THE ROLE OF CYCLIC-AMP IN THE REGULATION OF HEPATIC STEROID METABOLISM

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CONTENTS

1 INTRODUCTION .

SUMMARY

The hepatic monooxygenase system is responsible for the metabolism of xenobiotics and steroids (Kuntzman et al 1964) . In vivo studies in rats have shown that the activities of the constituent enzymes of the hepatic monooxygenase system are not constant but are under the control of the endocrine system and the hormones which have been found to influence enzyme activity are (a) testosterone (Yates et.al. 1958) , (b) oestrogen (Einarsson et al 1973) , (c) growth hormone (Wilson and Frohman 1974 ; Wilson and Spelsberg, 1976 ; Vockentanz and Virgo 1985) , (d) thyroxine (Kato and Gillette 1965a ; Kato et al 1970) , (e) insulin (Kato et al 1971 ; Weiner et al 1972a ; Reinke et al 1978 ; Past and Cook 1983) , (f) glucocorticosteroids (Kato and Gillette 1965a ; Kato et al 1971) , (g) glucagon (Weiner et al 1972a) and (h) catecholamines (Fouts 1962 ; Dixon et al 1964 ; Kato and Gillette 1965a) .

The intracellular mechanisms by which these hormones produce their effects , however , have been little studied and extrapolation from in vivo data is complicated by the fact that hormones appear to have different effects in male and female rats. One of the mechanisms by which hormones are known to produce their intracellular effects is by altering turnover of cyclic AMP and it has been reported that hormones which , when administered in vivo , increase intracellular cyclic AMP levels by a variety of mechanisms e.g. thyroxine (Müller and Seitz 1987) , adrenaline (Exton et al,1971; Studer and Borle 1984 ; Kunos et al 1984 ;

Kunos and Ishac 1987) tend to inhibit hepatic monooxygenase activity in the male rat but not in the female rat . Insulin , however , which decreases intracellular cyclic AMP levels by a variety of mechanisms (Heyworth et al 1985 ; O'Brien et al 1987) , stimulates hepatic monooxygenase activity when added to isolated rat hepatocytes (Hussin and Skett 1987) . In addition , in vivo administration of the cyclic AMP analogue , dibutyryl cyclic AMP , to male rats has also been reported to inhibit hepatic monooxygenase activity (Weiner et al 1972a ; Ross et al 1973) which provides more direct evidence for a role of cyclic AMP in hormonally-mediated inhibition of hepatic monooxygenase activity in the male rat . This effect , however , does not occur in the female rat .

It has recently been demonstrated that , in a reconstituted microsomal membrane system , the catalytic subunit of cyclic AMP-dependent protein kinase (PKa) can phosphorylate cytochrome P450 , the terminal oxidase in many hepatic monooxygenases (Pyerin et al 1983 , Pyerin et al 1986a ; Pyerin et al 1987) , resulting in its conversion to the inactive cytochrome P420 (Taniguchi et al 1985) with a concomitant inhibition of enzyme activity (Pyerin et al 1984) .

It is conceivable , therefore , that cyclic AMP , by activating PKa , phosphorylates cytochrome P450 and inhibits hepatic monooxygenase activity in the male rat but not in the female rat .

In contradiction to the above findings it has been reported that the induction of hepatic monooxygenases by Arochlor-1254

(Costa et al 1976) , phenobarbitone and 3-methylcholanthrene (Byus et al 1976) are accompanied by large increase in intracellular cyclic AMP levels and activation of PKa. PKa may be producing its effects by turning on gene expression (Teng et al 1971 ; Dokas and Kleinsmith 1971) .

The purpose of this present study was to attempt to elucidate the previously contradictory implications for a role of cyclic AMP in the regulation of hepatic monooxygenase activity and to examine the mechanisms by which cyclic AMP produces its effects .

The major problem of extrapolating from in vivo data is that the hormone may not necessarily be acting at the level of the liver but may be producing its effects by altering the synthesis or release of other hormones . Similarly the findings of in vitro studies may not be representative of the physiological situation . For the purpose of this study , therefore , we chose as our model the isolated rat hepatocyte which overcomes the major problems of in vivo and in vitro models . Cyclic AMP levels were elevated either indirectly using 8-bromo-cyclic AMP , adrenaline , isobutylmethylxanthine (IBMX) or forskolin in hepatocytes isolated from male or female (8-bromo-cyclic AMP only) rats , or directly by the addition of different concentrations of cyclic AMP to electropermeabilised hepatocytes isolated from male rats and subsequent effects upon the metabolism of the endogenous steroid , 4-androstene-3,17-dione , were determined after various periods of time .

We were able to show that elevation of intracellular cyclic AMP levels by both direct and indirect mechanisms had a time- and

concentration-dependent biphasic effect upon steroid-metabolising enzymes .

At low (physiological) concentrations of cyclic AMP produced indirectly by adrenaline , IBMX and the combination of adrenaline with IBMX , directly by 5 x 10^{-5}M cyclic AMP and mimicked by the cyclic AMP analogue 8-bromo-cyclic AMP (10^{-4}M) , non-specific inhibition of enzyme activity occurred in hepatocytes isolated from both male and female rats . The non-specific , non-sex dependent nature of the inhibition was thought to indicate that the in vivo hormonally-mediated maintenance of sex differentiation and refractoriness of female monooxygenases were due to complex interactions between components of the endocrine system and due to some hormonal influence which does not mediate intracellular effects by alteration of cyclic AMP levels .

At intermediate concentrations of cyclic AMP produced indirectly by forskolin and directly by 5 x 10^{-4}M cyclic AMP , the inhibitory effects upon enzyme activity were diminished and slight stimulation of enzyme activity occurred following inhibition .

At high (supraphysiological) concentrations of cyclic AMP produced indirectly by the combination of forskolin and IBMX and directly by 5 x 10^{-3}M cyclic AMP , only very slight inhibition of enzyme activity occurred and this was followed by marked stimulation of enzyme activity at the later time point .

Using cycloheximide we were able to show that the stimulation of enzyme activity was due to increase in protein synthesis and that recovery from enzyme inhibition at low concentrations of cyclic AMP was also due to weak stimulatory effects upon protein

synthesis . It was also apparent that at high concentrations of cyclic AMP marked inhibition occurred which was masked by the predominant stimulation and so it was concluded that inhibition and protein synthesis-dependent stimulation of enzyme activity occurred at all concentrations of cyclic AMP but the relative magnitudes of the effects and their changes with time resulted in apparent time- and concentration-dependent biphasic effects . Using the protein kinase inhibitors , KT5720 (specifically inhibits PKa) and K-252b (specifically inhibits protein kinase c (PKc)) (Kase et al 1986 ; Nakanishi et al 1986 ; Yasazawa et al 1986 ; Kase et al 1987) we were able to show that both inhibition and stimulation of enzyme activity were produced as a result of cyclic AMP activation of PKa . At low concentrations of cyclic AMP , however , PKa appeared to have no direct inhibitory effects upon enzyme activity but inhibition appeared to be mediated by secondary activation of PKc by PKa . At high concentrations of cyclic AMP , PKa did appear to have some direct inhibitory effects and the overall inhibition was due to a combination of the effects of PKa and PKc . Stimulation appeared to be due to a direct action of PKa at both low and high concentrations of cyclic AMP however at high concentrations of cyclic AMP the PKa-mediated stimulation of enzyme activity was too transient to account for the secondary stimulation of high concentrations of cyclic AMP and it was concluded that the PKa-mediated stimulation of enzyme activity may be prematurely terminated in the absence of PKc perhaps indicating that PKc activation is necessary for sustained stimulation to occur .

We also examined the effects of direct and indirect elevation of cyclic AMP upon whole cell cytochrome P450 levels and by using cycloheximide and K-252b to separate the components proposed to mediate the overall effects of cyclic AMP , we were able to show that the effects of low concentrations of cyclic AMP were not mediated by changes in the levels of whole cell cytochrome P450 levels . The overall effects and the constituent components of the effects of high concentrations of cyclic AMP , however , all reflected changes in whole cell cytochrome P450 content . The mechanisms by which high concentrations of cyclic AMP may produce these effects were not elucidated by this study .

To examine the possibility that the effects of physiological concentrations of cyclic AMP were due to reversible phosphorylation of cytochrome P450 , as has been implicated by "in vitro" studies (Pyerin et al 1987) , we examined the cyclic AMP-stimulated incorporation of (^{32}P) into cytochrome P450 isozymes in hepatocytes . We were unable to detect any incorporation of (^{32}P) into immunoprecipitated cytochrome P450 PB-2a (a constitutive isozyme which is male specific and thought to metabolise up to 90% of the hydroxylation of androstenedione at the 16α position (Waxman 1984)) , cytochrome P450 PB-3a (the major phenobarbitone-inducible isozyme) or cytochrome P450 MC-1b (the major 3-methylcholanthrene-inducible isozyme) following incubation with 5 x 10^{-5}M cyclic AMP and it is suggested that phosphorylation of cytochrome P450 may not be a physiological effect of cyclic AMP . The mechanism by which physiological concentrations of cyclic AMP mediate inhibition of enzyme activity

, therefore , has not been elucidated by this study .

From this study we have concluded that low concentrations of cyclic AMP mediate predominant inhibition of steroid-metabolising enzymes and these concentrations may correlate with those generated by hormones which have an inhibitory effect upon hepatic monooxygenase activity *in vivo* . High concentrations of cyclic AMP , however , mediate predominant stimulation of steroid-metabolising enzymes which we have shown to be due to induction of cytochrome P450 and the inductive effects of phenobarbitone , 3-methylcholanthrene and Arochlor-1254 *in vivo* may be mediated , at least in part , by elevation of cyclic AMP levels . It is proposed , therefore , that elevation of cyclic AMP may have a role in both hormonally-mediated inhibition of hepatic monooxygenases and in the actions of xenobiotics in hepatic monooxygenase induction .

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ABBREVIATIONS .

ACTH	: Adrenocorticotrophin
ALA	: Aminolaevulinic acid
5'-AMP	: Adenosine 5'-monophosphate
cyclic AMP	: Cyclic adenosine 3'5'-monophosphate
ATP	: Adenosine 5'-triphosphate
DAG	: Diacylglycerol
DNA	: Deoxyribonucleic acid
FSH	: Follicle stimulating hormone
GH	: Growth hormone
cyclic GMP	: Cyclic guanosine 3'5'-monophosphate
HO	: Haem oxygenase
IBMX	: Isobutylmethylxanthine
IP	: myo-Inositol 1-phosphate
IP_2	: myo-Inositol 1,4-bisphosphate
IP_3	: Inositol 1,4,5-trisphosphate
LH	: Luteinising hormone
NADH	: Reduced nicotinamide adenine dinucleotide
NADPH	: Reduced nicotinamide adenine dinucleotide phosphate
PA	: Phosphatidic acid
PI	: Phosphatidylinositol
PIP	: Phosphatidylinositol 4-phosphate
PIP_2	: Phosphatidylinositol 4,5-bisphosphate
PKa	: Cyclic AMP-dependent protein kinase
PKc	: Protein kinase c
PKg	: Cyclic GMP-dependent protein kinase
PMA	: 4β-Phorbol 12-myristate 13-acetate

PRL : Prolactin

RNA : Ribonucleic acid

TSH : Thyroid stimulating hormone

INTRODUCTION

1 INTRODUCTION

1.1 HISTORICAL BACKGROUND .

1.1(a) Discovery of hepatic metabolism .

It has been known for over a century that injested foreign compounds undergo a process of transformation or metabolism in the body prior to their excretion in the urine or faeces . The first of these biotransformations to be discovered was the formation of hippuric acid from injested benzoic acid which was subsequently excreted via the kidneys (Keller 1842) . In 1876 Bauman observed that the products excreted were less toxic than the injested compounds and twenty years later the proposal was put forward that the biotransformation process served as a chemical defence mechanism against toxicity (Lang 1894 ; Neumeister 1895). It is now accepted that metabolism and particularly hepatic metabolism , is essential for the excretion of lipid soluble compounds from the body (Brodie et al 1958) . The original work upon the body's biotransformation system was concerned with the detoxification of injested compounds , however it has subsequently been demonstrated that testosterone is also metabolised in the liver (Conney and Klutch 1963) and in 1964 Kuntzman et al postulated that the same enzymes were responsible for both endogenous steroid metabolism and the metabolism of exogenous compounds (xenobiotics) . It would appear , therefore , that the biotransformation of exogenous substrates occurs incidentally to the physiological role of these

enzymes .

1.1(b) Phase 1 and Phase 2 metabolism .

The process of metabolism occurs in two phases as classified by Williams (1959) . The first phase (phase 1) involves some alteration of the compound such as oxidation , reduction , hydration or hydrolysis whereas the second phase (phase 2) involves the combination of the transformed compound with a hydrophilic endogenous molecule such as sulphate , glucuronide or glutathione in a process known as conjugation . It is generally assumed that the transformation of the parent compound by phase 1 results in the production or the unmasking of a functional group which is essential for conjugation to occur (Caldwell 1982) and so the two processes must be thought of as two steps in a functionally single event .

The work undertaken and discussed in this thesis deals only with the mechanism of regulation of phase 1 metabolism . Reviews of phase 2 conjugation reactions , however , are listed in the reference section (Williams 1967 ; Caldwell 1982) .

1.1(c) Discovery of Hepatic Microsomal Metabolism .

Homogenisation and differential centrifugation of rat liver yields particles known as microsomes . These have been shown to be made up of small vesicles composed of pieces of the endoplasmic

reticulum (Porter et al 1945) and they retain the essential properties of the intact endoplasmic reticulum . In 1949 Mueller and Miller discovered that the microsomal fraction of rat liver could metabolise aminoazo dyes by reductive cleavage . In 1953 Mueller and Miller observed that the same dye could also be metabolised by N-demethylation by hepatic microsomes and that molecular oxygen , reduced nicotinamide adenine dinucleotide phosphate (NADPH) and reduced nicotinamide adenine dinucleotide (NADH) were required by the system for the reaction to occur at the optimum rate . This work was extended by Brodie and co workers who , working with rabbit liver microsomes , were able to show that the microsomal system was capable of metabolising a wide range of differing compounds (Brodie et al 1955) . In 1955 two independent groups showed that during oxidation of a compound one atom of molecular oxygen is transferred to the substrate and the other atom undergoes reduction to water (Mason 1957 ; Hayaishi 1964) . The oxidising enzymes were therefore given the name mixed function oxidases by one group (Mason 1957) and monooxygenases by the other group (Hayaishi 1964) . The proposal that the oxygen utilised in the metabolic biotransformation of the substrate was of molecular origin rather than derived from cellular water was later confirmed by Posner et al (1961) .

1.1(d) Discovery of cytochrome P450 .

In 1958 Klinkenberg discovered a pigment in rat hepatic microsomes which , when treated with carbon monoxide , absorbed light of an unusually long wavelength of 450 nm and postulated that the chromophore of the pigment contained a heavy metal ion as an integral component . In 1962 Omura and Sato showed that this carbon monoxide binding pigment was a cytochrome and in 1964 they presented evidence that the integral heavy metal was iron (Omura and Sato 1964a) . They subsequently named the pigment cytochrome P450 (pigment 450) . Although first discovered in hepatic microsomes , cytochrome P450 is also present in adrenocortical microsomes where it is involved in steroidogenesis (Hall 1987) and many of the first studies made to attempt to elucidate the function of cytochrome P450 were carried out upon adrenocortical , as opposed to hepatic , microsomes . In this manner a role for cytochrome P450 was implicated in the 21-hydroxylation of 17-hydroxyprogesterone in adrenocortical microsomes (Cooper et al 1963) and this was later followed by the recognition of a role for cytochrome P450 in the oxidation of acetanilide , benzo-a-pyrene , codeine and 3-methylaminoantipyrine by hepatic microsomes (Cooper et al 1965 ; Cooper et al 1977) .

1.2 CYTOCHROME P450 MONOOXYGENASES .

1.2(a) Structure of cytochrome P450 .

Cytochrome P450 is a unique haemoprotein which contains iron protoporphyrin lX (haem) as a prosthetic group (Maines and Anders 1973a) . The haem moiety is contained within a hydrophobic cleft in the polypeptide chain of the apoprotein and it differs from other haemoprotein (eg haemoglobin and myoglobin) in that the haem is not covalently bound to the protein (Hall 1987) but the iron of the prosthetic group is thought to be ligated at its fifth coordinate position to a sulphur atom contributed by a cysteine residue on the polypeptide chain of the cytochrome P450 apoprotein (Mason 1965 ; Waterman and Mason 1972) . Four of the six coordinate positions of the iron of the prosthetic group are ligated to pyrolle nitrogen but the nature of the sixth ligand has yet to be elucidated although both oxygen (in the form of water) (Griffin and Peterson 1975) and nitrogen (in the form of histidyl imidazole) (Sligar and Gunsalus 1979) have been implicated as the sixth ligand . The cytochrome P450 is associated with the smooth endoplasmic reticulum (Fouts 1962) and is anchored to the lipid bilayer by two NH_2-terminal transmembrane segments or signal peptides on the apoprotein which are strongly hydrophobic in nature (Szczesna-Skorupa et al 1988 ; Nelson and Strobel 1988) . The hydrophobic cleft containing the haem prosthetic group is on the membrane side of the cytochrome P450 with the haem positioned parallel to the membrane (Nelson

and Strobel 1988) .

1.2(b) Spectra .

In a similar manner to other haemoproteins , the haem readily combines with carbon monoxide in its reduced state and this complex has a unique absorption spectrum in that it absorbs at an unusually long wavelength of about 450nm (Omura and Sato 1964a). In addition the haem has a natural absorption spectrum which is produced in the absence of carbon monoxide . This is dependent upon the arrangement of the electrons in the d orbital of the iron. The d orbital electrons of the iron can exist in two configurations namely low spin (one unpaired electron) or high spin (five unpaired electrons) and at any given time a cytochrome P450 molecule exists in either the high or the low spin state . The overall spin state of the cytochrome P450 is dependent upon the sum of the effects of all of the individual cytochrome P450 molecules . Thus at no time does the cytochrome P450 exist in a state of 100% high spin configuration or 100% low spin configuration but the system exists in a state of dynamic equilibrium favouring a predominance of one state or the other . The equilibrium is determined by the conformation of the haem within the hydrophobic cleft of the protein and in the absence of a substrate the equilibrium favours a low spin conformation of electrons . This produces a major absorption peak (the Soret peak) at 420nm (Hall 1987) .

1.2(c) Function of cytochrome P450 .

Hepatic cytochrome P450 monooxygenase activity is restricted mainly , but not solely , to the parenchymal cells . Cytochrome P450-dependent activity has also been detected in the Kupffer cells and the reticuloendothelial cells but the ratios of the different enzyme activities may vary from those found in the parenchymal cells (La Franconi et al 1987) . The term monooxygenase indicates that it oxidises the substrate by utilising one atom of molecular oxygen and the second atom undergoes two equivalent reduction steps to produce water as a bi-product of the oxidation process (Mason 1957 ; Hayaishi 1964). During this process two electrons are donated to the reaction by two molecules of NADPH and the overall biotransformation is as follows :

$$RH + O_2 + 2H^+ + 2e^- \text{ ------- } ROH + H_2O$$

RH is the substrate and ROH is the oxidised product . The oxygen binds to the haem of the cytochrome P450 which is in its oxidised , ferric form and in oxidising the substrate the iron of the haem is reduced and is then reoxidised to complete the cycle.

1.2(d) NADPH - cytochrome P450 reductase .

The transfer of the two electrons from the NADPH to the cytochrome P450 does not occur directly but the electrons are passed first to an flavoprotein electron carrier and then from the carrier to the cytochrome P450 . This carrier is called NADPH -cytochrome P450 reductase and it contains two separate flavin moieties , namely : flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) (Iyanagi and Mason 1973) . The two flavin moieties appear to have separate functions in the transfer of electrons from NADPH to cytochrome P450 . FAD has been reported to act as the electron acceptor and , following the acceptance of electrons from NADPH , the electrons are passed to FMN and then transferred to the cytochrome P450 (Iyanagi et al 1981 ; Vermilion et al 1981 , Backes and Reker-Backes 1988) .

Analysis of the stoichiometry of the microsomal membrane has revealed that there are approximately 20-30 molecules of cytochrome P450 to every molecule of NADPH-cytochrome P450 reductase and the reductase , therefore , appears to act as an electron carrier for many molecules of cytochrome P450 . Two theories have been proposed for the organisation of these molecules in the endoplasmic reticulum . The "rigid arrangement" theory proposes that the reductase is surrounded by a cluster of cytochrome P450 molecules in a structurally organised formation (Peterson et al 1976) whereas the "fluid" model proposes that the reductase and the cytochrome P450 are both surrounded by a sheath of phospholipids and are able to move freely through the membrane

in a lateral manner (Yang et al 1977) . The criteria for both of these models have been proposed by Franklin and Estabrook (1971).

1.2(e) Sequence of events in cytochrome P450 dependent hydroxylation reactions .

Step 1 : Substrate binding .

Binding of the substrate occurs within the hydrophobic cleft which contains the haem prosthetic group (active site) (Schwarze et al 1988) . In 1967 , Schenkman et al divided substrates into three categories depending upon their mode of binding : type 1 ; type 2 and modified type 2 (Schenkman et al 1967a) . In all of these cases substrate binding occurs when the haem is in its oxidised , ferric state .

