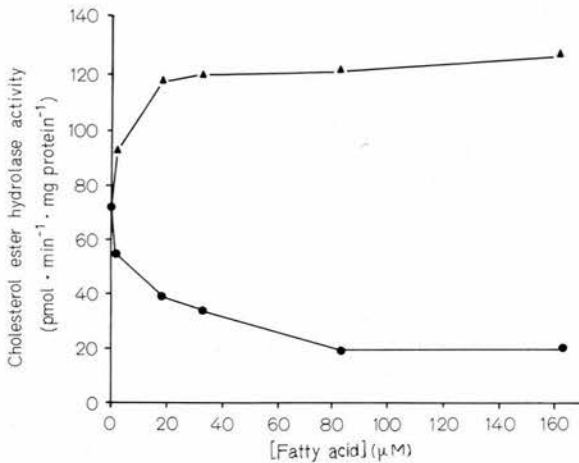
Fig. 1. *The effect of oleic acid (●) and erucic acid (▲) added* in vitro *on cholesterol ester hydrolase activity of 105000 × g supernatant from adrenals of rats fed stock diet.* Incubations were carried out as described in the Methods section. The acids were added in 10 μl acetone

Fig. 2. *The effect of oleic acid (●) and erucic acid (▲) added* in vitro *on cholesterol ester hydrolase activity of 105000 × g supernatant in the presence of cofactors from adrenals of rats fed stock diet.* Incubations were carried out as described in the Method section in the presence of 5 mM ATP, 5 mM MgCl$_2$ and 10 μM cyclic AMP. The acids were added in 10 μl of acetone

ester hydrolase activity had fallen from 70 pmol $\times$ min^{-1} $\times$ mg protein^{-1} to 20 pmol $\times$ min^{-1} $\times$ mg protein^{-1}.

Erucic acid gave a significant activation of cholesterol ester hydrolase activity over the same concentration range as oleic acid with a maximum stimulation to 128 pmol $\times$ min^{-1} $\times$ mg^{-1} found at a concentration of 160 μM.

When 5 mM ATP and 10 μM cyclic AMP were added, ester hydrolase activity was stimulated from 70 pmol $\times$ min^{-1} $\times$ mg^{-1} to 154 pmol $\times$ min^{-1} $\times$ mg^{-1}. Oleic acid again gave an inhibition until at a concentration of 160 μM the cholesterol ester hydrolase activity had again fallen to 22 pmol $\times$ min^{-1} $\times$ mg^{-1} (Fig. 2).

Erucic acid exhibited no further activation when added in the presence of ATP and cyclic AMP but unlike oleic acid, erucic acid did not exhibit any inhibition at a concentration of 33 μM and even at a concentration of 160 μM cholesterol ester hydrolase activity had not fallen below the unstimulated value of 70 pmol $\times$ min^{-1} $\times$ mg^{-1}.

The Activity of Adrenal 105000 × g Supernatant Protein Kinase in Rats Subjected to Olive Oil and Rape Seed Oil Diets

Fig. 3 shows the effect of a 10-min ether stress on rat adrenal supernatant protein kinase activity in rats fed olive oil and rape seed oil diets. Protein kinase activity was significantly increased ($P < 0.05$) in rats fed the olive oil diet when these animals were subjected to a 10-min ether stress. Rats fed the rape seed oil diet gave no significant increase in protein kinase activity.
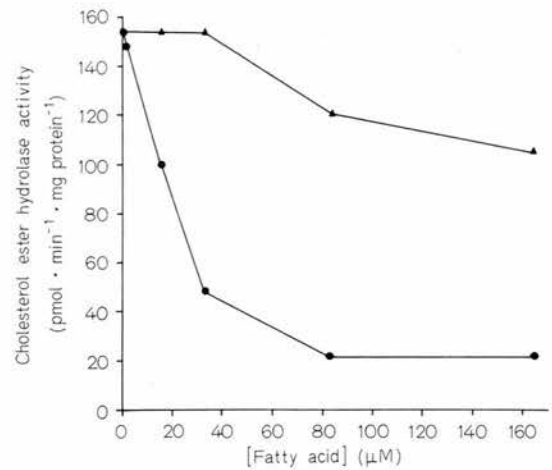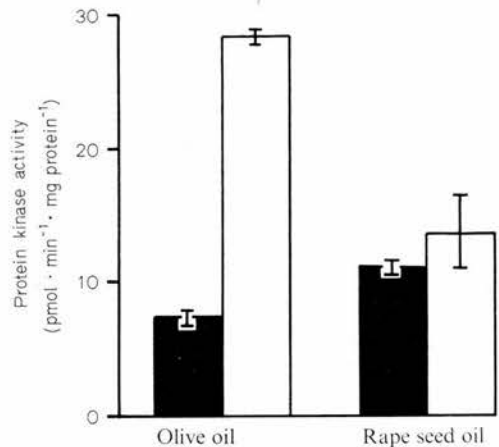