Type 1 substrates are thought to bind to the polypeptide chain of the apoprotein and the hydrophobic amino acids at positions 380-382 may have a role in this binding (Schwarze et al 1988) . This binding results in the displacement of the sixth ligand from the haem and a subsequent conformational change in the positioning of the haem within the hydrophobic cleft (White and Coon 1980) . This change alters the equilibrium position of the cytochrome P450 to favours transition to a predominantly high spin electron configuration and this results in a characteristic shift in the Soret peak from 420nm to 390nm and measurement of the spectral change shows a maximum at 390nm and a minimum at 420nm .

Type 2 substrates bind directly to the iron of the haem at the

sixth coordinate position (Schenkman et al 1967a) . This alters the equilibrium of the cytochrome P450 to favour a low spin electron configuration and measurement of the spectral change produced by the binding of a type 2 substrate produces a spectrum which is almost the mirror image of that produced by the binding of type 1 substrates i.e. maximum at 430nm and minimum at 395nm .

It is thought that modified type 2 (reverse type 1) substrates act by displacing some unknown endogenous compound which is bound to the cytochrome P450 at the type 1 substrate binding site or that they bind at the sixth coordinate position (Peterson et al 1971) . The spectral change which occurs with the binding of modified type 2 substrates is the exact reverse of that produced by the binding of type 1 substrates (hence reverse type 1) i.e. maximum at 420nm and minimum at 390nm .

Type 1 substrates (e.g. hexobarbitone and aminopyrine) and type 2 substrates (e.g. aniline) have been widely used in studies to determine the regulatory control of cytochrome P450 . The natural steroids are classified as type 1 substrates and so type 1 substrates can be considered to act in a more physiological manner than type 2 substrates .

Step 2 : Transfer of the first electron .

The first electron is passed from the reductase to the cytochrome P450 and is ultimately taken up by the iron which is reduced from the ferric to the ferrous form . This step is

accelerated by the prior binding of a type 1 substrate in the active site . The binding of a type 2 substrate , however , slows the rate of reduction by NADPH and may indicate that the oxidised cytochrome P450/type 2 substrate complex is reduced more slowly than the oxidised cytochrome P450 alone or that the transfer of the first electron occurs prior to the binding of a type 2 substrate (Gigon et al 1969) .

Step 3 : Binding of oxygen .

Following the reduction of iron to its ferrous form , molecular oxygen binds to the sixth coordinate position of the haem . This binding is promoted by the displacement of the sixth coordinate ligand by the binding of the substrate in step 1 . A threonine residue at position 302 on the apoprotein polypeptide chain may also be involved in stabilising the haem/oxygen complex (Wolf et al 1988) .

Step 4 : Insertion of the second electron and oxygen "activation" .

The second electron activates the oxygen to form a reactive species and internal rearrangement of the electrons re-oxidises the iron to its ferric form . Much work has been carried out to attempt to elucidate the nature of the reactive species of oxygen

and the mechanisms of activation but the process is still poorly understood . The reactive species of oxygen is assumed to be an oxygen anion and is equivalent to a ferric ion/peroxide ion complex however the actual electron distribution and protonation states are unknown and the identity of the reactive species is the subject of much debate as reviewed by White and Coon (1980) .

Subsequent steps in the cycle are poorly understood . The following sequence is a brief summary of the events which are thought to occur but the precise mechanism by which they occur is not presently known . More detailed discussion of the present theories concerning mechanisms are contained in a review by White and Coon (1980) .

Step 5 : Oxygen cleavage .

The activated oxygen cleaves and one atom combines with two hydrogen ions to form water whilst the second atom remains attached to the iron .

Step 6 : Oxygen insertion .

Hydrogen is removed from a carbon atom on the substrate . The hydrogen combines with the oxygen to form a hydroxyl radical and the loss of the hydrogen leaves a carbon radical on the

substrate. The two radicals recombine and the oxygen is thus transferred from the haem to the substrate to form a hydroxylated product .

Step 7 : Dissociation of the product .

The hydroxylated product dissociates from the active site and the haem in its ferric form is now ready to repeat the cycle with new substrate .

The whole cycle is summarised diagrammatically in figure 1

1.2(f) Other phase 1 biotransformations mediated by the cytochrome P450 monooxygenase system .

In the scheme shown above the substrate is metabolised to yield a hydroxylated product . In addition to hydroxylation cytochrome P450 has been shown to be involved in a wide range of other types of oxidative metabolism e.g. deamination , dealkylation , oxidation and sulphoxidation . Although the resultant products differ markedly in their substitutions most of the reactions proceed via intermediate hydroxylation steps which are then broken down to yield the product . The mechanisms of these reactions , therefore , proceed , at least initially , by the same pathway as that shown in figure 1 (Brodie et al 1958) .

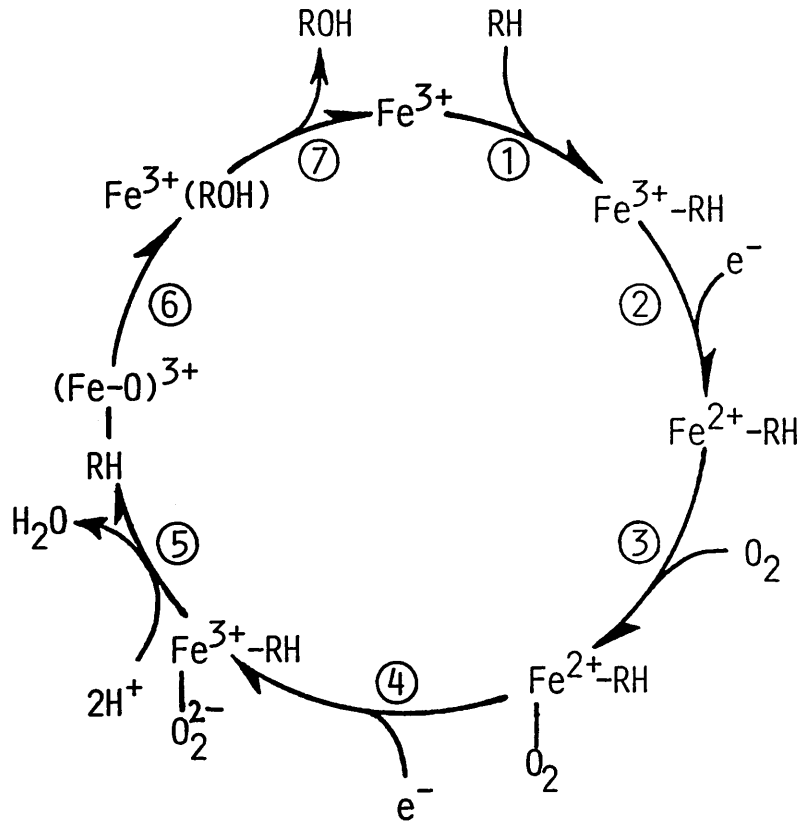

Figure 1 : Catalytic cycle of cytochrome P450 .

Two exceptions are epoxidation and N-oxidation which do not proceed via an intermediate hydroxylation step . Oxygen insertion occurs directly onto the nitrogen in the case of N-oxidation or across two carbon atoms in the case of epoxidation .

1.3 ADDITIONAL COMPONENTS OF THE CYTOCHROME P450 MONOOXYGENASE SYSTEM .

1.3(a) Lipids .

Lipids were first shown to be essential for the action of the cytochrome P450 monooxygenase system in 1968 when Lu and Coon demonstrated that a heat stable , lipid component was required for the optimal hydroxylation of fatty acids . In 1969 Lu et al extended this work to show that the same lipid component was required for the oxidative metabolism of both drugs and steroids. The lipid factor was postulated to be phosphatidylcholine by Strobel et al (1970) and Vore et al (1974) found that organic extraction of lypophilised rat microsomes resulted in 75% removal of phospholipids from the membrane and 10-25% loss of cytochrome P450 and NADPH-cytochrome P450 reductase . The enzymatic activity , however , was reduced to a much greater extent (30-50% of control) than could be explained by loss of the enzymatic components alone and the activity was fully restored by the readdition of both total lipid

extract and purified phosphatidylcholine . In similar manner , treatment of microsomes with phospholipase C resulted in 70% loss of total phospholipids and a substantial decrease in the metabolism of type 1 substrates (Chaplin and Mannering 1970 ; Eling and Di Augustine 1971) . However , in contrast to the study made by Vore et al (1974) , these workers found that full activity of the enzymes could not be restored by phosphatidylcholine alone . The exact nature of the lipid component , therefore , is unclear but it would appear that phosphatidylcholine is an essential component.

The lipid factor is thought to be essential for the transfer of electrons between NADPH and cytochrome P450 (Strobel et al 1970) and may facilitate the binding of type 1 substrates to cytochrome P450 although it appears to have no effect upon the binding of type 2 substrates (Chaplin and Mannering 1970 ; Eling and Di Augustine 1971) . The precise mechanism of its actions are unknown .

1.3(b) Cytochrome b_5 .

In the scheme outlined for cytochrome P450 catalysed oxidation, NADPH is assumed to be the source of both the first and the second electrons . The addition of NADH to an oxidising system, however , has a synergistic effect with NADPH (Conney et al 1957). Mueller and Miller (1953) found that both NADPH and NADH were required for the optimal metabolism of aminoazo dyes . The microsomal 9-desaturation of fatty acids is also dependent

upon either NADPH or NADH as an electron donor (Holloway et al 1963) and in a parallel system to cytochrome P450 , the desaturation of fatty acids is also a cytochrome-dependent process. The cytochrome involved is cytochrome b_5 and electrons are transferred from NADH to the cytochrome via NADH-cytochrome b_5 reductase (Strittmatter and Velick 1956) and in 1971 Hildebrandt and Estabrook attempted to explain the phenomenon of NADH stimulation of cytochrome P450 monooxygenase activity and postulated that cytochrome b_5 is a component of the hepatic cytochrome P450 monooxygenase system . Cytochrome b_5 is thought to be involved in the transfer of the second electron from NADH to cytochrome P450 . NADPH via NADPH-cytochrome P450 reductase can also supply the second electron but NADH is the preferential donor (Imai and Sato 1977) . It is generally accepted that NADH is unable to act as donor for the first electron since NADH alone cannot support drug oxidations (Correia and Mannering 1973) . Under certain circumstances , however , cytochrome b_5 may substitute for NADPH-cytochrome-P450 reductase in the transfer of electrons from NADPH (Vatsis et al 1980) and may participate in the transfer of the first electron from this donor (Golly et al 1988) . Similarly NADPH-cytochrome P450 reductase can participate in NADH supported monooxygenations in place of NADH-cytochrome b_5 reductase at high concentrations of NADH (Noshiro et al 1980) .

1.4 MULTIPLICITY OF CYTOCHROME P450 .

1.4(a) Discovery of multiple forms of cytochrome P450 .

The existence of multiple forms of cytochrome P450 in the liver has been recognised for over 20 years following the work of Conney et al (1959) who noted that benzo-a-pyrene administration increased the metabolism of benzo-a-pyrene and zoxazolamine , decreased the metabolism of pethidine and benadryl and had no effect upon the metabolism of chlorpromazine . In a similar manner, treatment of rats with either of the two classic inducing agents : phenobarbitone or 3-methylcholanthrene (section 1.9(a)) resulted in the induction of enzymes with different substrate specificities and reduced carbon monoxide absorption spectra (Alvares et al 1967 ; Kuntzman 1969 ; Sladek and Mannering 1969). In addition to studies examining the selective induction of cytochrome P450 by phenobarbitone and 3-methylcholanthrene and the properties of the enzymes induced by these agents , multiplicity has also been postulated on the grounds of different rates of turnover . Examination of the rates of haem synthesis has lead to the hypothesis that two forms of cytochrome P450 exist ; one with a fast rate of turnover and the other with a slow rate of turnover (Levin et al 1974) and studies using allylisopropylacetamide and furoxeme have suggested that the different cytochrome P450 isozymes are synthesised at different rates (Bradshaw et al 1978).

1.4(b) Purification of different cytochrome P450 isozymes .

The isolation of different forms of cytochrome P450 from phenobarbitone or 3-methylcholanthrene treated rats was first achieved in 1973 when Comai and Gaylor physically separated cytochrome P450 isozymes from phenobarbitone- , 3-methyl-cholanthrene-and ethanol-treated rats and showed that they differed in their cyanide binding ability . Simultaneously Fugita et al (1973) partially purified cytochrome P450 isozymes from phenobarbitone-and 3-methylcholanthrene-treated rats and showed that they differed in substrate specificity . Since this time a multitude of cytochrome P450 isozymes have been purified from induced and non-induced animals and these are listed in table 1 .

Evidence for distinct forms of cytochrome P450 has been presented based on the different properties of these purified isozymes :

(a) The isozymes have different mobilities on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) .

(b) The isozymes have different but overlapping substrate specificities .

(c) The isozymes can be separated by immunochemical techniques employing specific antibodies however cross reactivity

Levin	Waxman	Guengerich	Schenkman	Wolf	Comments
a	3	UT-F	-	UT-1	Steroid 7α-hydroxylase
b	PB-4	PB-B	PBRLM5	PB-3a	Major phenobarbitone-inducible isozyme .
c	BNF-B	BNF-B	-	MC-1b	Major 3-methylcholanthrene-inducible isozyme .
d	ISF-G	ISF-G	-	MC-1a	Major isosafrole-inducible isozyme also induced by methylcholanthrene .
e	PB-5	PB-D	PBRLM6	PB-3b	Phenobarbitone-inducible .
f	-	-	-	-	Constitutive isozyme , crossreactive with b
g	-	-	RLM3	-	Constitutive isozyme . Male-specific , strain-dependent .
h	2c	UT-A	RLM5	PB-2a	Male-specific isozyme. Steroid 16α-hydroxylase .
i	2d	UT-1	FRLM4	-	Female isozyme.Steroid sulphate 15β-hydroxylase .
j	-	-	RLM6	-	Ethanol-inducible .
k	PB-1	PB-C	RLM5a	PB-1b	Phenobarbitone-inducible . Hormone-independent .
p	PB-2a	PCN-E	-	-	Major Pregnenolone-16α-carbonitrile-inducible isozyme also induced by dexamethasone and triacetyloleandomycin .
-	2a	-	-	-	Male-specific , steroid 6β-hydroxylase

Table 1 . Nomenclature of different rat hepatic cytochrome P450 isozymes characterised by different groups (adapted from Waxman 1988) .

between different isozymes is common due to the conservation of many amino acid sequences in their structures .

(d) The isozymes can be distinguished from one another by peptide mapping and analysis of the amino acid sequences of the apoprotein .

1.5 METABOLISM OF STEROIDS .

Steroids are metabolised by a complex system of enzymes both cytochrome P450-dependent and cytochrome P450-independent . The androgenic steroid : 4-androstene-3,17-dione will be used to illustrate the complexity of the system and is the substrate which we chose to represent the steroids in examining the regulation of steroid-metabolising enzymes throughout this project.

The metabolism of androstenedione is shown in figure 2 . The parent molecule is hydroxylated at three positions : 16α , 6β and 7α by cytochrome P450-containing monooxygenases .

Purification of the male specific cytochrome P450 (P450 2c : see table 1) has shown that this cytochrome P450 isozyme catalyses up to 90% of the metabolism of androstenedione at the 16α position (Waxman 1984) and similarly hydroxylates testosterone at the 16α position to the same degree as assessed by purification studies (Morgan et al 1985a) and by assessing inhibition by specific monoclonal antibodies (Waxman et al 1987). It also hydroxylates testosterone at the 2α position (Waxman 1984

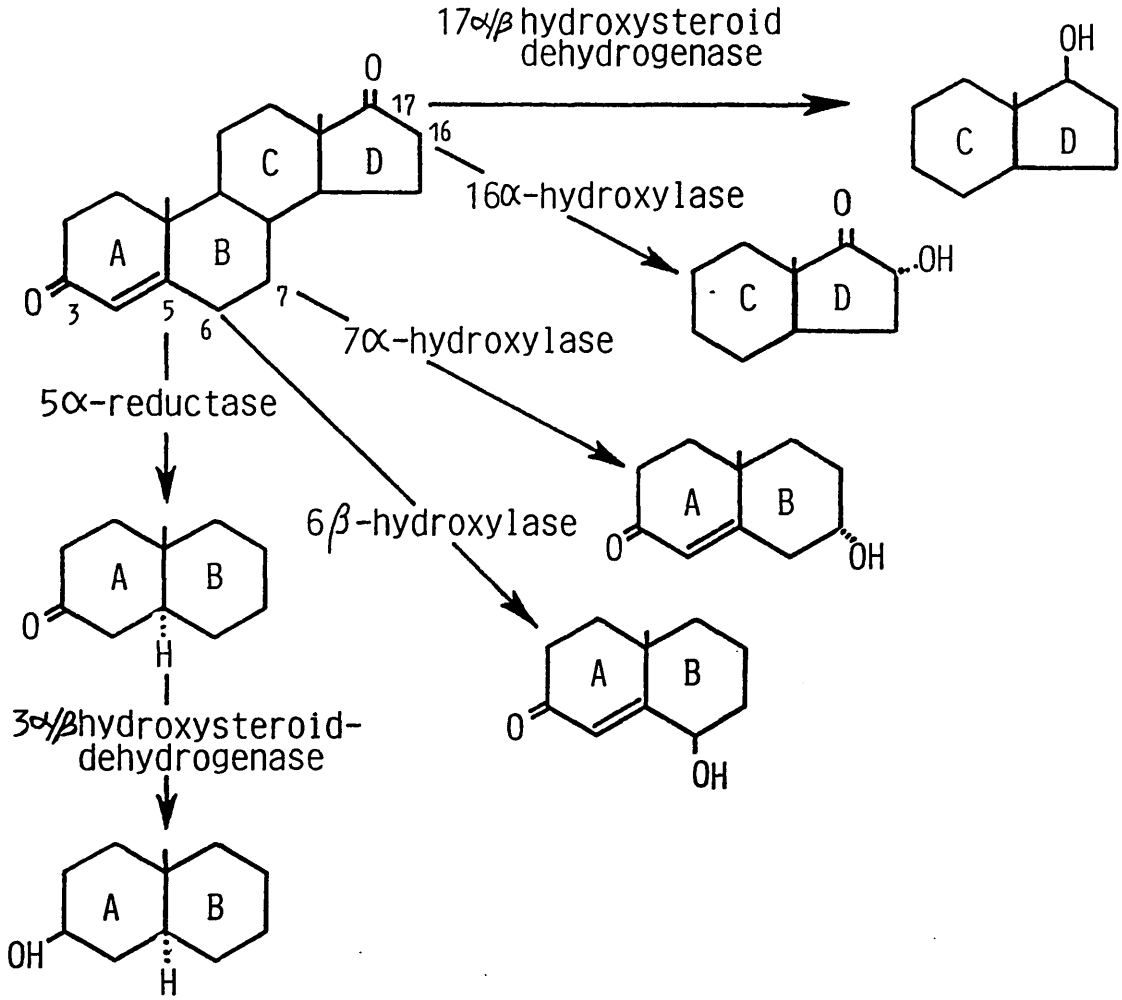

Figure 2 : Hepatic metabolism of 4-androstene-3,17-dione .

; Waxman et al 1983) but hydroxylation of androstenedione does not occur at this position .

Up to 85% of the hydroxylation of androstenedione at the 6β position is thought to be catalysed by cytochrome P450 2a (table 1) (Waxman et al 1985) although the cytochrome P450 isozyme is deactivated during the course of microsomal solubilisation and so direct assessment of the activity of the purified enzyme has not , so far , been possible .

Hydroxylation of androstenedione at the 7α position has been reported to be catalysed almost exclusively by cytochrome P450 3 (table 1) (Waxman et al 1987 ; Nagata et al 1986) .

It appears , therefore , that in the uninduced liver the hydroxylation of androstenedione is catalysed by three separate and specific cytochrome P450 isozymes .

5α reductase is also a membrane-bound microsomal enzyme and although it does not contain cytochrome P450 as an integral component , reconstitution studies have shown that it exists as part of a multi-component system which shows many similarities to the cytochrome P450 monooxygenase system . The electron transport chain is composed of three enzymes : steroid 5α reductase , coenzyme Q_{10} and NADPH-cytochrome oxidoreductase . Coenzyme Q_{10} is a cytochrome and electrons are passed from NADPH to coenzyme Q_{10} by NADPH-cytochrome oxidoreductase and then from coenzyme Q_{10} to the steroid substrate by steroid 5α reductase where they are utilised in the reduction of the double bond (Golf and Graef 1978). The system also contains phosphatidylcholine as an integral component in an analogous manner to cytochrome P450 monooxygenases

and it may perform similar functions (section 1.3(a)) .

17α/β and 3α/β hydroxysteroid dehydrogenases are also microsomal enzymes although details of their mode of action have been poorly documented . 3α hydroxysteroid dehydrogenase , however , has been the subject of a recent review by Penning et al (1987).

1.6 EXTRAHEPATIC FORMS OF CYTOCHROME P450 .

The best documented site of extrahepatic cytochrome P450 monooxygenases is the adrenal gland where the enzymes are involved in the biosynthesis of steroid hormones from cholesterol. These enzymes have been reviewed in detail by Hall (1987) and four distinct forms of cytochrome P450 have been purified and characterised (Waterman and Simpson 1985) . Cytochrome $P450_{scc}$ (cleaves the side chain of cholesterol to produce pregneneolone) and cytochrome $P450_{11\beta}$ (11β hydroxylation of 11-deoxycortisol to produce cortisol) are mitochondrial enzymes and cytochrome $P450_{17\alpha}$ (17α hydroxylation of progesterone) and cytochrome $P450_{C21}$ (21 hydroxylation of 17α hydroxyprogesterone to produce 11-deoxycortisol) are microsomal enzymes . The mitochondrial electron transport system differs from that of the liver and adrenal microsomal systems in that it utilises two carrier proteins in the transport of electrons from NADPH to the cytochrome P450 . The first carrier is a flavoprotein , NADPH-adrenodoxin reductase , and this then passes the electrons to an iron-sulphur protein , adrenodoxin , and then the electrons

are passed from adrenodoxin to the cytochrome P450 .

Extrahepatic steroidogenic forms of cytochrome P450 also exist in the testes and in the ovaries . In addition to cytochrome $P450_{scc}$ and cytochrome $P450_{17\alpha}$, the testes also contain cytochrome $P450_{C17-C20lyase}$ which catalyse the synthesis of androstenedione from 17α hydroxyprogesterone and the ovaries contain both cytochrome $P450_{C17-C20lyase}$ and cytochrome $P450_{aromatase}$ which catalyses the synthesis of oestradiol from androstenedione .

In addition to the liver and the steroidogenic organs , cytochrome P450 monooxygenases have also been reported in many other organs including the kidneys (Orrenius et al 1973) , the lungs (Bend et al 1973) and the brain (Näslund et al 1987 ; Sugita et al 1987) .

1.7 SYNTHESIS OF CYTOCHROME P450 .

Due to the fact that cytochrome P450 consists of two components i.e. the apoprotein and the haem prosthetic group , the synthesis of the holoenzyme is a complex process .

The haem biosynthetic pathway , which is probably identical in all cells , is initiated by the condensation of glycine and succinyl coenzyme A in the presence of δ-aminolaevulinic acid synthase (ALA-synthase) and pyridoxyl phosphate to form aminolaevulinic acid (ALA) (Granick and Urata 1963) . ALA-synthase is the rate limiting enzyme in the synthesis of haem

and is located on the matrix of the mitochondria (McKay et al 1969) . The ALA then passes out of the mitochondria into the cytosol and is converted to phosphobilinogen in the presence of ALA-dehydratase . Phosphobilinogen is then converted to protoporphyrin IX by a series of uncharacterised enzyme steps and then the protoporphyrin IX passes back into the mitochondria and is converted to haem (iron protoporphyrin IX) by chelation with iron in the presence of protohaem ferrolyase (ferrocheletase) .

The apoprotein of the cytochrome P450 confers isozyme specificity and current evidence suggests that the genes encoding the amino acid sequences form a superfamily consisting of at least five gene families as classified by their susceptibility to different classes of inducing agent . The apoprotein is synthesised from the specific genetic code for the isozyme and then the haem is incorporated to yield the holoenzyme . The incorporation of haem into the apoprotein occurs outside the mitochondria , however the site of haemoprotein production and the mechanism by which the haem passes out into the cytosolic haem pool are unknown .

1.8 DEGRADATION OF CYTOCHROME P450 .

1.8(a) Cytochrome P420 .

When microsomes are treated with a variety of solubilising agents e.g. lipases , proteases and detergents , cytochrome P450 is converted to an inactive form and this is accompanied by a

characteristic shift in the reduced carbon monoxide absorption peak from 450nm to 420nm . The absorption peak has led to it being named cytochrome P420 (pigment-420) (Omura and Sato 1964a) . In 1978 Dawson et al , in an attempt to elucidate the nature of the fifth coordinate haem ligand in cytochrome P450 , concluded that thiolate was the likely fifth ligand in cytochrome P450 but that this ligand was absent in cytochrome P420 . Thus cytochrome P420 may be formed from cytochrome P450 by loss or disruption of the fifth coordinate thiolate (sulphur) linkage between the haem and the apoprotein . The conversion of cytochrome P450 to cytochrome P420 is generally assumed to be a non-physiological transformation , however Taniguchi et al (1985) have postulated that this may be the underlying mechanism in the inhibition of monooxygenase activity mediated by the phosphorylation of cytochrome P450 and so may represent a physiological mechanism of degradation .

1.8(b) Haem oxygenase .

Haem oxygenase (HO) is a mixed function oxidase localised in microsomal membranes and it consists of two immunologically distinguishable isozymes : HO-1 and HO-2 (Maines et al 1986) . Both forms required NADPH , NADPH-cytochrome P450 reductase and molecular oxygen in an analogous manner to cytochrome P450 but the two isozymes have different induction profiles in that only the HO-1 isozyme is inducible . Both isozymes cleave the iron

protoporphyrin IX (haem) ring at the α meso bridge to form a linear α isomer of biliverdin (a tetrapyrolle) (Tenhunen et al 1969 ; Maines et al 1986) . Biliverdin is then converted to bilirubin by reduction of the methane bridge of biliverdin by a cytosolic enzyme known as biliverdin reductase in the presence of NADH or NADPH to supply reducing equivalents (Ellis-Bell and Maines 1988) . Of these two steps the cleavage of the iron protoporphyrin IX ring by haem oxygenase is rate limiting in the degradation of haem to bilirubin (Tenhunen et al 1969) . One argument against a role of haem oxygenase-catalysed conversion of haem to bilirubin as a major route of cytochrome P450 degradation in the liver is that haem oxygenase is localised in the hepatic reticuloendothelial cells (where it is responsible for the breakdown of haemoglobin derived haem) and very little exists in the hepatic parenchymal cells (Guengerich 1977) . Maines and Anders (1973b) , however , proposed that the cytochrome holoenzyme is transported to the haem oxygenase by a transfer protein and both albumin and hemopexin (the two known plasma haem binding proteins) may perform this function . They also found , however , that only cytochrome P420 and not cytochrome P450 could bind to these transfer proteins . Similarly , Kutty et al (1988) found that a major phenobarbitone-inducible cytochrome P450 isozyme , cytochrome P450c was not susceptible to haem oxygenase-mediated degradation unless first converted to the analogous cytochrome P420 . This may indicate that the importance of haem oxygenase as a major route of cytochrome P450 degradation is dependent upon prior conversion of the cytochrome P450 to the

analogous cytochrome P420 and may provide further implications for the physiological significance of this latter conversion .

1.8(c) Lipid peroxidation .

In the presence of NADPH and molecular oxygen , NADPH-cytochrome P450 reductase can degrade the haem of cytochrome P450 , cytochrome P420 and cytochrome b_5 (Guengerich 1977) . This has been shown to be due to the production of hydrogen peroxide or another oxygen derived species at the site of the haem which results in the production of lipid peroxides (Schacter et al 1973) and the process overcomes two points which argue against haem oxygenase as a physiological mechanism of haem breakdown , namely (a) cytochrome P450 can be degraded without prior conversion to cytochrome P420 and (b) the cytochrome P450 is degraded at the site of the parenchymal cell endoplasmic reticulum and does not require transportation to the reticuloendothelial cells .

The major route of haem degradation in the untreated liver is unresolved and arguments have been offered for and against both haem oxygenase-mediated and lipid peroxide-mediated degradation . Both systems appear to have physiological significance and the ultimate effect is loss of haem which may increase the susceptibility of the apoprotein to proteolytic breakdown (Abraham

et al 1988) .

1.9 XENOBIOTIC-MEDIATED INDUCTION OF HEPATIC MONOOXYGENASES .

1.9(a) Inducing agents .

The ability of phenobarbitone to stimulate the metabolism of itself and other drugs has been known for many years and is a dose-dependent effect (Tavoloni et al 1983) . One of the first studies upon the mechanism of phenobarbitone's actions was conducted in 1965 by Ernster and Orrenius and it was reported that pretreatment with phenobarbitone increased drug metabolism and this was accompanied by concomitant induction of both cytochrome P450 and NADPH-cytochrome P450 reductase . In 1975 Hutterer et al reported that , in addition to induction of cytochrome P450 , phenobarbitone also increased the size of the liver , the total microsomal protein content and the phospholipid content of the liver and the effects of phenobarbitone , therefore , are not confined to the cytochrome P450 monooxygenases but it has a general proliferative effect upon the liver . Despite this general proliferative effect phenobarbitone has a selective induction effect upon some cytochrome P450 isozymes , minimal effect upon other isozymes and has also been reported to decrease the microsomal content of some isozymes (Waxman et al 1985 ; Ioannides and Parke 1987) . Other inducing agents have selective isozyme-induction profiles differing from that of phenobarbitone and it is now recognised that three major classes of inducing

agents exist and each mediates a characteristic induction profile.

The three major groups are (i) phenobarbitone-like ; (ii) polycyclic aromatic hydrocarbons and (iii) synthetic steroids .

(i) Phenobarbitone-like .

This group includes , in addition to phenobarbitone , chlordane ; SKF-525A ; trans stilbene oxide ; glutethimide and mephenytoin . All of these agents induce two major isozymes termed cytochromes P450b and P450e (table 1) of which cytochrome P450b is the major phenobarbitone-inducible isozyme . Phenobarbitone-like inducers also induce cytochrome P450a (table 1) which is responsible for steroid hormone 7α-hydroxylase activity and cytochrome P450-PCN which is the major synthetic steroid-inducible isozyme (Ioannides and Parke 1987) . It has also been reported to decrease the levels of the male-specific isozyme (termed P450 h , P450 2c , P450 UT-A and P450 RLM5 by differing groups : see table 1) (Waxman et al 1985) but the overall hydroxylation of testosterone is increased (Kuntzman et al 1966) and this effect may be augmented by the ability of cytochrome P450b to compensate for the loss of testosterone-16α-hydroxylase activity attributable to decrease in the levels of the male-specific cytochrome P450 isozyme , by hydroxylating the steroid at the 16β position (Waxman et al 1983) .

(ii) Polycyclic aromatic hydrocarbons .

This group is usually represented by the prototype hydrocarbon : 3-methylcholanthrene but the group also includes β-naphthoflavone , benzo-a-pyrene and 2,3,7,8-tetrachlorodibenzo -p-dioxin (TCDD) . These agents induce two major cytochrome P450 isozymes termed cytochrome P450c and cytochrome P450d (table 1) and these isozymes are also collectively known as cytochrome P448 due to the fact that the reduced CO-absorption spectrum peak occurs at the slightly shorter wavelength of 448nm (Ioannides and Parke 1987) . The major isozyme inducible by the polycyclic aromatic hydrocarbons is cytochrome P450c . In a similar manner to the phenobarbitone-like inducers , the polycyclic aromatic hydrocarbons also increase the levels of cytochrome P450a but to a lesser extent than cytochromes P450c and P450d (Yeowell et al 1987a) and decrease the levels of the male-specific cytochrome P450 isozyme (Yeowell et al 1987b) . They also suppress the levels of cytochrome P450b and the other phenobarbitone-specific isozymes in both untreated and phenobarbitone-treated rats (Dannan et al 1983 ; Yeowell et al 1985) .

Isosafrole also induces cytochromes P450c and P450d in a similar manner to the polycyclic aromatic hydrocarbons but unlike these agents , cytochrome P450d is the major isosafrole-inducible isozyme and cytochrome P450c is induced to a lesser extent . Isosafrole also induces cytochrome P450a to a limited extent and

unlike the polycyclic aromatic hydrocarbons , cytochrome P450b is also induced by isosafrole but to a lesser extent than its induction by phenobarbitone (Levin et al 1980) . Isosafrole , therefore , appears to have a similar induction profile to the polycyclic aromatic hydrocarbons but shows characteristic differences and it has been suggested that isosafrole is a unique inducer of microsomal monooxygenases (Dickens and Bridges 1978). It also differs from the other classes of inducing agents in that isosafrole-induced cytochrome P450d but not cytochromes P450a , P450b or P450c , is purified as an isosafrole metabolite /cytochrome P450 complex with the metabolite bound to the haem of the cytochrome (Levin et al 1980) .

(iii) Synthetic steroids .

The prototype of this group is pregneneolone-16α-carbonitrile (PCN) but dexamethasone is also thought to induce monooxygenases in an identical manner . PCN induces a cytochrome P450 isozyme which is distinct from those induced by either phenobarbitone or 3-methylcholanthrene and which is called cytochrome P450-PCN (Elshourbagy and Guzelian 1980) . This isozyme can also be selectively induced by the macrolide antibiotic : triacetyloleandomycin and has , therefore , also been called cytochrome P450-TAO (Wrighton et al 1985) . Triacetyloleandomycin also shows similarities to isosafrole in

that its metabolite binds to the cytochrome P450 and forms a reversible complex (Pessayre et al 1981) .

It has been proposed that inducing agents tend to induce the cytochrome P450 isozymes which metabolise them and induction , therefore , represents a form of homeostasis . The characteristics of phenobarbitone-inducible and 3-methylcholanthrene-inducible isozymes have been reviewed by Ioannides and Parke (1987) . The phenobarbitone-inducible isozymes tend to be induced by non planar, bulky molecules and substrates are similarly non planar, globular molecules . Endogenous steroids , therefore , are good substrates for the phenobarbitone-inducible isozymes . The oxidations catalysed by these cytochrome P450 isozymes produce good substrates for conjugation and these isozymes tend to be located in the centrilobular region of the liver which is also the region in which the conjugating enzymes are most prevalent . 3-methylcholanthrene-inducible isozymes , however , are induced by rigid , planar molecules and their substrates similarly tend to be rigid and planar . Endogenous steroids , therefore , are poor substrates for the 3-methylcholanthrene-inducible isozymes . The oxidised products are poor substrates for conjugation and these isozymes are found in the periportal region of the liver which is low in conjugating enzymes . Much of the work upon the structure/activity relationships of the phenobarbitone-like and 3-methylcholanthrene-like inducers has utilised Arochlor-1254 which is a mixture of polychlorinated biphenyls and is a mixed phenobarbitone-like and 3-methylcholanthrene-like inducer . The

ability to induce phenobarbitone-inducible or 3-methyl-cholanthrene-inducible isozymes has been found to be dependent upon the degree of substitution of the molecule and specifically at the position ortho to the biphenyl bridge . As the degree of substitution increases the molecule becomes more sterically hindered and therefore less planar and so the ability of the molecule to act as a 3-methylcholanthrene-like inducer decreases with a concomitant increase in its ability to act as a phenobarbitone-like inducer (Dannan et al 1983 ; Parkinson et al 1983) .

1.9(b) Induction of apoprotein synthesis .

The mechanism of action of the inducing agents is , as yet , unknown and is an area of intensive research .

The major phenobarbitone- and 3-methylcholanthrene-inducible isozymes are induced in hepatocytes in a similar manner to the whole animal (Daujat et al 1987 ; Hishinuma et al 1987) and so the effect of inducing agents upon specific isozymes appears to occur at the level of the liver cells .

The polycyclic aromatic hydrocarbons bind to a cytosolic receptor , an effect which may involve sulphydryl groups on the receptor , and binding of the ligand has been proposed to produce some conformational change in the receptor which results in its activation (Henry et al 1988) . The inducer/receptor complex then translocates into the nucleus where it associates with a

regulatory gene as a complex with the inducer molecule (Fujisawa-Sehara et al 1987) . This leads to an increase in gene transduction and a subsequent increase in cytochrome P450 messenger RNAs and , in cells which do not accumulate the inducer/receptor complex in the nucleus , these effects do not occur (Israel and Whitlock 1984) .

No equivalent receptor has been discovered for phenobarbitone-like inducers and the mechanism of action of these agents is unknown . They appear , however , to increase gene transcription in a similar manner to the polycyclic aromatic hydrocarbons (Pike et al 1985 ; Shephard et al 1987) . Iversen et al (1987) , however , reported that phenobarbitone and 3-methylcholanthrene synergistically superinduced the messenger RNAs for phenobarbitone-inducible isozymes but this superinduction was not accompanied by a corresponding rise in the activity of the isozymes and so it was proposed that the regulatory step of induction occurred at a level beyond gene transcription . Similarly the isosafrole-mediated induction of apo-cytochrome P450 was found to occur with no detectable increase in gene transcription (Shephard et al 1987) . It appears therefore that inducing agents induce the apoprotein of cytochrome P450 isozymes by increasing gene transcription and/or stabilising post-transcriptional events .

1.9(c) Effects upon haem synthesis .

Both phenobarbitone and 3-methylcholanthrene have been reported to increase the activity of δ-aminolaevulinic acid synthase in chick embryo liver and this leads to a subsequent increase in the levels of haem (Shedlofsky et al 1987 ; Lincoln et al 1988) . It has been postulated that this increase in haem leads to an increase in the expression of cytochrome P450 genes as inhibitors of haem synthesis (cobalt chloride and thioacetamide) block the induction of the apoprotein of cytochrome P450 by phenobarbitone (Dwarki et al 1987 ; Satyabhama et al 1986) and it has been proposed that haem is a general positive regulator of cytochrome P450 gene expression which may act by promoting DNA transcription and messenger RNA elongation (Jayarama Bhat and Padmanaban 1988) but the inducing agent imparts isozyme specificity (Ravishankar and Padmanaban 1985) . Elevation of haem in the haem pool , however , does not appear to be a necessary precedent for apo-cytochrome P450 gene transcription as increase in the gene transcription has been reported to occur at an earlier time point following administration of phenobarbitone than its effects upon haem synthesis (Rajamanickam et al 1975 ; Correia and Meyer 1975) . Kumar et al (1980) , however , found that the administration of cobalt chloride in conjunction with phenobarbitone had no effect upon phenobarbitone-mediated induction of messenger RNA levels but decreased the synthesis of apo-cytochrome P450 and it was proposed , therefore , that the rate of apo-cytochrome P450 synthesis is dependent upon the rate

of endogenous haem synthesis and that haem produces its effects by acting at some post-transcriptional site .

1.9(d) Effects upon cytochrome P450 degradation .

In addition to an increase in δ-aminolaevulinic acid synthase and gene transcription/messenger RNA stabilisation , both phenobarbitone and 3-methylcholanthrene have been reported to increase haem oxygenase in chick embryo liver and this is thought to occur secondary to the increase in aminolaevulinic acid synthase and an increase in haem levels (Shedlovsky et al 1987 ; Lincoln et al 1988) . In addition , 3-methylcholanthrene has also been proposed to accelerate the degradation of haem by uncoupling the cytochrome P450 system which leads to an increase in the production of hydrogen peroxide (De Mattheis 1988) . These effects may all contribute to the decrease in cytochrome P450 half life which has been shown to occur in phenobarbitone-and 3-methylcholanthrene-induced rat liver (Sadano and Omura 1983) . It has also been reported that phenobarbitone-induced membranes are rapidly removed from the cell following cessation of treatment and this is thought to be due to an increase in autophagic vacuoles induced by phenobarbitone and the increase in the levels of intracellular membranes . The autophagic vacuoles are thought to engulf the membrane and breakdown subsequently occurs via the action of proteolytic enzymes (Bolender and Weibel 1973) .

1.10 XENOBIOTIC-MEDIATED INHIBITION OF HEPATIC MONOOXYGENASES

Xenobiotic inhibition is a much more diverse subject than is induction and many xenobiotics have specific toxic actions upon the cytochrome P450 monooxygenases which are related to the specific mode of action of the individual drug . Such toxic actions are outside the subject of this thesis and will not be discussed .

1.10(a) Destruction of cytochrome P450 .

The best documented of the mechanisms of cytochrome P450 destruction are those mediated by xenobiotics containing olefinic or acetylenic groups e.g. allylisopropylacetamide (AIA) and 3,5-dicarbethoxy-2,6-dimethyl-4-ethyl-1,4-dihydropyridine (DDEP). These drugs are classified as "suicide substrates" as metabolism by cytochrome P450 is a necessary precedent for their degradative actions . These xenobiotics give rise to the formation of "green pigments" in the liver and these have been shown to be alkylated-or substrate-haem fragments derived from the native prosthetic group of the cytochrome P450 (Ortiz de Montellano et al 1981 ; De Matteis et al 1981) . The major site of action of these drugs has been proposed to be directly at the haem locus and the effects of AIA have been reported to be readily reversible by the administration of exogenous haem (Bornheim et al 1987) . Cytochrome P450p (table 1) , however , is refractory to the

addition of haem and similarly the administration of DDEP has been reported to alkylate the haem at the active site of cytochrome P450p and then the subsequently produced alkylated-haem fragments bind to the apoprotein of the cytochrome and increase its susceptibility to proteolytic breakdown (Correia et al 1987) . These agents , therefore , may have additional secondary degradative effects upon some cytochrome P450 isozymes by increasing susceptibility to proteolytic enzymes .

1.10(b) Xenobiotics which form inactive complexes with cytochrome P450 .

These drugs include amphetamines , cimetidine , methadone and isoniazid and unlike the drugs in category (a) they do not appear to share any common structural reactive groups . They are similar to the drugs in category (a) , however , in that they require metabolism by cytochrome P450 to exert their full inhibitory effects and this metabolism is thought to yield an intermediate or product which forms an inactive complex with cytochrome P450 (Testa and Jenner 1981) . They differ from the drugs in category (a) , however , in that the complexes thus formed are reversible and do not lead to degradation of cytochrome P450 . Triacetyloleadomycin (TAO) and isosafrole also appear to form complexes with cytochrome P450 but in addition induce specific cytochrome P450 isozymes and , thus , isosafrole both induces and forms an inactive complex with cytochrome P450d (Levin et al 1980) and TAO both induces cytochrome P450-TAO and forms an

inactive complex with this and other cytochrome P450 isozymes (Pessayre et al 1981) and so the overall effect of these inducing agents is time dependent inhibition of their specifically induced isozymes .