Fig. 3. *The effect of ether stress on protein kinase activity of 105000 × g supernatant from adrenals of rats fed olive oil and rape seed oil diets.* Incubations were carried out as described in the Methods sections. 12 rats were used in each group. Unshaded bars represent protein kinase activity in animals subjected to a 10-min ether stress. Shaded bars represent protein kinase activity in control animals

DISCUSSION

Carrol [2] has shown that feeding Sprague Dawley rats a diet of 45% by weight rape seed oil for four weeks produced an increase in adrenal size and an increase in the absolute amount of adrenal cholesterol compared with a control where the oil was absent. The oil produced nearly the same effect when fed as 25% of the diet and the adrenals had the same white appearance which was also noted when the whole seed was fed. The growth rate of rats fed 25% rape seed oil was lower than the control animals although the growth rate was much better than when 45% rape seed oil was given.

Beare *et al.* [25] using Wistar rats found no difference in adrenal weights after animals had been fed rape seed oil at 20% for four weeks. Animals fed 20% rape seed oil failed to gain weight at the same rate as a group of animals fed 20% corn oil for four weeks. Beare *et al.* [25,26] have attributed this slow weight gain to a lower food consumption of the rape seed diet and possibly to the low level of saturated acids particularly palmitic acid.

Our findings confirmed those of Beare *et al.* [25] in that although the adrenals of animals fed 25% by weight rape seed oil appeared larger and had a white appearance there was no increase in total adrenal weight compared with animals fed 25% by weight olive oil. Animals fed 25% rape seed oil also failed to gain weight compared with animals fed 25% by weight olive oil. Animals fed 25% rape seed oil also failed to gain weight at the same rate as the controls.

Walker and Carney [10] have reported that when rats are subjected to a cold stress for 30 min total adrenal cholesterol esters in animals receiving an olive oil diet decrease by 34% of the control value. In contrast, a cold stress applied to animals receiving ethyl erucate resulted in an insignificant decrease in cholesterol esters. Animals fed ethyl erucate showed a significant decrease in the concentration of the 18:1 ester when subjected to a 30-min cold stress, while animals fed olive oil showed decreases in 16:1, 18:2, 20:1, 20:4 and 22:4 cholesterol esters.

In our experiments when animals were subjected to a 10-min ether stress, decreases in the concentration of 14:0, 16:0, 16:1, 18:1, 18:2 and 20:4 cholesterol esters were observed in animals fed rape seed oil, while animals fed olive oil showed decreases in 16:0, 18:1 and 18:2 esters. As reported by Carrol [2] we found a high accumulation of cholesterol erucate in the adrenals of rats fed rape seed oil for four weeks but little of this ester accumulated in rats fed olive oil or stock diet for the same period of time.

In our experiments ether stress, which increases plasma adrenocorticotropin concentrations [17], caused an increase in the activity of cholesterol ester hydrolase in both animals fed stock diet or olive oil but in animals fed rape seed oil no increase in activity was found, and in fact in two experiments a significant decrease in activity was observed. These results explain Carney and Walker's findings [10] that animals fed ethyl erucate show little decrease in cholesterol ester content of the adrenal when the animals are subjected to a cold stress and also explain the findings of Carrol and Noble [11] that large doses of adrenocorticotropic hormone failed to reduce the concentration of total adrenal cholesterol to low levels in animals fed rape seed oil.