1.10(c) Xenobiotics which alter rates of haem synthesis and degradation .

The best documented of these agents is cobalt which both inhibits δ-aminolaevulinic acid synthase and induces haem oxygenase (Drummond and Kappas 1982) whereas the well documented hepatotoxic effects of carbon tetrachloride are thought to be mediated by an increase in the formation of lipid peroxides (Davies et al 1985) . As stated in the previous sections the classic inducing agents , phenobarbitone and 3-methylcholanthrene also appear to induce haem oxygenase and/or uncouple the cytochrome P450 system leading to an increase in the production of hydrogen peroxide and this may contribute to the increase in turnover rates of the isozymes induced by these agents .

1.11 PHYSIOLOGICAL REGULATION OF HEPATIC BIOTRANSFORMATION .

1.11(a) Hormonal control of hepatic enzyme activity

Hormonal fluctuation has been shown to be one of the major mechanisms by which the body can physiologically regulate hepatic enzyme activity . Steroids have been shown to be the natural substrates for the hepatic monooxygenases (Kuntzman et al 1964) and this regulation , therefore , may constitute a major pathway of hormonal homeostasis . The major hormones which have been found to influence enzyme activity are : (1) the sex steroids : testosterone and oestrogen ; (2) the pituitary hormones acting both directly at the level of the liver or indirectly by stimulating other endocrine organs ; (3) thyroxine ; (4) insulin ; (5) glucocorticosteroids ; (6) glucagon and (7) catecholamines . The regulation of enzyme activity by each of these agents will be dealt with under separate headings .

The metabolism of type 1 substrates (including steroids) is sensitive to hormonal regulation by the above agents . The metabolism of type 11 substrates , however , is relatively refractory to hormonal changes .

1.11(b) Sexual differentiation of enzyme activity .

Short term fluctuation in the level of any particular hormone can occur in response to a number of physiological stimuli and constitutes an important mechanism of body homeostasis . In

addition , hormone levels are subject to long term (fixed) variation which can occur in pathological states such as hyperthyroidism and diabetes and under physiological conditions is dependent upon the age and sex of the animal . It follows , therefore , that the hepatic monooxygenases , under the influence of these hormones , are also subject to fixed variation of their activities depending upon the age and sex of the animal . The onset of sexually differentiated enzyme activity occurs at puberty and this corresponds to sexual differentiation of pituitary hormone release . In the rat this sexual differentiation of enzyme activity is marked and has been well documented for both xenobiotics and steroids as shown in table 2 .

Type 1 substrates e.g. hexobarbitone and aminopyrine , have been found to be more susceptible to hormonal regulation than type 2 substrates e.g. aniline and zoxazolamine , and it follows , therefore , that sexually differentiation in metabolism tends to occur with type 1 substrates under the influence of sexually differentiated hormone profiles whereas the metabolism of type 2 substrates tends to occur independently of the age or sex of the animal .

In addition to the rat , sexual differentiation of hepatic monooxygenase activity also occurs in the mouse and in the hamster . Both of these species exhibit sex-dependent metabolism of steroids , however , in both cases , the sexual differentiation appears to be in the opposite direction to that seen in the rat in that steroid hydroxylases are more active in the female than in the male of the species (Niedermayer and Shapiro 1988 ; Miura et

Substrate	Differentiation	Reference
Xenobiotics		
Hexobarbital oxidase	male > female	Kuntzman et al 1964
Aminopyrine-N-demethylase	male > female	Schenkman et al 1967
Ethylmorphine-N-demethylase	male > female	Rumbaugh et al 1984
Lignocaine-N-deethylase	male > female	Skett et al 1980a
Imipramine-N-demethylase	male > female	Skett et al 1980a
Aniline hydroxylase	male = female	Kato and Gillette 1965b
Zoxazolamine hydroxylase	male = female	Kato and Gillette 1965b
Steroids		
4-Androstene-3,17-dione/ testosterone :		
(a) 5α-reductase	male < female	Gustafsson and Stenberg 1974a
(b) 3α-hydroxysteroid-dehydrogenase	male = female	" "
(c) 17α-hydroxysteroid-dehydrogenase	male > female	" "
(d) 6β-hydroxylase	male > female	" "
(e) 16α-hydroxylase	male > female	" "
(f) 7α-hydroxylase	male = female	" "
5α-Androstane-3,17-diol :		
(a) 2α-hydroxylase	male > female	" "
(b) 7α-hydroxylase	male > female	" "
(c) 18-hydroxylase	male > female	" "
Steroid sulphate-15β-hydroxylase	male < female	Gustafsson and Ingelman-Sundberg 1974
Cortisol 20β-hydroxylase	male > female	Denef and De Moor 1968
Cholesterol 7α-hydroxylase	male = female	Einarsson et al 1973

Table 2 : Examples of substrates which are metabolised by the rat hepatic monooxygenase system and illustration of sex differentiation of the metabolism of some xenobiotics and steroids .

al 1988) . Sex differences in metabolism also occur in the human and show similar differences to those seen in the rat although less marked (Pfaffenberg and Horning 1977) .

The sex differences in hepatic monooxygenase activity in the rat have been postulated to be due to 3 factors (a) differences in the constitutive cytochrome P450 isozymes (Kamataki et al 1983); (b) greater substrate affinity for cytochrome P450 in the male than in female (Schenkman et al 1967b) ; (c) slower turnover of cytochrome P450 in the male than in the female (Levin et al 1974). Recent reports have indicated that sex differences also exist in the lipid component of the hepatic monooxygenase system (section 1.3(a)) . It has been shown that the activity of a purified cytochrome P450 isozyme which catalyses N-demethylation of lignocaine and which is thought to correspond to the male-specific cytochrome P450 isozyme (P450 2c , P450 UT-A , P450h , P450 RLM5 or P450 PB-2a : table 1) , is much higher when reconstituted in a system composed of male derived lipids than when reconstituted with female derived lipids (Meftah and Skett 1987) . This may indicate that differences in the lipid environment of the cytochrome P450 may be a fourth factor which contributes to the overall sexual differentiation of the hepatic monooxygenase system .

1.11(c) Imprinting .

The sexual differentiation of both hormone profiles and hepatic enzyme activity in the rat do not occur until puberty .

Few sex differences exist in the genome except for the Y/X chromosome , the major function of which is to determine testicular / ovarian differentiation , and it is the differences in the secretory products of the gonads which are responsible for sexual dimorphism (Bardin and Catterall 1981) . Neonatal exposure to androgens imprints both the central nervous system (MacLusky and Naftolin 1981) and the metabolising enzymes of the liver (Denef and De Moore 1968) to produce "maleness" in the postpubertal animal . Imprinting of the hypothalamus results in sexual differentiation of hormone release profiles from the pituitary and this results in sexual differentiation of hepatic enzyme activity .

The presence of oestrogen does not appear to imprint sexual dimorphism at this levels although an exception may be the difference in the secretory patterns of thyroxine (which is under the direct control of the pituitary gland) . The higher plasma levels of thyroxine in the female appear to be dependent upon oestrogen but this difference can be reversed , at least in part, by oestrogen administration to the adult male (Donda et al 1987) and so does not appear to be due to imprinting of the pituitary gland/hypothalamus by neonatal oestrogen .

The full male pattern of postpubertal hepatic activity in the rat is determined by direct imprinting by neonatal testosterone at the level of the hypothalamus and maintenance of a male pattern hormone profile by postpubertal testosterone .

In addition it was found that neonatal testosterone was necessary for testosterone responsiveness in the postpubertal male

rat (Gustafsson and Stenberg 1974b) and females were reported to be unresponsive to postpubertal increase in their testosterone levels (Bitar and Shapiro 1987) . This further implies that imprinting of the hypothalamus by neonatal testosterone is necessary for postpubertal maintenance of the male hormone profile by testosterone . Powell-Jones et al (1981) and Sloop et al (1983) , however , postulated that neonatal testosterone also had an imprinting effect upon the liver to permanently program higher levels of the oestrogen-binding protein HCLA (also known as HASP or male SBP : section 1.11(e)) in the postpubertal male rat than in the postpubertal female rat . It is conceivable , therefore , that neonatal testosterone imprints the male rat liver in a similar manner to that proposed for the oestrogen-binding protein , HCLA . The absence of hepatic androgen receptors , therefore , may be an alternative explanation for the lack of effect of testosterone in the postpubertal female rat (Bitar and Shapiro 1987) and in the neonatally castrated , postpubertal male rat (Gustafsson and Stenberg 1974b) .

1.11(d) Testosterone .

The precise role of testosterone in the postpubertal regulation of hepatic monooxygenase activity is unclear . Although castration in the male rat leads to increase in the activity of hepatic steroid 5α reductase (normally higher in the female) and this effect can be reversed by testosterone (Yates et al 1958) . Kramer et al (1975) reported that the masculinising effect of

testosterone upon ethylmorphine metabolism did not occur in the absence of the pituitary gland . Other workers , however , have reported that hypophysectomy is unable to mimic the abolition of the male pattern of steroid metabolism produced by castration (Lax et al 1974) . This may imply that testosterone has some pituitary-independent effects at the levels of the liver . Skett (1978) , however , found that although hypophysectomy had no effect upon steroid metabolism in the male rat , hypophysectomy of female rats resulted in masculinisation of steroid metabolism . It is now thought , therefore , that the effects of testosterone upon hepatic monooxygenase activity are due to inhibition of the synthesis or release of a pituitary "feminising factor" (section 1.11(f)) and testosterone has little direct at the levels of the liver .

Androgen receptors , however , are present in the liver (Sarkar et al 1987) although their physiological significance is obscure . The levels of the hepatic androgen receptors are under the control of the Tfm locus . In mice deficient in the locus (Tfm/Y) the androgen receptor is absent from the liver and testosterone administration fails to masculinise the metabolism of ethylmorphine (Brown et al 1978) . Similarly the levels of a hepatic androgen-binding protein have been correlated to the ability of testosterone to produce a male pattern of xenobiotic metabolism in rats (Rumbaugh et al 1984) . These findings imply that testosterone does have some direct effects at the level of the liver in addition to suppressing the synthesis or release of some pituitary factor .

The sex differences in hepatic monooxygenase activity correlate with differences in the levels of the constitutive cytochrome P450 isozymes (Kamataki et al 1983) . Sex differences also exist in the binding of type 1 substrates to cytochrome P450 in that binding is twice as high in the male rat than in the female rat (Schenkman et al 1967b) and in the ratio of fast to slow turnover types of cytochrome P450 in that male rats have a higher ratio of slow turnover cytochrome P450 which contributes to the higher activities of the cytochrome P450 isozymes in the male than in the female (Levin et al 1974) . The administration of testosterone increases the affinity of type 1 xenobiotic substrates for their respective cytochrome P450 isozymes but has little effect upon cytochrome P450 levels (Kato and Onoda 1970). This may imply that a direct effect of testosterone at the level of the liver and mediated by the androgen receptor , is an increase in the affinity of the substrate for cytochrome P450 whereas the indirect effects of testosterone via the pituitary gland , are necessary for the maintenance of the levels of constitutive cytochrome P450 isozymes and their turnover rates .

1.11(e) Oestrogen .

Physiological concentrations of oestrogen appear to have little direct effect at the level of the liver . Administration of oestrogens to male rats leads to feminisation of enzyme activity (Einarsson et al 1973) . This effect , however , does not occur in the hypophysectomised rat (Gustafsson and Stenberg 1976) . It

has been postulated , therefore , that the feminising effects of oestrogens in the male rat are due to an indirect effect at the level of the pituitary gland .

Oestrogen binding proteins , however , are present in the liver and appear to fall into two categories . Both male and female livers contain equal quantities of a classic oestrogen receptor (Rumbaugh et al 1983) . In addition , a second oestrogen binding protein exists which is present at much higher concentrations in the male than in the female rat . This binding protein has been investigated by a number of workers and has been designated : HASP (Lax 1987) ; HCLA (Sloop et al 1983 ; Powell-Jones et al 1981) and male SBP (Powell-Jones et al 1980) . Another male-specific oestrogen binding protein , MEB may also be identical to the above but appears to differ from them in some of its properties (Rogerson and Eagon 1986) . These male-specific oestrogen binding proteins have been proposed to act as a sink for oestrogen by binding oestrogen and preventing its action at the oestrogen receptor (Rumbaugh et al 1983) or alternatively may influence the translocation of the oestrogen/receptor complex into the nucleus (Powell-Jones et al 1980) . Little evidence exists , however , for a direct hepatic action of oestrogen , at physiological concentrations , in feminising hepatic monooxygenase activity and at physiological concentrations the receptors are thought to be non functional (Lax 1987) . Inhibition of 3β-hydroxysteroid dehydrogenase by oestrogen , however , can be blocked by tamoxifen which indicates that some of the effects of oestrogen are mediated via an

oestrogen receptor (Lax et al 1980) . This study , however , did not indicate the site of the proposed oestrogen receptors . Oestrogen receptors are present in the pituitary and are thought to be involved in the oestrogen-mediated alteration of pituitary function (Lax et al 1983) . This may be the mechanism , therefore , by which tamoxifen prevents the oestrogen-mediated inhibition of 3β-hydroxysteroid dehydrogenase activity rather than by blocking hepatic oestrogen receptors . Pharmacological levels of oestrogen , however , do have a direct effect at the level of the liver , presumably via the oestrogen receptor , and produce masculinisation of steroid metabolism (Lax 1987) .

1.11(f) Pituitary hormones .

The anterior pituitary gland is responsible for the synthesis and secretion of a number of hormones . Follicle-stimulating hormone (FSH) ; luteinising hormone (LH) ; thyroid stimulating hormone (TSH) and adrenocorticotrophin (ACTH) all act on peripheral endocrine organs to cause the secondary release of their respective hormones . Growth hormone (GH) and prolactin (PRL) have direct effects upon target tissues . In addition , growth hormone may mediate indirect effects upon target tissues by stimulating the synthesis of somatomedins (section 1.16(e)) . The alteration of hepatic monooxygenase activity which accompanies hypophysectomy i.e. feminisation of hepatic monooxygenase activity in the male rat (Skett et al 1980) and the masculinisation of hepatic monooxygenase activity in the female rat (Skett 1978)

may be mediated by alteration in the levels of any one or a combination of the above or their respective peripheral hormones . The implantation of a pituitary gland under the kidney capsule in the hypophysectomised male or female rat was found to feminise hepatic monooxygenase activity regardless of the sex of the animal (Denef 1974) . In this model hypothalamic control of pituitary function is absent and so these results imply that the pituitary gland from either sex is capable of secreting a hormone (or combination of hormones) which feminises hepatic monooxygenase activity however in the male rat the secretion of this pituitary fraction is suppressed by some hypothalamic factor . This pituitary fraction was termed " the feminising factor " and the inhibitory male-specific hypothalamic factor as " feminostatin " . It has be postulated that a specific centre in the hypothalamus is irreversibly imprinted at birth by neonatal testosterone and at puberty this centre is " turned on " in the male only and secretes "feminostatin" under the influence of testosterone which inhibits the pituitary secretion of " feminising factor " . In the female the absence of " feminostatin " results in unopposed secretion of " feminising factor " (Gustafsson et al 1979 ; Gustafsson et al 1981) . Much work has been carried out to establish the hormonal nature of the " feminising factor " . Both LH and FSH increase ethylmorphine-N-demethylase activity in the castrated rat indicating a non-gonadal mode of action (Schreifers et al 1975). This increase in ethylmorphine metabolism , however , represents masculinisation rather than feminisation and neither FSH or LH , therefore , is likely to be the feminising factor

although a role in the pituitary control of hepatic metabolising enzyme activity cannot be ruled out . Implantation of a pituitary mammotrophic tumour which secreted ACTH , GH and PRL inhibited xenobiotic metabolism (Wilson 1968) however treatment of rats with 2-bromo-α-ergocriptine which inhibits the release of PRL from the pituitary gland , had no influence upon the feminising effects of an ectopic pituitary on steroid or xenobiotic metabolism (Skett et al 1978) . It seems unlikely , therefore , that PRL is the feminising factor . Injection of GH inhibited the metabolism of ethylmorphine and aminopyrine (Wilson 1970) and a pituitary mammotrophic tumour which secreted GH and PRL but very little ACTH had the same effect upon steroid metabolism as that secreting GH , PRL and ACTH (Eneroth et al 1976) . In addition purification of the " feminising factor " from rat pituitary has lead to a product identical to GH (Mode et al 1983) and so the identity of the " feminising factor " is now assumed to by GH . Furthermore , the feminising effects of GH on both xenobiotic and steroid metabolism has been confirmed by Kramer and Colby (1976) . Injection of ACTH , however , does result in a sex-dependent / GH-independent inhibition of the metabolism of hexobarbitone and aminopyrine in the male rat (Kato and Gillette 1965a) and may augment the action of GH in feminising hepatic monooxygenase activity .

1.11(g) Growth hormone .

Although growth hormone is secreted by the pituitary in both males and females there is a sex difference in the secretory

dynamics . In the female rat GH levels are constant whereas male rats exhibit large peaks in plasma levels with intervening undetectable levels of the hormone (Edén 1979) . The pattern of GH secretion is now assumed to be the regulator of hepatic monooxygenase activity in the rat (Mode et al 1982) and levels of steroid 16α hydroxylase have been directly correlated to changes in the diurnal pattern of GH secretion i.e. feminisation (decrease) or masculinisation (increase) (Morgan et al 1985b). Similarly constant infusion of growth hormone , mimicking female secretory dynamics , results in complete feminisation of 3α hydroxysteroid dehydrogenase and steroid 5α reductase activities in male rats (Lax et al 1983) . The sex differences in the secretory dynamics are neonatally imprinted by testosterone (Jansson et al 1984) and the resulting pulsatile release of GH in the male has be postulated to be due to interaction between two pituitary factors : growth hormone releasing hormone (GHRH) and growth hormone release-inhibiting hormone (GHRIH or somatostatin) (Weiss et al 1987) . In an in vivo study , lesions of the paraventricular hypothalamus or an injection of somatostatin antiserum led to a drop in somatostatin release in the male and this resulted in a change of the GH secretory dynamics to a female pattern concomitant with feminisation of hepatic monooxygenase activity (Gustafsson et al 1983) . If GH is assumed to be the " feminising factor " , therefore , somatostatin appears to correspond to " feminostatin".

The inhibitory effects of GH upon xenobiotic metabolism may be

mediated by a reduction in the flow of reducing equivalents to cytochrome P450 (Wilson and Frohman 1974) or by reducing levels of cytochrome P450 (Wilson and Spelsberg 1976) . Later work , however , has shown that the GH-mediated feminisation of the hepatic monooxygenase system was due to a specific decrease in the synthesis of the male specific cytochrome P450 probably regulated at the level of translation (Vockentanz and Virgo 1985) and that the higher level of steroid 15β hydroxylase activity in the female than in the male was due to the selective induction of the female-specific cytochrome P450 by GH at a step which appears to precede translation (MacGeoch et al 1987) . Thus it appears that the alteration of hepatic monooxygenase activity may reflect differential changes in the content of specific cytochrome P450 isozymes .

The major problem which arises when studying the effects of GH in vivo are the complex interactions which exist between the components of the endocrine system . Diabetes is associated with elevation of somatostatin and suppression of GH release (Tannenbaum 1981) and thyroxine stimulates the synthesis of GH by a direct action at the level of the pituitary gland (Mirell et al 1987 ; Santos et al 1987) . Similarly hypothyroidism , which may occur secondary to diabetes , leads to suppression of GH release (Ortiz-Caro et al 1984 ; Shapiro 1983) . GH may also attenuate the effects of insulin by suppression of the affinity of hepatic insulin receptors (Davidson and Melmed 1983) . When isolated hepatocytes were incubated with GH in an attempt to examine the direct effects of GH at the level of the liver ,

steroid metabolism was unaffected (P. Gulati : personal communication) . This contrasts markedly with the feminising effects seen in vivo and indicates that feminisation of the hepatic monooxygenases is a complex interactive process which may involve more than one component of the endocrine system .

1.11(h) Thyroxine

Thyroxine is subject to fixed , sexually differentiated variation in its secretion . Females have higher plasma levels of triiodothyronine (the active form of thyroxine) than do males and also have a higher number of pituitary triiodothyronine receptors (through which triiodothyronine negatively regulates its own production by attenuating the production of TSH) . This sexually differentiated secretory pattern may be due to higher oestrogen levels in females than in males as oestrogen administration to male rats elevates the number of pituitary triiodothyronine receptors (Donda et al 1987) . The effects of thyroxine upon the activity of the hepatic monooxygenases are also sex dependent . Administration of thyroxine to male rats (hyperthyroidism) impairs the metabolism of the type 1 substrates , hexobarbitone and aminopyrine and slightly enhances the metabolism of the type 2 substrate , aniline (Kato and Gillette 1965a) . It also inhibits the hydroxylation of testosterone (Kato et al 1970) . Inhibition of both type 1 xenobiotic metabolism and the hydroxylation of steroids is dependent upon sex and neither occur in the female rat . These

effects , however , cannot be fully explained by the sexually differentiated secretory pattern of thyroxine and paradoxically thyroidectomy also inhibits the hydroxylation of testosterone (Kato et al 1970) . These heterologous effects upon hepatic monooxygenase activity similarly cannot be adequately explained purely in terms of an alteration of the levels of gonadal or pituitary hormones although the inhibitory effects of thyroxine upon hepatic monooxygenase activity are abolished by castration (Kato and Gillette 1965a) and thyroxine stimulates the release of LH (Wang et al 1987) and the synthesis of GH (Mirell et al 1987 ; Santos et al 1987) in the pituitary gland . Thyroxine may, however , have a permissive effect upon the hepatic actions of testosterone (section 1.11(d)) by altering the concentrations of the androgen receptor (Lax et al 1979) and this may explain the lowering of the substrate affinity which accompanies the inhibition of hepatic monooxygenase activity in the thyroidectomised rat (Kato et al 1970) . One possible explanation for the heterologous effects of thyroxine i.e. inhibitory effects upon hepatic monooxygenase activity in both hyperthyroid and thyroidectomised rats , is that physiological levels of thyroid hormones induce hepatic δ-aminolaevulinate synthase whereas higher concentrations of thyroxine induce haem oxygenase (Smith and Drummond 1988) . Inhibition of enzyme activity in the thyroidectomised rat , therefore , may be due to a decrease in cytochrome P450 synthesis whereas inhibition in the hyperthyroid rat may be due to an increase in cytochrome P450 degradation .

In addition to any direct effects exerted at the level of the liver and effects produced by alteration of pituitary function , thyroxine may also produce effects upon enzyme activity by altering pancreatic function . Close correlation exists between pancreatic function and thyroxine secretion and thyroxine stimulates the release of glucagon from pancreatic α cells which then stimulates the pancreatic β cells and produces a secondary rise in insulin secretion (Wolf and Eisenstein 1981) . Furthermore hypothyroidism attenuates the cellular response to insulin by reducing insulin binding and receptor number (Sterman et al 1983) .

1.11(i) Insulin .

Acute diabetes or starvation , both of which result in a lowering of plasma insulin have both a sex-dependent and a sex-independent effect upon hepatic monooxygenases .

The sexually differentiated enzymes which metabolise type 1 substrates (including steroids) are inhibited in the male rat but stimulated in the female rat . This represents a loss of sexual differentiation (Kato and Gillette 1965a ; Kato and Gillette 1965b) and this effect can be reversed by insulin administration (Weiner et al 1972a ; Reinke et al 1978) . The non-sexually differentiated enzymes which metabolise type 2 substrates are stimulated to an equal extent in both male and female rats (Past and Cook 1982 ; Past and Cook 1983) . Similarly steroid 7α hydroxylase activity is also stimulated by

diabetes in the male rat and this effect can be reversed by insulin administration (Reinke et al 1978) .

The increase in the metabolism of aniline (a type 2 substrate) in both male and female rats has been correlated with the appearance of a diabetes-specific cytochrome P450 which metabolises aniline (Past and Cook 1982 ; Past and Cook 1983 ; Favreau et al 1987 ; Bellward et al 1988) . The decrease in the activity of steroid 16α hydroxylase activity in the male rat has been correlated with loss of the male-specific cytochrome P450 (P450 2c , P450 UT-A , P450h , P450 RLM5 or P450 PB-2a : table 1) (Favreau and Schenkman 1987) and it has been postulated that the changes in hepatic monooxygenase activity caused by diabetes reflect differential changes in the specific cytochrome P450 isozyme levels (Past and Cook 1980) . This may reflect a direct effect of insulin at the level of the hepatocyte and in the isolated rat hepatocyte it has been found that insulin does have a direct stimulatory effect upon steroid metabolism producing two peaks of activity at half an hour and 24 hours . These effects , however , do not appear to be due to protein synthesis as evaluated by measuring whole cell cytochrome P450 levels , but may indicate a direct effect at the level of the cytochrome P450 (Hussin and Skett 1987) . The effects of insulin in hepatocytes, however , differ from those that would have been expected from the findings of previous in vivo studies in that the effects of insulin are not specific for particular isozymes and this may indicate that the effects of insulin in vivo are due , at least in part , to effects upon the release of hormones from other

endocrine organs .

Insulin has many effects upon other hormones and similarly diabetes results in changes in the release patterns of many hormones in addition to a fall in insulin levels . The feminising effects of diabetes upon the activity of the hepatic monooxygenases in the male rat can be partially reversed by administration of testosterone (Skett et al 1984) which implies that the effects of insulin are not mediated directly at the levels of the liver but are due to alterations in testosterone secretion . Testosterone secretion , however , is not consistently lowered in diabetes and diabetes , therefore , may be producing its effects by altering pituitary function (Kato and Gillette 1965a ; Skett et al 1984) . Diabetes does , however , result in a decrease in the spectral change associated with the binding of the substrate to cytochrome P450 which may represent impairment of an androgen-dependent regulatory mechanism for binding due to inhibition of testosterone secretion . Diabetes also suppresses plasma levels of thyroxine by inhibiting its secretion (Ortiz-Caro et al 1984) and the elevated levels of glucagon in diabetes leads to a secondary loss in hepatic triiodothyronine receptors (Dillman et al 1978 ; Wiersinga et al 1982 ; Jolin 1987) which further suppresses the effects of thyroxine . Diabetes also suppresses pituitary release of GH which may occur secondary to a rise in somatostatin (Tannenbaum 1981) or to suppression of thyroxine levels (Ortiz-Caro et al 1984) and this is accompanied by a decrease in the ability of the liver to synthesise somatomedin c in response to GH (Scott and Baxter

1986) (see section 1.16(e)) . The in vivo effects of insulin/diabetes upon hepatic monooxygenase activity could , therefore , be mediated by the secondary alteration in the levels of these other hormones in addition to any direct effects of insulin at the level of the liver . In addition , Bellward et al (1988) reported that the levels of a diabetes-induced cytochrome P450 isozyme which metabolises aniline , cytochrome P450j (table 1) , in spontaneously diabetic rats did not correlate with the plasma levels of insulin but did correlate with the levels of ketone bodies . The changes in hepatic monooxygenase activity in diabetes , therefore , may occur , at least in part , by impairment of cellular processes in addition to a fall in insulin levels and effects upon other endocrine organs .

The effects of acute diabetes upon hepatic monooxygenase activity are diminished in chronically diabetic rats (Skett and Joels 1985) and , similarly , hepatocytes which have been isolated from chronically diabetic rats are refractory to the direct stimulatory effects of insulin (Hussin and Skett 1988) .

1.11(j) Glucocorticosteroids .

Glucocorticosteroids are synthesised and released from the adrenal cortex under the control of adrenocorticotrophin (ACTH). Their effects upon hepatic monooxygenase activity are complex and in general the natural glucocorticosteroids inhibit drug metabolism whereas synthetic glucocorticosteroids induce metabolism . Dexamethasone has a similar enzyme induction profile

to PCN (see section 1.9(a)) . This is not thought to be due to its steroidal properties (Skett 1987) but it may be acting in a similar manner to the classic inducing agents as it has been shown that the DNA fragments which are enhanced in their activity by the presence of 3-methylcholanthrene are similar in base structure to the fragments which respond to induction by glucocorticosteroids (Fujisawa-Sehara et al 1987) . Adrenalectomy also decreases the metabolism of type 1 substrates but has no effect upon the metabolism of type 2 substrates in the male rat . This represents a feminisation of metabolism in the male rat and adrenalectomy has no effect upon the metabolism of either type 1 or type 2 substrates in the female rat (Kato and Gillette 1965a) . The decrease in the metabolism of type 1 substrates may be due to a decrease in the affinity of the substrate for cytochrome P450 rather than a decrease in cytochrome P450 levels as adrenalectomy decreases the magnitude of the spectral change associated with the binding of type 1 substrates to the cytochrome P450 (Kato et al 1971) . Adrenalectomy also inhibits the activities of steroid 3α-hydroxysteroid dehydrogenase and 17β-hydroxysteroid dehydrogenase but has no effect on the activity of 5α reductase . These enzymes do not contain cytochrome P450 as an integral component (see section 1.4) and unlike the effects of adrenalectomy upon the metabolism of type 1 xenobiotics , these enzymes are inhibited to an equal extent in rats of both sexes (Lax et al 1979) . One possible mechanism by which adrenalectomy might lead to a decrease in monooxygenase activity is by the induction of haem oxygenase as has been reported in the chick embryo liver (Sassa et al 1980) .

Glucocorticosteroids reduce hepatic insulin binding and the number of hepatic insulin receptors (Sterman et al 1983) and also stimulate the synthesis of GH in the pituitary gland (Gick and Bancroft 1987) . Some of the effects of glucocorticosteroids/ adrenalectomy upon hepatic monooxygenase activity , therefore , may be produced by a secondary alterations in the levels of these other hormones .

1.11(k) Glucagon .

In vivo administration of glucagon to rats increases the hexobarbitone sleeping time indicating that glucagon inhibits the activity of the hepatic monooxygenases (Weiner et al 1972a) . Addition of glucagon to isolated rat hepatocytes in culture inhibits the metabolism of androstenedione in a nonspecific manner and does not appear to have a direct feminising effect upon steroid metabolism . This effect of glucagon is maximal at physiological concentrations of glucagon (10^{-8}M) but is lost at supraphysiological concentrations indicating that the inhibitory effects of glucagon upon hepatic monooxygenase activity are dose dependent (Hussin et al 1988) . Glucagon has been reported to increase cell autophagy in the liver and thus one of its mechanisms may include changes in lysosomal fragility and increase in the proteolytic breakdown of the microsomal proteinaceous components (Deter and DeDuve 1967) .

1.11(1) Catecholamines .

Multiple injections of noradrenaline into rats inhibit the metabolism of hexobarbitone and aminopyrine (Dixon et al 1964) and multiple injections of adrenaline inhibit the metabolism of hexobarbitone and chlorpromazine (Fouts 1962) . All of the substrates used in the above studies are type 1 substrates and the inhibitory effects of catecholamines appear to be specific for type 1 substrates . In vivo administration of catecholamines have no effect upon the metabolism of type 2 substrates (Kato and Gillette 1965a) . The inhibitory effects of the catecholamines appear to be sex-dependent and no inhibitory effects upon either type 1 or type 2 substrates have been noted in the female rat (Kato and Gillette 1965a) .

1.12 INTRACELLULAR SECOND MESSENGERS FOR HORMONAL TRANSDUCTION

The two major second messenger systems in target cells are (a) adenylate cyclase activation and cyclic adenosine 3',5' -monophosphate (cyclic AMP) generation and (b) phosphatidyl-inositol hydrolysis which leads to inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) generation . The ultimate result of the generation of any of these second messengers is generally thought to be due to the activation of their respective protein kinases and the phosphorylation of intracellular proteins . Hydrolysis of inositol phospholipids also leads to an increase in the release of arachidonic acid and an increase in the concentration of another nucleotide second messenger , cyclic guanosine 3',5'-monophospate (cyclic GMP) which may also contribute to the intracellular effects of hormones which stimulate this pathway .

1.12(a) Cyclic AMP .

Cyclic AMP was first discovered by Sutherland and Rall in 1960 and is produced within the cell from adenosine 5'-triphosphate (ATP) in the presence of magnesium ions by the action of the enzyme adenylate cyclase . Adenylate cyclase is located in the plasma membrane of the cell and was originally thought to be composed of two components , namely a receptor for the hormone located on the extracellular surface of the membrane and a catalytic unit located on the cytosolic side of the

membrane, and it was proposed that the hormone receptor activated the catalytic unit by direct interaction (Perkins 1973). In 1971 , however , Rodbell et al found that guanosine triphosphate (GTP) was essential for the activation of the catalytic subunit by the receptor and , in 1975 , Rodbell and co-workers reported that hormone receptor binding enhanced this interaction . This led to the identification of a GTP binding protein as a component of adenylate cyclase in 1977 (Pfeuffer 1977) and it is now thought that the adenylate cyclase system consists of at least three separate components : the hormone receptor at the outer surface of the membrane , the catalytic subunit at the inner surface of the membrane and a GTP binding protein (N) which mediates the effects of hormones upon the catalytic subunit (Rodbell 1980) . Later work has shown that at least two different GTP binding proteins can act as transducers between the hormone receptor and the catalytic subunit . Ns is associated with hormone-induced stimulation of the catalytic subunit whereas Ni is associated with inhibition of the catalytic subunit (Ross and Gilman 1980 ; Jakobs et al 1984) . Both of these proteins are composed of three non-identical subunits . Two of the subunits designated β and γ appear to be identical in both Ns and Ni , however the α subunits vary in molecular size (Houslay 1984) .

In the resting state it is thought that the GTP binding proteins exist as aggregates with the receptors and are separated from the catalytic units (Rodbell 1980) . When a stimulatory hormone binds to its receptor , GTP binds to the α subunit of Ns

and causes it to dissociate from the β and ϒ subunits . The α subunit of Ns bound to GTP then interacts with and activates the catalytic unit of adenylate cyclase (Gilman 1984 ; Schramm and Selinger 1984) . Activity is terminated by the hydrolysis of the GTP to guanosine diphosphate (GDP) by an intrinsic GTPase (Jakobs et al 1984) .

The mechanism by which Ni mediates the effects of inhibitory hormones upon adenylate cyclase is less well understood . Hormone binding to the receptor results in the binding of GTP to the α subunit and its dissociation from the β and ϒ subunits as with Ns and Toro and Birnbaumer (1987) have proposed that in an analogous manner to Ns , the α subunit of Ni mediates the inhibitory effects of the hormone by interacting with the catalytic subunit . Other workers , however , have proposed that the β and ϒ subunits of Ni and not the α subunit interact with the α subunit of Ns and thereby inhibit the catalytic subunit indirectly (Cerione et al 1985 ; Hekman et al 1987) .

Cyclic AMP is thought to produce the majority of its effects in the cell by the activation of cyclic AMP-dependent protein kinase (PKa) . The elevation of cyclic AMP also activates a cyclic AMP phosphodiesterase (Corbin et al 1985) , which then breaks down cyclic AMP to the inactive adenosine 5'-monophosphate (5'-AMP) and , therefore , terminates the signal . The sequence of events in the hormonally-stimulated generation of cyclic AMP and its subsequent degradation is shown in figure 3 .

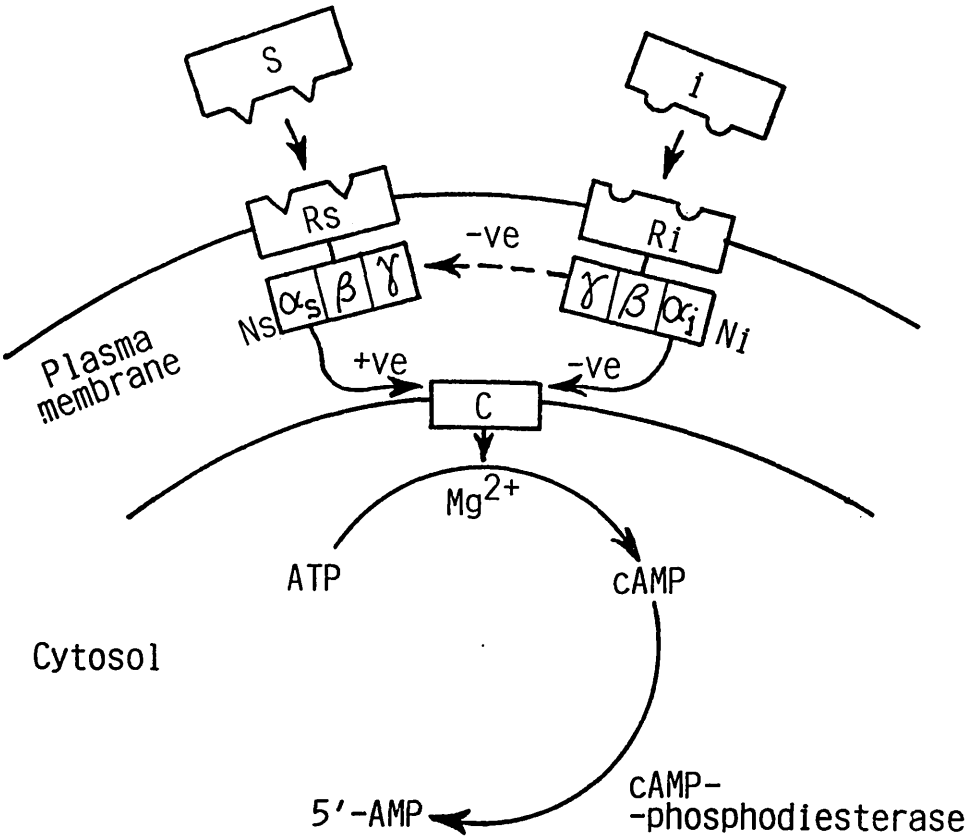

Figure 3 : The sequence of events in hormonal stimulation of cyclic AMP generation and subsequent degradation , where s is the stimulatory hormone , i is the inhibitory hormone , Rs is the stimulatory hormone receptor and c is the catalytic subunit of adenylate cyclase . Other abbreviations are as follows : ATP (adenosine 5'-triphosphate) , cAMP (adenosine 3',5'-cyclic monophosphate or cyclic AMP) and 5'-AMP (adenosine 5'-monophosphate) .

1.12(b) Cyclic AMP-dependent protein kinase .

PKa is associated with the smooth and rough endoplasmic reticulum and also with ribosomes (Jergil and Ohlsson 1974) and it consists of two different types of subunit : a regulatory (R) subunit and a catalytic (C) subunit . It is activated by the dissociation of the two subunits and the release of the free C subunit (Kumon et al 1970) . The enzyme is not a single species but is a family of closely related enzymes with identical C groups but slightly differing R groups with regards to amino acid sequence (Robinson-Steiner et al 1984) . These enzymes are classified as either type 1 or type 2 depending upon their stability in the holoenzyme form . Type 1 enzymes are less stable as holoenzymes and dissociate in the presence of histone or physiological concentrations of sodium chloride . They ,therefore, are less dependent upon cyclic AMP than are type 2 enzymes (Corbin et al 1975) . The liver contains both type 1 and type 2 protein kinases with a slightly greater proportion of type 1 .

The PKa holoenzyme exists as a quaternary aggregate composed of two R subunits and two C subunits (Beavo et al 1975) and each of the two R subunits of the holoenzyme are able to bind two molecules of cyclic AMP (four molecules bound in total) at two distinct binding sites called site 1 and site 2 (Beebe et al 1986) . Both of the binding sites are found on the carboxy-terminal two thirds of the polypeptide chain and have differing cyclic AMP dissociation rates (Doskeland 1978) . The binding of cyclic AMP to the two sites shows positive

cooperativity (Robinson-Steiner and Corbin 1983) and this has been attributed to a tyrosine residue in site 2 , as substitution of this residue with phenylalanine leads to loss of the positive cooperativity (Bubis et al 1988) . Binding of the C subunit to the R subunit in the holoenzyme occurs at a different site to the binding of cyclic AMP and it has been found that the R subunit is a substrate for the catalytic activity of the C subunit (Corbin et al 1978) . First and Taylor (1988) reported that the phosphorylation of the serine residue at position 95 on the R subunit by the C subunit increases the affinity of the R subunit for the C subunit and postulated that this amino acid was the site of contact between the R and the C subunit and that the R subunit inhibits the catalytic activity of the C subunit in the holoenzyme by competing with the substrate for the active site of the C subunit . It has also been reported that the cysteine residues at position 97 in the R subunit and at position 199 in the C subunit form a disulphide linkage and that , as these positions coincide with the proposed linkage between the serine 95 and the active site of the C subunit , this disulphide bond may also contribute to the association of the two subunits in the holoenzyme (First et al 1988) .

Cyclic AMP generated by the activation of adenylate cyclase , therefore , binds to the regulatory subunit of the PKa holoenzyme at four binding sites and induces some conformational change which leads to the disruption of the bonds between the regulatory and the catalytic subunits and the dissociation of the holoenzyme to yield free catalytic subunits .

1.12(c) Inositol phospholipid hydrolysis .

The physiological role of inositol phospholipid hydrolysis in the intracellular transduction of hormones has only recently been realised . The ability of hormones to alter the composition of plasma membrane phosphoinositides , however , has been known for over twenty years following the work of Hokin and Hokin (1953) who showed that acetylcholine was able to stimulate the rapid incorporation of (^{32}P) into phosphatidylinositol (PI) and phosphatidic acid . It was not until 1975 , however , that Michell reported that this incorporation of (^{32}P) was due to the hormonal stimulation of breakdown and resynthesis of inositol phospholipids (PI turnover) . This theory was later modified by Michell et al in 1981 when they reported that the breakdown of phosphatidylinositol 4,5-bisphosphate (PIP_2) was the primary event in hormonal effects and not PI breakdown and resynthesis and this was verified by Creba et al in 1983 . Two enzymes are known to mediate hydrolysis of PIP_2 (Irvine 1982a) ; a phosphomonoesterase cleaves the phosphate at position 5 to yield phosphatidylinositol 4-phosphate (PIP) and is important in the interconversion of phosphatidylinositols in the plasma membrane , the second is a phosphodiesterase known as phospholipase c which is activated by occupation of the hormone receptor . This enzyme hydrolyses PIP_2 in the presence of calcium ions to produce two products : diacylglycerol (DAG) and inositol 1,4,5,-trisphosphate (IP_3) which are thought to mediate the intracellular effects of hormone binding .