Cofactor addition *in vitro* of cyclic AMP and ATP stimulated cholesterol ester hydrolase activity in stressed and unstressed animals fed stock diet or olive oil. In only one case was a significant ($P < 0.05$) stimulation of cholesterol ester hydrolase activity achieved *in vitro* in animals fed rape seed oil.

It has been inferred that activation of cholesterol ester hydrolase in the bovine adrenal cortex is achieved by phosphorylation of an inactive cholesterol ester hydrolase by a cyclic-AMP-dependent protein kinase in the presence of ATP [16] and it has been suggested that a similar system occurs in the rat [9]. Protein kinase activity in the rat adrenal $105000 \times g$ supernatant from animals fed stock or olive oil diet was elevated as reported previously [9] when animals were subjected to ether anaesthesia. Animals fed rape seed oil did not exhibit the usual stimulation of protein kinase activity on stress.

It has been reported [3] that ATP production in heart mitochondria isolated from rats fed rape seed oil is inhibited and there is an inverse linear relationship between the amount of erucic acid injected and ATP production. Swarttouw [27] has also shown that erucic acid is oxidised more slowly by rat heart mitochondria than palmitic and oleic acid. If it is true that ATP is necessary in the activation of cholesterol ester hydrolase, then a reason for the enzyme not being activated when the animal is subjected to an ether stress could be that ATP production from adrenal mitochondria is impaired and thus ATP levels are not sufficiently high to activate the enzyme. We found a significant stimulation of cholesterol ester hydrolase on addition of cyclic AMP and ATP in only one experiment, so it seems unlikely that the failure of cholesterol ester hydrolase to respond normally in rats fed rape seed oil is due to a lower concentration of ATP in the adrenals of these animals.

It is possible that feeding rape seed oil may impair the ability of the protein kinase to phosphorylate the cholesterol ester hydrolase or may even cause an inability of the pituitary to release adrenocorticotropin. Griesbach [28] showed that pronounced changes in pituitary cell structure occurred when rats were fed rape seed oil for seven days.

Free erucic acid as shown by our results does not exhibit any inhibitory properties on cholesterol ester hydrolase unlike oleic acid which strongly inhibits at μM concentrations. Erucic acid in fact activated cholesterol ester hydrolase *in vitro* and it is possibly this activating property of the free acid which resulted in the quiescent animals fed rape seed oil having a higher basal level of cholesterol ester hydrolase activity than animals fed stock diet. Electron microscopy showed no differences in the structure of the adrenals from animals fed rape seed oil or olive oil.

As our results have shown, cholesterol erucate is hydrolysed *in vitro* at only 25% of the rate of cholesterol oleate in animals fed rape seed oil and this together with the inability of cholesterol ester hydrolase to respond normally to a stress situation explains why cholesterol erucate accumulates in adrenals of animals fed rape seed oil.

Rats fed olive oil diet, which respond normally to a stressful situation to give an increase in cholesterol ester hydrolase activity, hydrolyse cholesterol erucate *in vitro* at only 30% of the rate of cholesterol oleate. It would seem therefore that in rats fed rape seed oil diet the initial cause of the accumulation of cholesterol erucate is an inability of the cholesterol ester hydrolase to hydrolyse the cholesterol erucate at a sufficient rate to stop its accumulation. On a prolonged diet of rape seed oil the adrenal cholesterol ester hydrolase system seems to lose its ability to respond to a stressful situation. No increase in cholesterol ester hydrolase activity can be observed *in vitro* and this would favour further accumulation of cholesterol erucate.

The activation mechanism of cholesterol ester hydrolase, and the inhibition of this activation system in animals fed a prolonged diet of rape seed oil is not fully understood and further experimentation is required to answer such questions.

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