Recent studies have shown that the activation of phospholipase c by hormone occupation of the receptor may be mediated via a GTP binding protein in a manner analogous to adenylate cyclase (Molski et al 1984 ; Okajima and Ui 1984) and the presence of GTP analogues has been reported to lower the calcium requirement of phospholipase c and increase its activity at saturating concentrations of calcium (Taylor and Exton 1987) . The nature of the postulated GTP binding protein is , however , unresolved and Bonjanic et al (1987) have proposed that guanine nucleotide stimulation of PIP_2 hydrolysis may be a nonspecific effect .

IP_3 , produced by the hydrolysis of PIP_2 , is released into the cytoplasm and is thought to mediate the mobilisation of intracellular calcium stores . DAG is insoluble in water and remains within the plasma membrane where it is thought to directly activate protein kinase c (the mechanisms of each of these second messengers in producing their effects will be dealt with in greater detail under separate headings) . IP_3 is broken down to myo-inositol 1,4-bisphosphate by a specific phosphomonoesterase which exists predominantly in the plasma membrane (Seyfred et al 1984) . Myo-inositol 1,4-bisphosphate is subsequently broken down to myo-inositol 1-phosphate and myo-inositol by two consecutive hydrolysis steps . DAG is metabolised to phosphatidic acid by the action of diacylglycerol kinase , the activity of which may by regulated by an endogenous inhibitor (Jeng et al 1988) . This is then converted to PI in the endoplasmic reticulum by combination with inositol and is subsequently transported to the plasma membrane by a PI exchange

protein . Resynthesis of PIP_2 occurs by consecutive phosphorylation steps by the ATP-requiring enzymes : PI kinase and PIP kinase (reviewed by Berridge 1981) . The sequence of events in hormonal stimulation of inositol phospholipid hydrolysis and subsequent resynthesis is shown in figure 4 .

1.12(d) Elevation of intracellular free calcium .

The breakdown of inositol phospholipids has been linked to the elevation of intracellular free calcium from two main sources. An early effect is the mobilisation of calcium from intracellular calcium stores which appears to be mediated by IP_3 . The main site of the calcium stores mobilised by IP_3 is the endoplasmic reticulum (Joseph and Williamson 1983 ; Burgess et al 1984 ; Joseph et al 1984 ; Thomas et al 1984) and a receptor for IP_3 has been discovered in the permeabilised guinea pig hepatocyte which has been postulated to mediate the calcium ionophore-effects of IP_3 (Spät et al 1986) . This effect may also involve transduction via a GTP binding protein (Mullaney et al 1987 ; Codina et al 1988) . Calcium can also be mobilised from the mitochondria (Murphy et al 1980) and it has been proposed by Joseph and Williamson (1983) that , at low calcium concentrations , calcium is mobilised predominantly from the endoplasmic reticulum whereas at high calcium concentrations it is mobilised predominantly from the mitochondria . Joseph and Williamson (1981) also reported that in addition to mobilisation of internal calcium stores from either the endoplasmic reticulum

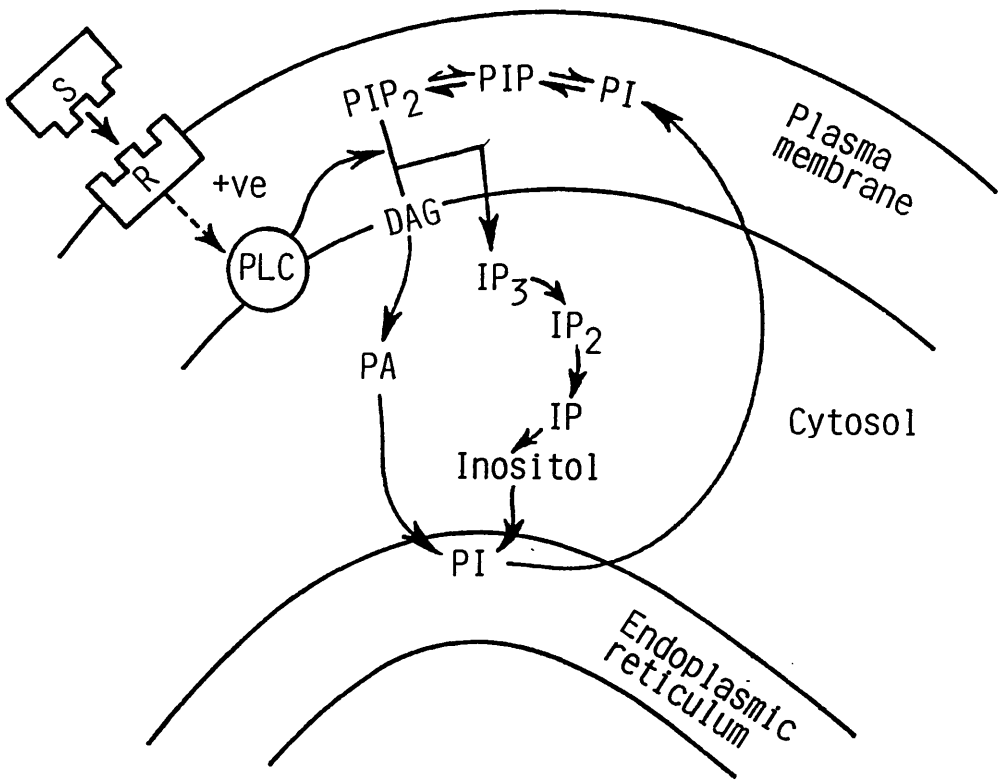

Figure 4 : The sequence of events in hormonal stimulation of inositol phospholipid hydrolysis and subsequent resynthesis , where s is the stimulatory hormone , and R is the hormone receptor . Other abbreviations are as follows : PLC (phospholipase c) , PIP_2 (phospatidylinositol 4,5-bisphosphate) , PIP (phosphatidylinositol 4-phosphate) , PI (phosphatidylinositol) , DAG (diacyglycerol) , IP_3 (inositol 1,4,5-trisphosphate) IP_2 (myo-inositol 1,4,-bisphosphate) IP (myo-inositol 1-phosphate) and PA (phosphatidic acid) .

or the mitochondria , inhibition of calcium efflux from the cell by inhibition of the calcium efflux pump was another early event . The second major source of calcium is from the extracellular medium and influx of extracellular calcium occurs secondary to the mobilisation of calcium from internal stores (Mauger et al 1984). The precise mechanism of calcium influx is at present unknown but it appears that increase in the permeability of the plasma membrane to calcium occurs independently of IP_3 generation (Joseph et al 1984) .

Some workers have argued against a role of inositol phospholipid hydrolysis in elevation of intracellular calcium . In 1982 , Prpic et al postulated that the breakdown of inositol phospholipids occurred too slowly to mediate the rapid elevation of intracellular free calcium and that inositol phospholipid hydrolysis may occur secondary to changes in intracellular calcium. Similarly the phorbol ester , phorbol myristate acetate (PMA) was found to attenuate the effects of the calcium mobilising hormone , angiotensin II upon IP_3 production but to have no effects upon the calcium mobilising effects of the hormone (Johnson and Garrison 1987) . It has been calculated , however , that the increase in IP_3 production necessary to elicit maximum cytosolic free calcium elevation is only 10-15% above control (Thomas et al 1984) . Thus one explanation for the discrepancy noted above by Prpic et al (1982) is that although the maximum elevation of IP_3 occurred at a later time point than calcium mobilisation , the increase in IP_3 concentrations necessary to elicit maximum mobilisation of calcium i.e. 10-15% above basal

levels was sufficiently rapid to account for calcium mobilisation. Similarly , although the angiotensin-mediated elevation of IP_3 concentrations was greatly attenuated by the presence of PMA in the study conducted by Johnson and Garrison (1987) , the levels of IP_3 were still sufficient to elicit maximum mobilisation of calcium and it is now generally accepted that , although inositol phospholipid hydrolysis has a minimum requirement for calcium , it is not mediated by prior elevation of intracellular calcium and is a necessary prerequisite for calcium mobilisation to occur (Thomas et al 1983) although stimulation of calcium influx may be mediated by inositol phospholipid-independent mechanisms .

Following elevation of cytosolic free calcium the signal is terminated by the efflux of calcium from the cell (Williamson et al 1985) and by uptake into the mitochondria (Nicchita and Williamson 1984) and the endoplasmic reticulum where its uptake and internal binding may be controlled in a similar manner to the uptake and binding of calcium to calciquestrin in the sarcoplasmic reticulum of muscle (Damiano et al 1988) .

1.12(e) Calmodulin and calcium/calmodulin-dependent protein kinase .

Many of the effects of calcium in the cell occur following the binding of calcium to a calcium-binding protein known as calmodulin to form a calcium/calmodulin complex . In the absence of calcium , calmodulin is conformationally inactive . It is able to bind up to four calcium ions per molecule and this binding

induces a conformational change in the molecule and allows it to interact with a number of intracellular receptor proteins (Klee et al 1980) . A number of structural features of the receptor protein have been postulated to be necessary for the binding of the calcium/calmodulin complex , namely the ability to form an α helix (Malencik and Anderson 1983) , clusters of basic residues and a hydrophobic region adjacent to the basic residues (Mallencik and Anderson 1982) . The first receptor protein discovered for the calcium/calmodulin complex was cyclic AMP phosphodiesterase (Cheung 1967) . The binding of the complex leads to activation of the enzyme and , since 1967 , calcium/calmodulin has been reported to activate many other cellular receptor proteins e.g. adenylate cyclase (Katada et al 1987) . The activation of adenylate cyclase will lead indirectly to the activation of PKa by elevating cyclic AMP . The calcium/calmodulin complex has also been reported to directly activate a number of protein kinases . The first of these kinases to be discovered were myosin light chain kinase and phosphorylase kinase both of which have very narrow substrate specificities within the cell . Later work , however , has shown that calcium/calmodulin is able to activate a specific calcium/calmodulin-dependent protein kinase which has a much broader substrate specificity and is thought to mediate many of the actions of calcium within the cell (Bennett et al 1983 ; Nairn et al 1985) .

The enzyme is present mainly in brain tissue but is also found in other organs and is composed of two different isozymes : α (Mr 50.000) and β/β' (Mr 58,000-60,000) (McGuiness et al

1985) . The amino acid sequences of both of these isozymes have been elucidated (Hanley et al 1987 , Bennett and Kennedy 1987) and they have been reported to be very similar in structure and function .

The protein kinase isozymes appear to exist as aggregates of twelve kinase molecules consisting of 9 α isozymes and 3 β/β' isozymes (Bennett et al 1983) arranged as two hexameric rings stacked one above the other (Woodgett et al 1983) .

The calcium/calmodulin-dependent protein kinase differs from PKa in that the inhibition of the kinase in its non-activated state is intrinsic to the same molecule that contains the catalytic site and is not mediated by a separate regulatory protein (Payne et al 1988) .

Calcium/calmodulin binding proteins are also found in the nucleus of hepatocytes and although not bound directly to DNA itself they are associated with active DNA and may regulate its function by some mechanism which appears to result in the decrease of the phosphorylation of proteins in some fractions but which is not thought to involve the activation of a protein phosphatase (Bachs and Carafoli 1987) (see section 1.15) .

1.12(f) Diacylglycerol and protein kinase c .

Diacylglycerol (DAG) is water insoluble and is produced in the plasma membrane where it is retained within the lipid bilayer (Takai et al 1979) . In 1977 a proteolytically-activated protein kinase was identified and named protein kinase c (PKc)

(Takai et al 1977) . This protein kinase was later shown to be dependent upon calcium ions and a phospholipid for its activation (Nishizuka 1983) . DAG was shown to increase the affinity of the enzyme for calcium ions and produce full activation of the protein kinase without a net increase in calcium concentration (Kaibuchi et al 1983) . Later work has shown that PKc is not a single enzyme but is a family of subspecies with closely related structure , mode of activation , kinetic properties and substrate specificities (Ido et al 1987 ; Sekiguchi et al 1987) .

One of the problems encountered when studying the activation of PKc by DAG is the inability of DAG to penetrate the plasma membrane . The tumour promoting phorbol esters , such as phorbol myristate acetate (PMA) , however , are structurally very similar to DAG and have been shown to activate PKc in vitro and in vivo (Castagna et al 1982) . It is now generally accepted that the receptor for the phorbol ester is PKc (Kikkawa et al 1983 ; Niedel et al 1983) and like DAG , phorbol esters activate PKc by increasing the affinity of the enzyme for calcium . Much of the work carried out upon the activation and the intracellular mechanisms of PKc , therefore , have utilised the PKc-activating properties of the phorbol esters . One problem with the use of the phorbol esters in the study of PKc activation and mechanisms is their stability to breakdown within the cell in contrast to DAG , which is present only transiently in membranes . The phorbol esters , therefore , may extend a phase of cellular response which is limited under physiological conditions . This , to some extent, has been overcome by the development of synthetic

diacylglycerols , such as 1-oleoyl-2-acetylglycerol , which are able to penetrate the cell membrane and activate PKc in a manner analogous to hormonally-stimulated production of DAG and are metabolised rapidly to their corresponding phosphatidic acids and probably to inositol phospholipids (Yamanishi et al 1983) . Activation of PKc by both PMA and hormones which stimulate the plasma membrane production of DAG has been shown to be associated with the translocation of PKc from the cytosol to the plasma membrane (Hernandez-Sotomayor and Garcia-Sainz 1988) although the precise mode of activation is unknown . The enzyme is composed of a single polypeptide chain which contains both a hydrophobic domain , thought to be involved in the binding of the protein kinase to the plasma membrane , and a hydrophilic domain containing the active site (Kikkawa et al 1982) . The membrane phospholipid composition also appears to be critical for activation . Phosphatidylserine has been shown to be indispensible but phosphatidylethanolamine shows positive cooperativity with phosphatidylserine for kinase activation whereas phosphatidylcholine is inhibitory (Kaibuchi et al 1981). Activation of PKc , therefore is favoured by assymetric distribution of phospholipids in the lipid bilayer .

1.12(g) Generation of free arachidonic acid .

Most of the phosphatidylinositol found in mammalian tissues contain arachidonic acid at position 2 and , thus , diacylglycerol and phosphatidylinositol may both liberate free

arachidonic acid directly by two differing routes (Reviewed by Irvine 1982) . Phosphatidylinositol (and other phospholipids such as phosphatidylethanolamine and phosphatidylcholine) is cleaved at the sn-2-acyl bond to produce equimolar amounts of lysophospholipid and free fatty acids (including arachidonic acid) by phospholipase A_2 (Chang et al 1987) . Diacylglycerol produced by hormonal stimulation of inositol phospholipid hydrolysis by phospholipase c can be further degraded by diacylglycerol lipase to yield glycerol and free fatty acids (including arachidonic acid) . Arachidonic acid has been reported to mobilise intracellular calcium from internal stores in a manner similar to IP_3 (Chan and Turk 1987) and , as arachidonic acid is the precursor for the production of prostaglandins , the elevation of prostaglandins may also contribute to the intracellular transduction of hormones which stimulate inositol phospholipid hydrolysis . Prostaglandins may also be produced indirectly as a consequence of PKc activation as has been reported in the 3T3 tumour cell line (Butler-Gralla et al 1983).

1.12(h) Cyclic GMP .

Cyclic guanosine 3'5'-monophosphate (cyclic GMP) is produced in the cell in an analogous manner to cyclic AMP by the activation of guanylate cyclase and it similarly activates a specific cyclic GMP-dependent protein kinase (PKg) . Guanylate cyclase can be activated by the binding of hormones to specific receptors linked to the enzyme e.g. hepatic muscarinic receptors (Illiano et al

1973) and also by the hormonal stimulation of inositol phospholipid hydrolysis . The exact mechanism of elevation of cyclic GMP by PIP_2 hydrolysis is unclear but it has been proposed that arachidonate peroxide and prostaglandin endoperoxides serve as activators of guanylate cyclase (Graff et al 1978) . Elevation of cyclic GMP and the subsequent activation of PKg , therefore , may contribute to the intracellular effects of hormones which stimulate inositol phospholipid hydrolysis .

1.13 PROTEIN PHOSPHORYLATION AS A REGULATOR OF INTRACELLULAR ACTIVITY .

Activation of specific protein kinases by their corresponding ligands leads to the phosphorylation of intracellular proteins utilising the terminal phosphate of ATP in the presence of magnesium ions . Many key regulatory proteins can be phosphorylated on seryl , threonyl or occasionally tyrosyl residues and this leads to a conformational change occurring within the protein and subsequent alteration of its biological properties . The list of the intracellular phosphate-accepting proteins is extremely long and the proteins currently thought to be phosphorylated by PKc have been reviewed by Nishizuka (1986) and , similarly , Cohen (1985) has reviewed the enzymes which are phosphorylated by PKa and calcium/calmodulin-dependent protein kinases .

PKa , PKc and calcium/calmodulin frequently phosphorylate the

same protein substrates and , in 1978 , Garrison reported that treatment of cells with cyclic AMP analogues increased the phosphorylation of 12 cytosolic proteins and that at least 10 of the same protein substrates could also be phosphorylated by hormones which stimulated inositol phospholipid hydrolysis . It was also reported that four of these proteins could also be phosphorylated by addition of the calcium ionophore , A23187 . In a separate study it was reported that 7 of the 10 proteins phosphorylated by stimulation of inositol phospholipid hydrolysis were also phosphorylated by addition of A23187 whereas the remaining 3 were phosphorylated by addition of PMA indicating that their phosphorylation was mediated by activation of PKc (Garrison et al 1984) . Although phosphorylation may occur on the same protein substrates , the phosphorylation sites are usually , although not always , distinct and so the biological changes caused by phosphorylation , attributed to the different protein kinases , may not be identical . For example PKa , PKc and calcium/calmodulin all increase the phosphorylation of glycogen phosphorylase (Cohen 1985 ; Nishizuka 1986) , an important glycogenolytic enzyme . Phosphorylation by PKa and calcium/ calmodulin results in activation of the enzyme . PKc , however , has been reported to have no effect upon the activation of glycogen phosphorylase (Garrison et al 1984) but may have a synergistic effect upon the activation of this enzyme with calcium (Fain et al 1984) .

Extensive work has shown that the amino acid sequence in the vicinity of the phosphorylation site is a primary factor for

substrate recognition (Reviewed by Krebs and Beavo 1979) and phosphorylation of differing proteins or of differing sites on the same protein may , therefore , be attributable to the different substrate recognition sites of the protein kinases . The best documented of the protein kinase recognition sites is that for PKa which is thought to require two or more adjacent basic residues amino-terminal to the phosphorylatable residue (Cohen 1985) . Information about recognition sites for the other protein kinases , however , is limited .

1.14 THE ROLE OF PROTEIN PHOSPHORYLATION IN SECOND MESSENGER TRANSDUCTION .

In addition to mediating the response of the cell to hormones and their corresponding second messenger systems , phosphorylation of proteins is also thought to be the underlying mechanism mediating the effects of second messengers upon their own transduction (feedback) and upon the transduction of other second messengers (interaction or "cross-talk") .

1.14(a) Negative feedback systems .

Both PKa and PKc exert a negative feedback effect upon the pathways that generate them .

PKa is thought to phosphorylate and activate cyclic AMP phosphodiesterase (Loten et al 1978 ; Heyworth et al 1983 ; Corbin et al 1985) and phosphorylate and inhibit adenylate

cyclase (Constantopoulos and Najjar 1973) . The overall effect , therefore , will be the lowering of cyclic AMP levels . PKa may also have an inhibitory or desensitising effect upon the transmembrane transduction of cyclic AMP-elevating hormones (β adrenergic agonists/glucagon) by phosphorylating the receptor (Sibley et al 1984) . This is thought to modulate the interaction between the receptor and Ns (Heyworth and Houslay 1983a ; Heyworth et al 1984a ; Bouvier et al 1987) and/or decrease agonist binding (Refnes et al 1987) .

In similar manner to PKa , PKc is also thought to inhibit the hormonal stimulation of inositol phospholipid hydrolysis (α_1 adrenergic agonists) (Garcia-Sainz et al 1985 ; Van de Werve et al 1985) and this has been reported to be due to down regulation of the receptor (Cooper et al 1985 ; Corvera et al 1986) perhaps involving phosphorylation of the putative transducing GTP binding protein (Blackmore and Exton 1986) and uncoupling of the receptor from phospholipase c (Leeb-Lundberg et al 1985) . Cyclic GMP produced as a result of PIP_2 hydrolysis also appears to exert a negative feedback effect upon further hydrolysis of PIP_2 to DAG by stimulating the phosphorylation of two proteins (Takai et al 1981) and this is thought to be a major route of negative feedback in platelets and smooth muscle (Schultz et al 1977) . Its relevance in other tissues and mechanisms of action , however , are unclear .

1.14(b) Interactions between inositol phospholipid-degrading and cyclic AMP-generating pathways .

Many workers have reported that cyclic AMP elevation leads to an increase in calcium influx (Mauger et al 1985 ; Staddon and Hansford 1986) and this has been proposed to be due to the phosphorylation of a site near to or on the calcium gating channels (Poggioli et al 1986) . Cyclic AMP may also increase the receptor binding of hormones which stimulate inositol phosphate hydrolysis (α_1 adrenergic agonists) (Morgan et al 1984) although reports of the effects of cyclic AMP upon inositol phosphate hydrolysis directly , vary . In hepatocytes cyclic AMP has been reported to stimulate PIP_2 hydrolysis by phosphorylation of a GTP binding protein (Blackmore and Exton 1986) , inhibit PI turnover by inhibiting PI kinase (see section 1.12(c)) (O'Shea et al 1987) , although this was thought to be a non-specific effect of adenine nucleotides , and to have no effect upon PIP_2 hydrolysis (Kaibuchi et al 1982) , although the same study reported inhibition of PIP_2 hydrolysis in lymphocytes . Cyclic AMP has also been reported to have an inhibitory effect upon the mobilisation of calcium from internal stores perhaps by stimulating mitochondrial uptake of calcium (Morgan et al 1983a). Interaction also exists between PKa and the protein kinases activated as a result of inositol phospholipid hydrolysis . PKa inhibits the activation of PKc in skeletal muscle myoblasts (Narindrasorasak et al 1987) . In the GH_4 pituitary cell tumour line , however , cyclic AMP has been postulated to produce some of

its nuclear effects by stimulating the activity of a calcium-dependent protein kinase although the exact nature of this protein kinase is unclear (Waterman et al 1985) .

In rat hepatocytes , PKc (activated by PMA) inhibits the activation of adenylate cyclase (Heyworth et al 1985) and the hormonal (glucagon) stimulation of cyclic AMP accumulation (Garcia-Sainz et al 1985) . This effect is thought to be mediated by disruption between the catalytic subunit of adenylate cyclase and Ns (Heyworth et al 1984b) . In turkey erythrocytes phorbol esters also desensitise the effects of stimulatory hormones (β-adrenergic agonists) upon adenylate cyclase by phosphorylation of the receptor (Kelleher et al 1984) . Hydrolysis of PIP_2 may also have an indirect negative effect upon hormonal stimulation of cyclic AMP accumulation by the secondary elevation of arachidonic acid and prostaglandins (Bronstad and Christoffersen 1981 ; Brass et al 1987) although this is unlikely to represent a protein phosphorylation-mediated interaction between inositol phospholipid hydrolysis and cyclic AMP generation . Phorbol esters have also been reported to inhibit cyclic AMP phosphodiesterase in hepatocytes by phosphorylating the enzyme (Irvine et al 1986) . This is in direct contrast to the effect of PKa-mediated phosphorylation upon the same enzyme , which has been reported to activate the phosphodiesterase (Corbin et al 1985) and so may be another example of dual control of an enzyme by phosphorylation at different sites . In contrast to the effects in hepatocytes , PKc activation by PMA has been reported to enhance the activation of adenylate cyclase in response to cyclic AMP-elevating hormones in

S49 lymphoma cells perhaps by enhancing the interaction between Ns and the catalytic subunit (Bell et al 1985) , and directly activate adenylate cyclase in adipocytes (Naghshineh et al 1986).

1.15 PROTEIN PHOSPHATASES .

The role of protein phosphatases in cellular function has been reviewed by Cohen (1985) and Ingebritsen and Cohen (1983). In addition to phosphorylation of proteins by the activation of the protein kinases , the overall phosphorylation state of any protein *in vivo* is also controlled by the activation states of protein phosphatases which dephosphorylate the phosphoprotein . In contrast to the large number of different protein kinases , relatively few protein phosphatases participate in cellular regulation and these , therefore , have broad substrate specificities . Four protein phosphatases have been identified which are capable of dephosphorylating seryl or threonyl residues. These have been roughly divided into two groups namely protein phosphatase-1 (PP-1) and protein phosphatase-2 (PP-2) which is composed of three subspecies designated PP-2a , PP-2b and PP-2c . PP-1 , PP-2a and PP-2c account for nearly all of the protein phosphatase activity in the liver . PP-1 differs from the group 2 protein phosphatases in that it is a membrane bound enzyme whereas PP-2a and PP-2c are both cytosolic enzymes and also because it can be inhibited by two inhibitor proteins termed inhibitor-1 and inhibitor-2 . Inhibitor-2 exhibits spontaneous inhibitory activity (Goris et al 1978) but inhibitor-1 is

inactive unless phosphorylated at specific threonine residues by PKa . Cyclic AMP , therefore , in addition to activating PKa , also activates an inhibitor of the membrane bound PP-1 which will further enhance the protein phosphorylation effects of PKa . PP-2b (also known as calcineurin) appears to be active mainly in the dephosphorylation and subsequent inactivation of inhibitor-1 . PP-2b consists of a catalytic subunit and a calcium binding subunit which is structurally related to calmodulin (Aitkin et al 1984) and the binding of calcium is essential for the activation of PP-2b (Tonks and Cohen 1983) . Thus calcium antagonises the effects of PKa and other protein kinases upon protein phosphorylation by the activation of the membrane bound PP-1 . In addition to the indirect effects of PP-2b upon the activation of PP-1 , Yang et al (1987) have also reported the existence of a directly acting activator of PP-1 in the liver which has been called Fa and which has been found to be a membrane-bound protein kinase . This mechanism , therefore , may represent a form of negative feedback between activation of the kinase and termination of the phosphorylation effects by activation of the phosphatase . The exact nature of the endogenous ligand which activates Fa , however , was not apparent in this study .

1.16 INTRACELLULAR TRANSDUCTION OF HORMONES .

1.16(a) Hormonal action at target cells .

Hormones can be divided into three categories depending upon their mode of action in target cells .

(1) Hormones acting at a plasma membrane receptor . These hormones are unable to penetrate the plasma membrane and produce their effects by alteration of the synthesis , release or activation of intracellular second messengers stimulated by the binding of the hormone to a plasma membrane receptor . This category includes adrenaline , glucagon , insulin and the pituitary hormones including growth hormone .

(2) Hormones acting at an intracellular receptor and having no effect upon intracellular second messengers . These hormones penetrate the plasma membrane and bind to a cytosolic/nuclear receptor and the intracellular effects are produced by the interaction between the hormone/receptor complex and DNA to induce (or inhibit) the synthesis of intracellular proteins . This category includes testosterone and oestrogen and , in most target organs , glucocorticosteroids . The transduction of the hormones will not be discussed in detail but have been reviewed (King 1987 ; Lax 1987 ; Eisenfeld and Aten 1987) .

(3) Hormones acting at an intracellular receptor and having additional effects upon second messenger responsiveness to plasma membrane hormones . These hormones have identical effects upon protein synthesis as listed in category (2) but , in addition , have indirect effects upon second messenger systems . Hormones in this category include glucocorticosteroids in the liver and thyroid hormones .

1.16(b) Catecholamines .

Three different types of receptor for the catecholamines exist on the plasma membrane of liver cells . β_2-Adrenergic receptors have a stimulatory effect upon the activity of adenylate cyclase via Ns and agonist binding , therefore , results in the elevation of intracellular cyclic AMP (Exton et al 1971) . α_1-Adrenergic receptors are linked to stimulation of inositol phospholipid hydrolysis and agonist binding , therefore, results in the subsequent mobilisation of calcium and activation of PKc (Birnbaum and Fain 1977 ; Tolbert et al 1980 ; Studer and Borle 1982) . α_2-Adrenergic receptors have an inhibitory effect upon adenylate cyclase activity and subsequent cyclic AMP accumulation and act through the inhibitory GTP binding protein , Ni (Jard et al 1981) . Adrenaline has agonist activity at each of these receptors and the overall effect upon second messenger systems is dependent upon the relative densities of each of the receptors on the plasma membrane and/or their sensitivities . Noradrenaline , however , is much more specific

for α_1-receptors and therefore produces its effects by stimulation of inositol phospholipid hydrolysis rather than by elevation of cyclic AMP (Garrison and Wagner 1982) . Sex differences exist in the hepatic cyclic AMP-elevating effects of adrenaline (Bitensky et al 1970) and this is thought to be due to differences in the relative densities of α_1- and β_2-receptors on the hepatocyte plasma membrane in that females have a much higher ratio of β_2-receptors to α_1-receptors than do males , or that the sensitivity of the hepatocyte β_2-receptors is lower in the male than in the female (Studer et al 1984) , and therefore the cyclic AMP accumulation produced by adrenaline is much higher in the female than in the male . Close links exist between the coupling of β_2- and α_1-receptors to their respective intracellular pathways and it has been reported that in calcium-depleted hepatocytes , α_1-receptors become linked to the cyclic AMP-generating pathway (Chan and Exton 1977) and this has been attributed to calcium-mediated inhibition of the coupling of α_1-receptors to adenylate cyclase in calcium-replete cells . A similar effect has been reported in the aging male rat (Morgan et al 1983b ; Morgan et al 1983c) and may be due to a drop in testosterone levels (Bitensky et al 1970) . In these models , noradrenaline may elevate cyclic AMP via its actions at α_1-receptors .

In 1971 Exton et al reported that in the perfused male rat liver , adrenaline mediated a 4 fold increase in cyclic AMP levels after 1 minute which rapidly declined back to basal levels and they postulated that the stimulation of glycogenolysis by

adrenaline was mediated via β_2 receptors and a rise in cyclic AMP . It is now more generally accepted , however , that in the adult male rat adrenaline-mediated elevation of cyclic AMP is functionally unimportant for the stimulation of glycogenolysis (Birnbaum and Fain 1977 ; Studer and Borle 1982 ; Morgan et al 1983b ; Studer et al 1984) . Elevation of cyclic AMP , however , is thought to mediate adrenaline-stimulated induction of ornithine decarboxylase and its associated messenger RNA (Evoniuk et al 1985) and adrenaline-stimulated phosphorylation and subsequent activation of fructose-1,6-biphosphatase (Nilsson-Ekdahl and Ekman 1987) .

1.16(c) Glucagon .

It is well established that one of the actions of glucagon is the stimulation of adenylate cyclase and elevation of cyclic AMP (Exton et al 1971) and in the isolated perfused male rat liver, glucagon (10^{-8}M) was reported to increase the levels of cyclic AMP 11-fold after one minute and 60-fold after 5 minutes . Both the metabolic effects upon glycogen and glucagon-mediated induction of ornithine decarboxylase (Evoniuk et al 1985) have been attributed to the cyclic AMP-elevating effects of glucagon.

More recently it has been shown that glucagon also increases intracellular free calcium levels (Studer et al 1984 ; Staddon and Hansford 1986) by both promoting its mobilisation from the endoplasmic reticulum (Bond et al 1987) and also by stimulating the influx of extracellular calcium (Mauger et al 1985 ; Poggioli

et al 1986) . Glucagon may produce its effects upon intracellular calcium by either a cyclic AMP-dependent or a cyclic AMP-independent mechanism . It has been shown that the ability of glucagon to elevate intracellular calcium levels can been mimicked by forskolin (which directly activates adenylate cyclase) and by the cyclic AMP analogue , 8-bromo-cyclic AMP (Staddon and Hansford 1986) . Angiotensin II acting at the A_2 receptors (linked by Ni to the inhibition of adenylate cyclase) blocks both the glucagon-mediated elevation of cyclic AMP and its effects upon calcium levels (Morgan et al 1983d) . Similarly the cyclic AMP antagonist : (Rp)-cAMP also blocks the effects of glucagon upon calcium levels (Connelly et al 1987) and in 1983 Creba et al reported that glucagon had no effect upon the hydrolysis of the inositol phospholipids and thus appears to have no direct effects upon the pathway which would lead to the generation of IP_3 . These findings imply that the glucagon-mediated elevation of calcium occurs secondary to elevation of cyclic AMP .

In contradiction to the above reports it has been suggested that the stimulation of glycogen phosphorylase activity by glucagon occurs at a lower concentration than that required to elevate cyclic AMP levels (Birnbaum and Fain 1977) and it has also been reported that the activation of PKc by PMA inhibits the glucagon-mediated elevation of cyclic AMP but not its effects upon ureogenisis (Garcia-Sainz et al 1985) . Similarly the glucagon analogue , (1-N^{α}-trinitrophenylhistidine,12-homoarginine)glucagon (TH-glucagon) mimics the glucagon-mediated stimulation of glycogenolysis (Corvera et al 1984) and

inhibition of steroid metabolism (Hussin et al 1988) but has no stimulatory effect upon cyclic AMP levels . These finding imply that the calcium-mobilising effects of glucagon occur independently of its effects upon cyclic AMP levels and it has been proposed that two classes of plasma membrane receptor mediate the intracellular effects of glucagon , namely : GR-1 linked to inositol phospholipid hydrolysis and GR-2 linked to cyclic AMP generation (Wakelam et al 1986) .

1.16(d) Insulin .

One of the short term effects of insulin is a lowering of intracellular cyclic AMP mediated by stimulation of cyclic AMP phosphodiesterase (Loten et al 1978 ; Heyworth et al 1983 ; Heyworth et al 1984c ; Beebe et al 1985 ; Heyworth et al 1985) and inhibition of adenylate cyclase (Heyworth and Houslay 1983b ; O'Brien et al 1987) . Both of these effects are thought to be mediated via a GTP binding protein although they do not appear to share a common pathway (Weber et al 1987) . Insulin also lowers the effects of cyclic AMP by inhibiting the effects of PKa (Marks and Parker-Botelho 1986 ; Malchoff et al 1987) and by activation of one or several protein phosphatases (Thomas et al 1985) which oppose the cyclic AMP-mediated phosphorylation of proteins .

Insulin also affects other intracellular transduction pathways in addition to attenuation of cyclic AMP levels . In the liver insulin increases the levels of cyclic GMP and this effect

is not blocked by atropine which indicates that insulin-mediated elevation of cyclic GMP is not due to actions at the muscarinic receptor (Illiano et al 1973) and some of the effects of insulin in rat adipocytes have been proposed to be mediated by activation of PKc (Cherqui et al 1987 ; Pershadsingh et al 1987 ; Smal and De Meyts 1987) . This is accompanied by activation of a phospholipase c (Koepker-Hobelsberger and Wieland 1984) . However , it has been reported that insulin does not itself stimulate inositol phospholipid degradation in adipocytes but may enhance the increase in PIP_2 hydrolysis mediated by α_1 agonists by stimulating the de novo synthesis of PI and PIP_2 (Pennington and Martin 1985) . In hepatocytes , however , insulin appears to have no effect upon α_1 agonist-mediated PIP_2 hydrolysis (Creba et al 1983 ; Pushpendran et al 1984) and attenuates their effects upon intracellular calcium levels (Strickland et al 1980 ; Thomas et al 1985) although it has no effect upon the calcium mobilisation mediated by either vasopressin or A23187 (Dehaye et al 1981) .

In addition to activation of phosphatases , insulin also activates a number of protein kinases e.g. ribosomal protein S6 kinase , protease-activated kinase and a cytosolic serine kinase (Blackshear et al 1987 ; Yu et al 1987) and increases the phosphorylation of a number of hepatic proteins (Le Cam 1982) although it has been reported to affect only cytosolic proteins (Vargas et al 1982) .

The mechanism by which insulin mediates its effects is , as yet , unclear . The insulin receptor is composed of two subunits

, the α subunit which binds insulin (Yip et al 1978) and the β subunit which possesses tyrosine kinase activity (Shia et al 1983) . Phosphorylation by the β subunit may mediate the activation of an inhibitory GTP binding protein as has been reported for purified Ni from rat brain and may have some relevance in insulin-mediated inhibition of adenylate cyclase (O'Brien et al 1987) . A number of reports have been made that the insulin-mediated attenuation of cyclic AMP levels can be mimicked by a peptide or glycopeptide intracellular mediator which can be isolated from insulin treated cells (Caro et al 1983 ; Zhang et al 1983 ; Malchoff et al 1987) . Alternatively a phospho-oligosaccaride polar head group of an insulin-sensitive glycolipid has been isolated from hepatocytes which mimics insulin-mediated inhibition of glucagon-stimulated cyclic AMP levels (Alvarez et al 1987) and inhibits PKa (Villalba et al 1988) . A unifying theory of insulin action has been proposed by Brautigan and Kuplic (1988) which postulates that phosphorylation of a GTP binding protein by the tyrosyl kinase activity of the insulin receptor β subunit , leads to the activation of a specific phospholipase c and the generation of two signals (a) myristate diacyglycerol which may activate PKc in a manner identical to arachidonyl diacylglycerol produce by hydrolysis of PIP_2 ; and (b) inositol phosphoglucosamine glycan which may activate a number of enzymes including cyclic AMP phosphodiesterase and protein disulphide isomerase . This latter enzyme may catalyse rearrangement of the disulphide bonds in phosphatase-1 (PP-1) thereby stabilising and subsequently

activating it .

1.16(e) Growth hormone .

Although growth hormone receptors are present on the plasma membrane of liver cells (Baxter and Zaltzman 1984) little is known of the intracellular transduction mechanism for this hormone . The growth stimulatory effects of growth hormone are thought to be mediated by the growth hormone-stimulated induction of somatomedins in the liver and other tissues . One of these somatomedins , somatomedin c is also known as insulin like growth factor-1 (IGF-1) (Isaksson et al 1985) . However , although the liver is the main site of IGF-1 production , production of IGF-1 can be directly stimulated in the brain by a direct action of growth hormone and is thought to mediate the effects of growth hormone upon various neuronal growth processes (Hynes et al 1987). In the liver , growth hormone is thought to act directly to elevate levels of IGF-mRNA (Norstedt and Möller 1987) perhaps by derepression of chromatin DNA and enhancement of RNA polymerase activity (Spelsberg and Wilson 1976) . Although the growth stimulatory effects of growth hormone are thought to be mediated by somatomedins , the other actions of growth hormone are thought to be mediated directly by somatomedin-independent mechanisms . Receptor populations for growth hormone may be heterologous as there appear to be different structure/activity requirements for direct effects and IGF-inducing effects of growth hormone (Isaksson et al 1985) . Growth hormone increases the

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phosphorylation states of a number of protein substrates in isolated rat hepatocytes and this action is not thought to be due to stimulation of IGF production (Yamada et al 1986) . The identity of the protein kinase (or combination of protein kinases) which mediates growth hormone-stimulated protein phosphorylation has not been elucidated although growth hormone has been reported to mediate some of its direct actions in adipocytes by activation of PKc (Smal and De Meyts 1987) . Growth hormone has also been reported to increase the levels of cyclic GMP which may lead to activation of PKg . The elevation of cyclic GMP occurs concomitant with a decrease in the levels of cyclic AMP and these effects are thought to be due to direct interaction with adenylate cyclase or guanylate cyclase (Isaksson et al 1985) .

1.16(f) Glucocorticosteroid effects in the liver .

The glucocortisteroids are identical to the sex steroids in that they enter the cell and bind to a cytosolic receptor . This receptor is similar to the receptor for the sex steroids (reviewed by King 1987) and can be phosphorylated . The role of receptor phosphorylation is unclear , however , and although phosphorylation has been proposed to be important for some of the actions of the glucocorticosteroids (Perisic et al 1987) , phosphorylation by PKa is not required for the function of the receptor in the induction of tyrosine aminotransferase (Schmid et al 1987) . The receptor is , however , able to interact with

ATP and this enhances its activation by the glucocorticosteroids but this effect is not thought to be due to phosphorylation as it can be mimicked by non-hydrolysable analogues of ATP (Diehl and Schmidt 1987) . Once binding has occurred the glucocorticosteroid/receptor complex is translocated into the nucleus and increases transcription by interacting with regulatory DNA regions 5' to the structural gene (King 1987) . Unlike the sex steroids, however , glucocorticosteroids exert permissive control over cyclic AMP generation and inositol phospholipid degradation in determining the responses of these intracellular events to circulating catecholamines . This permissive control is thought to be mediated via inhibition of phospholipase A_2 and a subsequent decrease in the metabolism of the phospholipids to arachidonic acid and prostaglandins and it has been postulated that this is the mechanism by which β_2 adrenergic receptor activity is suppressed in the male rat (Kunos and Ishac 1987) . This suppression may be mediated by Ni (Itoh et al 1984) or alternatively may be due to a deficiency of Ns linking the β_2 receptor to adenylate cyclase (Goodhardt et al 1982) . When hepatocytes are cultured or the animal is adrenalectomised , this inhibitory effect is lost and the activity of β_2 adrenergic stimulation of adenylate cyclase and the elevation of cyclic AMP is enhanced with a concomitant decrease in the activity of the α_1 receptors (Studer and Borle 1984 ; Kunos et al 1984 ; Kunos and Ishac 1987) . This effect can be reversed by the administration of glucocorticosteroids (Bitensky et al 1970) and does not appear to be due to a change in the coupling of α_1 receptors from

PIP_2 hydrolysis to adenylate cyclase (Chan et al 1979) . It differs , therefore , from the mechanism proposed for the changes in the receptor coupling which occur in the aging rat and in calcium-depleted hepatocytes (see section 1.16(b)) and has been proposed to be due to changes in the levels of the GTP binding proteins which are postulated to couple the receptors to their respective intracellular pathways (Goodhardt et al 1982) .

1.16(g) Thyroid hormones .

Thyroid hormones have little direct effect upon intracellular cyclic AMP levels or PKa activation (Müller and Seitz 1987) but increase the sensitivity of the target cells to the cyclic AMP-elevating effects of the circulating catecholamines and glucagon . This is well documented in the heart (Tsai and Chen 1978) and adipocytes (Malbon et al 1978) where the β adrenergic responses to catecholamines are blunted in hypothyroidism and enhanced in hyperthyroidism . In the liver , however , it has been reported that the effects of thyroid hormones are opposite to those seen in the heart and in adipocytes (Malbon 1980) and that thyroid hormones blunt the activation of glycogen phosphorylase by adrenaline (Malbon and Lo Presti 1981). This may , however , be due to the exhaustion of substrates for these metabolic pathways as Müller and Seitz (1987) reported that , in the presence of unlimited substrate , hyperthyroidism resulted in enhancement of glucagon-mediated effects upon gluconeogenesis in the liver which correlates with the effects of

hyperthyroidism in the heart and in adipocytes . The changes in the sensitivity of target cells to glucagon and catecholamines may be due to (a) alteration in receptor number and binding which is increased in hyperthyroidism and decreased in hypothyroidism (Tsai and Chen 1978 ; Ali et al 1987) ; (b) alteration in the levels of the α subunit of Ni which is elevated in hypothyroidism (Milligan et al 1987) and (c) alteration in the activity of cyclic AMP phosphodiesterase which is enhanced by hypothyroidism (Gumaa et al 1977) and blunted by hyperthyroidism (Morgan et al 1982) .

Thyroid hormones also exert permissive control over α_1 adrenergic function and upon the effects of calcium-mobilising hormones (e.g. vasopressin and angiotensin II) . α_1 adrenergic receptors and receptors for the calcium mobilising hormones are reduced (Preiksaitis et al 1982 ; Ali et al 1987) and the calcium mobilising actions of these hormones are slower and of less magnitude in the hypothyroid rat (Storm and Van Hardeveld 1986) .

Thyroxine does not appear to produce its effects by binding to a plasma membrane receptor but has been reported to be taken up into the cell by a saturable , stereospecific , sodium ion-independent carrier system (Blondeau et al 1988) . Receptors for triiodothyronine (the active form of thyroxine) are attached to the DNA nuclear matrix (Venkatraman and Lefebvre 1987) and are concentrated in fractions which contain the most endogenous RNA polymerase activity (Spindler et al 1975) . Binding differs from that of the steroids in that cytosolic binding does not occur prior to triiodothyronine action in the nucleus and is not

necessary for its action (Degroot et al 1976) . By binding to its nuclear receptors , triiodothyronine induces an increase in the number of messenger RNAs in the liver , the synthesis of which are blocked by cycloheximide thus implying that short lived proteins act as intermediate transducers of triiodothyronine action (Hamblin et al 1987) . Thyroxine stimulation of gene transcription has been proposed as the mechanism by which triiodothyronine increases the activity of a number of enzymes involved in carbohydrate metabolism in the liver (Böttger et al 1970) and stimulates the synthesis of growth hormone in the pituitary gland (Mirell et al 1987 ; Santos et al 1987) . No evidence exists , however , for a role of protein induction in the membrane effects of thyroxine/triiodothyronine e.g. increase in adenylate cyclase sensitivity to hormones and subsequent cyclic AMP accumulation . In rat thymocytes thyroxine promotes the rapid influx of external calcium and it has been proposed that calcium/calmodulin mediates the extranuclear/plasma membrane effects of thyroxine in these cells (Segal 1988) . Thyroxine-stimulated influx of calcium , therefore , may be a mechanism by which thyroxine mediates its plasma membrane effects in other cell types .

1.17 INDICATIONS FOR A ROLE OF CYCLIC AMP IN HEPATIC MONOOXYGENASE REGULATION .

1.17(a) Inhibition .

It is apparent from the previous sections that hormones or hormone deficiency states which lead to feminisation or inhibition of monooxygenase activity (thyroid hormones , diabetes , adrenalectomy , glucagon and adrenaline) tend to increase intracellular cyclic AMP levels directly or indirectly as one of their actions whereas insulin which increases monooxygenase activity when added directly to hepatocytes (Hussin and Skett 1987) has been reported to decrease intracellular cyclic AMP levels (Heyworth et al 1985 ; O'Brien et al 1987) (section 1.16(f)) . More direct evidence for a role of cyclic AMP in the regulation of monooxygenase activity was provided by Weiner et al (1972a) who reported that *in vivo* administration of the cyclic AMP analogue , dibutyryl-cyclic AMP inhibited the metabolism of hexobarbitone . Similarly *in vivo* administration of dibutyryl-cyclic AMP has also been reported to inhibit the phenobarbitone-mediated induction of cytochrome P450 (Hutterer et al 1975) and has been implicated as the intracellular mediator of the inhibitory effects of the Walker 245 carcinoma toxohormones upon the metabolism of p-chloro-N-methylaniline (Olson and Weiner 1980) . One of the problems encountered when using *in vivo* studies is the possibility that cyclic AMP may be producing its effects indirectly by altering hormone levels . This problem was

overcome by Weiner et al (1972b) using the perfused rat liver and liver slices and they reported that in these models , administration of dibutyryl-cyclic AMP inhibited the metabolism of hexobarbitone and p-chloro-N-methylaniline . Similarly , Banhegyi et al (1988) reported that dibutyryl-cyclic AMP inhibited the biotransformation of aminopyrine in isolated mouse hepatocytes . These models eliminate the possibility of indirect effects and indicate that dibutyryl-cyclic AMP produces effects directly at the levels of the liver . Weiner et al (1972b) also noted that the effects of dibutyryl-cyclic AMP upon drug metabolism did not occur in whole liver homogenates and it was postulated that dibutyryl-cyclic AMP did not have a direct effect upon the enzymes themselves but inhibited enzyme activity indirectly via some intracellular intermediate . As cyclic AMP is thought to produce most of its actions through activation of PKa (see section 1.12(a)) , it is conceivable that the identity of this postulated intermediate is PKa .

Pyerin et al (1983) have recently shown that incubation of the purified rabbit cytochrome P450 , P450 LM_2 , with the catalytic subunit of PKa in a reconstituted system , leads to phosphorylation of the cytochrome P450 and the identity of the phosphorylated residue has subsequently been shown to be serine 128 (Pyerin et al 1986a) which is on the cytoplasmic surface of the cytochrome P450 (Nelson and Strobel 1988) . It has been proposed by Jansson et al (1987) that the phosphorylatable residue may be at the site of interaction of cytochrome P450 with cytochrome b_5 . The phosphorylation of the cytochrome P450 has

been reported to convert the cytochrome P450 to cytochrome P420 (Taniguchi et al 1985) with a concomitant decrease in the metabolism of benzphetamine and ethoxycoumarin (Pyerin et al 1984) . These in vitro findings provide further evidence of an indirect effect of cyclic AMP upon monooxygenase activity via activation of PKa . In a later study Pyerin and co-workers (1987) reported that the phosphorylation of cytochrome P450 was not restricted to rabbit cytochrome P450 but that PKa can also phosphorylate several cytochrome P450 isozymes purified from the liver of the rat . In the same study , however , it was reported that many of these same isozymes could also be phosphorylated by PKc and some isozymes could be phosphorylated by PKc and not by PKa . In vitro phosphorylation of cytochrome P450 , therefore , does not appear to be a PKa-specific effect . Furthermore it has also been reported that activation of PKc utilising PMA or 1,2-dioleoylglycerol in isolated rat hepatocytes inhibits the metabolism of androstenedione in a nonspecific manner (Allan and Skett 1988) but mobilisation of intracellular calcium with A23187 (Hussin et al 1988) and incubation of isolated rat hepatocytes with vasopressin or angiotensin (C.J. Allan : personal communication) was reported to have no effect upon the metabolism of steroids . Banhegyi et al (1988) reported , however , that A23187 inhibited the metabolism of aminopyrine in isolated mouse hepatocytes . It appears , therefore , that PKa activation , PKc activation and calcium may all contribute to inhibitory effects upon monooxygenase activity although calcium appears to mediate inhibition in the mouse but not in the rat and

may represent a species difference in regulatory mechanisms .

Paradoxically Pyerin et al (1983) also reported that incubation of rabbit liver cytochrome P450 LM_2 with phosphatases in a reconstituted system inhibited the biotransformation of ethoxycoumarin and this inhibitory action was later proposed to be due to inactivation of NADPH-cytochrome P450 reductase (Pyerin et al 1986b) by the conversion of FMN to riboflavin (Taniguchi and Pyerin 1987) .

Theoretically , therefore , cyclic AMP may inhibit hepatic monooxygenase components by :

(a) Activation of PKa and direct phosphorylation of cytochrome P450 .

(b) Interaction with the components of the phosphatidylinositol hydrolysis pathway and subsequent activation of corresponding protein kinases .

(c) Activation of PKa and secondary activation of phosphatases perhaps involving elevation of calcium as an intermediate step .

A similar mode of regulation for another cytochrome P450 has been reported for the action of PKa and phosphatases upon the activity of cholesterol-7α-hydroxylase , a cytochrome P450-containing microsomal monooxygenase which catalyses the rate limiting step in the conversion of cholesterol to bile acids .

The regulation of this enzyme , however , differs from that proposed for the regulation of xenobiotic/steroid metabolising enzymes in that the enzyme is activated by PKa-mediated phosphorylation and deactivated by phosphatase-mediated dephosphorylation (Sanghvi et al 1981 ; Goodwin et al 1982 ; Holsztynska and Waxman 1987) .

1.17(b) Induction .

It has been reported that the in vivo induction of cytochrome P450 by Arochlor-1254 (Costa et al 1976) , 3-methylcholanthrene and phenobarbitone (Byus et al 1976) is accompanied by elevation of cyclic AMP and activation of PKa . Furthermore , the activation of PKa and the increase in cytochrome P450 levels mediated by 3-methylcholanthrene both required the presence of the polycyclic aromatic hydrocarbon receptor whereas the phenobarbitone-mediated PKa activation and induction of cytochrome P450 occurred independently of the receptor (Manen et al 1978) . The effects of Arochlor-1254 upon cytochrome P450 induction were augmented by the presence of the cyclic AMP phosphodiesterase inhibitor , aminophylline (Costa et al 1976) and it was concluded, therefore , that the elevation of cyclic AMP and the activation of PKa is the mechanism by which inducing agents produce their effects . One of the mechanisms by which cyclic AMP is thought to turn on gene expression is by phosphorylation of acidic nuclear proteins (Johnson and Allfrey 1972) and this is thought to stimulate transcription as correlation exists between the binding of

phosphoproteins to specific initiation sites and the subsequent enhancement of RNA synthesis (Teng et al 1971) which has been reported to occur in the liver within one hour following the *in vivo* administration of cyclic AMP (Dokas and Kleinsmith 1971) . It is interesting to note that phenobarbitone has similarly been reported to stimulate the phosphorylation of acidic nuclear proteins in rat liver nuclei (Blankenship and Bresnick 1974) .

Cyclic AMP has also been implicated in the induction of a number of extrahepatic forms of cytochrome P450 and is thought to mediate ACTH-stimulated induction of cytochrome $P450_{17\alpha}$ (Zuber et al 1985) and of cytochrome $P450_{11\beta}$ (John et al 1985) in bovine adrenocortical cells , LH-mediated induction of cytochrome $P450_{17\alpha}$ and cytochrome $P450_{C17-C20\ lyase}$ in mouse leydig cells (Hales et al 1987 ; Malaska and Payne 1984 ; Anakwe and Payne 1987) and FSH-mediated induction of cytochrome $P450_{aromatase}$ in human adipose stromal cells (Evans et al 1987 ; Steinkampf et al 1987) .

1.18 AIMS OF THE PRESENT PROJECT .

(a) To assess the direct effects of elevating cyclic AMP upon the metabolism of 4-androstene-3,17-dione using indirect methods (8-bromo-cyclic AMP , adrenaline , isobutyl-methylxanthine and forskolin) in isolated rat hepatocytes.

(b) To assess the effects of different concentrations of cyclic AMP upon the metabolism of 4-androstene-3,17-dione when added directly to electropermeabilised rat hepatocytes .

(c) To elucidate the roles of the protein kinases in the actions of cyclic AMP using specific protein kinases inhibitors .

(d) To elucidate the role of protein synthesis in the actions of cyclic AMP .

(e) To determine if the effects of cyclic AMP upon the metabolism of 4-androstene-3,17-dione were due to alteration in the levels of whole cell cytochrome P450 .

(f) To determine if the effects of cyclic AMP upon the metabolism of 4-androstene-3,17-dione were due to changes in the phosphorylation states of the cytochrome P450 isozymes .

We therefore hoped to elucidate the effects of cyclic AMP upon steroid-metabolising enzymes in the liver and the mechanisms by which it produces its effects and in doing so we hoped to determine the physiological and pharmacological roles of cyclic AMP in the regulation of the hepatic biotransformation system .

MATERIALS AND METHODS

2 MATERIALS AND METHODS

Methods used can be separated into three main categories .

(1) Assessment of agent-generated cyclic AMP / cyclic AMP analogue effects upon androstenedione metabolism and cytochrome P450 levels in intact rat hepatocytes .

(2) Assessment of cyclic AMP effects upon steroid metabolism and cytochrome P450 levels in electropermeabilised rat hepatocytes .

(3) Assessment of cyclic AMP effects upon protein phosphorylation in electropermeabilised rat hepatocytes .

The methods used in each of these three categories are summarised in figure 5 .

2.1 ANIMALS .

Mature Wistar rats , bred in the department and weighing 250g-300g were used throughout the study . Male rats were used in every case with the exception of section 2.7 in which female rats were used . The animals were housed in light- and temperature-controlled conditions (lights on 0700-1900 ; 19 ± 1°C) allowed free access to food (CRM Nuts , Labsure , Croydon) and tap water .

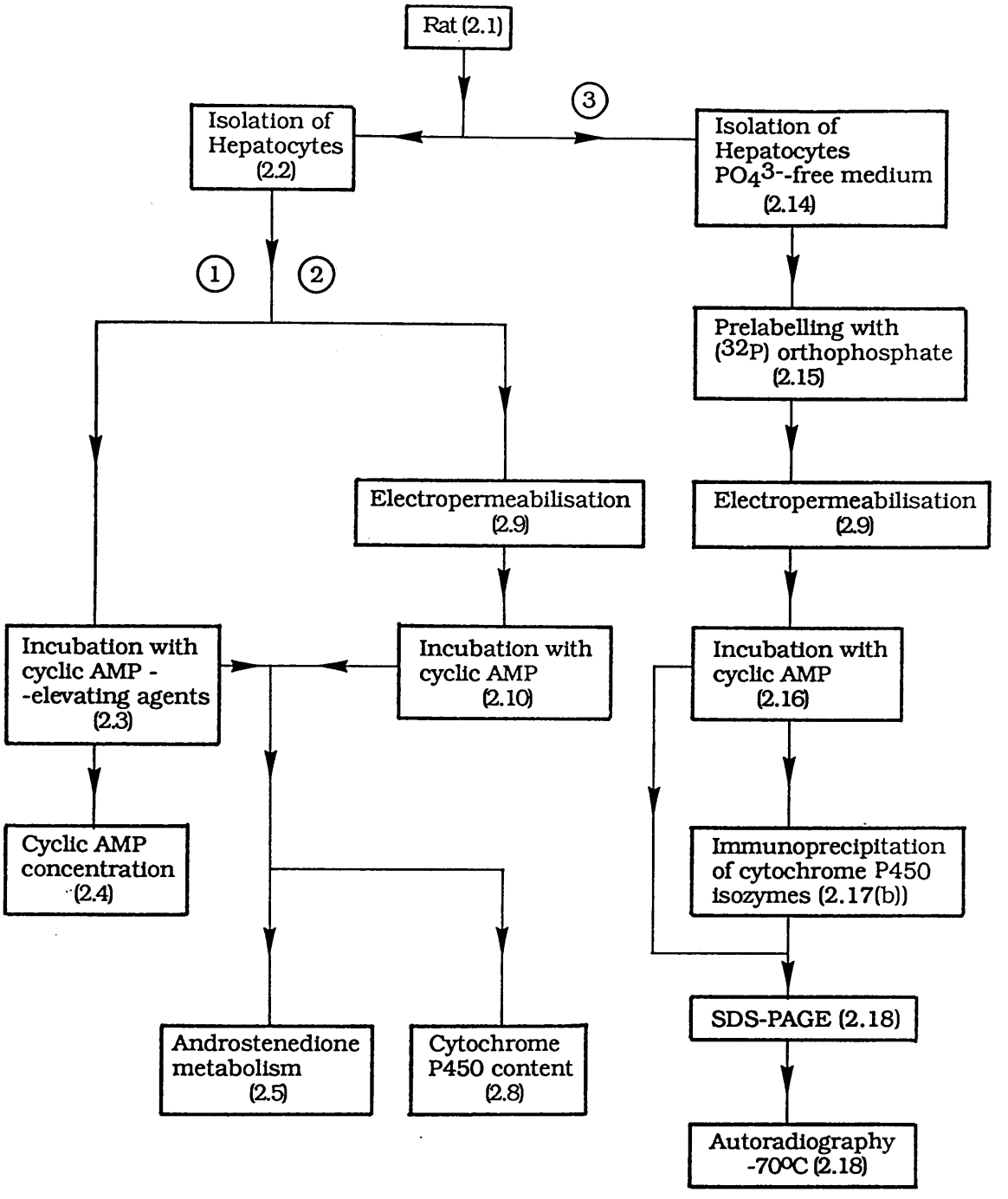

Figure 5 : Summary of methods (Numbers in brackets refer to sections of this thesis in which more detailed explanation of the methods are given) .

To avoid introducing effects due to diurnal variation in hormonal secretory patterns , the rats were sacrificed between the hours of 0900 and 1100 in all cases .

2.2 ISOLATION OF HEPATOCYTES .

Prior to the isolation of hepatocytes , the rat was placed under anaesthesia using halothane 3.5% in oxygen/nitrous oxide 0.4L/0.8L per minute . Hepatocytes were isolated essentially by the two step collagenase perfusion technique of Seglen (1973) . Prior to hepatocyte isolation approximately 500ml of calcium-free Hanks B.S.S. (section 2.19(a)) was gassed for 30 minutes with 95%:5% O_2/CO_2 at 37°C . Throughout the perfusion , temperature of the perfusate was maintained at 37°C and the perfusate was gassed with O_2/CO_2 in the ratio stated above . Following cannulation of the hepatic portal vein , the liver was perfused "in situ" with calcium-free Hanks B.S.S. at a flow rate of 90ml per minute to remove the blood . Following puncture of the vena cava the liver was perfused for a further 7-8 minutes at a flow rate of 60ml per minute to allow break down of the calcium-dependent bonds between the cells . The perfusing buffer was then changed and the liver was perfused for 10-15 minutes with a collagenase buffer containing 60mg of collagenase in 120ml of calcium-free Hanks B.S.S. resupplemented with 4mM of $CaCl_2$. During this time the collagenase buffer was recirculated through the liver . When the liver was judged to be sufficiently digested, the liver was removed from the rat carcase and following the

removal of the liver capsule , the hepatocytes were "combed out" into calcium-free Hanks B.S.S. The hepatocyte suspension was filtered through gauze to remove connective tissue and portions of the liver which remained undigested following perfusion with collagenase and then centrifuged at 300g for 3 minutes in a Damon-IEC DPR 6000 centrifuge to sediment intact cells . The supernatant , containing extraneous cell debris , was removed and the cells were resuspended in Hams F10 culture medium supplemented with 0.1% bovine serum albumin and 100IU penicillin/streptomycin per ml (Hussin & Skett 1987) at a cell density of 3 x 10^7 cells per ml . This method yielded approximately 5 x 10^7 cells per g wet weight liver with a viability , as assessed by trypan blue exclusion , of greater than 97% and negligible contamination by non-parenchymal cells .

2.3 INCUBATION OF INTACT HEPATOCYTES WITH CYCLIC AMP ELEVATING AGENTS .

Freshly isolated hepatocytes were incubated in the presence of the cyclic AMP-elevating agents for times ranging from 2 minutes to 24 hours . For studies involving incubation times of ½ to 24 hours the hepatocytes were plated out into sterile 10cm diameter culture dishes (Nunclon , Denmark) with 10^7 cells in 10ml of Hams F10 supplemented with 0.1% bovine serum albumin and 100IU penicillin/streptomycin per ml . The cultures were placed in an incubator set at 5% CO_2 , 98% humidity and 37°C for 30 minutes before proceeding to allow the cells to recover from the

isolation process . For studies involving incubation times of 2 to 20 minutes difficulties arose when using an incubator for cell incubations . These were overcome by incubating the cell samples as suspensions in 25ml conical flasks maintained at 37°C in a shaking water bath . The cell density and medium was the same as that used during the longer incubation periods and the cell suspensions were similarly placed for 30 minutes in an incubator under identical conditions to recover from the isolation process before being transferred to the water bath . The following additions were added to the cells either in culture dishes or in conical flasks and left for the designated times of between 2 minutes and 24 hours ; (a) 8-bromo-cyclic AMP 10^{-4}M ; (b) adrenaline 10^{-7}M ; (c) isobutylmethylxanthine (IBMX) 10^{-3}M ; (d) adrenaline and IBMX 10^{-7}M/10^{-3}M ; (e) forskolin 10^{-5}M ; (f) forskolin and IBMX 10^{-5}M/10^{-3}M . Incubation with adrenaline (10^{-7}M) was repeated in the presence of prazocin (10^{-5}M) and propranolol (10^{-5}M) to differentiate between α_1- and β-adrenergic receptor-mediated responses . Adrenaline was dissolved as the bitartrate in ascorbic acid solution (10^{-7}M) and forskolin in dimethylsulphoxide (DMSO) . All other compounds were dissolved in distilled water . Control cells received vehicle alone except for those used as control for the addition of adrenaline where sodium tartrate (10^{-7}M) in ascorbic acid solution was used . Following incubation with the agents the cells were removed from the culture plates or flasks and centrifuged at 300g for 3 minutes in a Damon-IEC DPR-6000 centrifuge . The supernatant was then removed and the cells washed in incubation medium (section

2.19(b)) . Viability , as assessed by trypan blue exclusion , was in excess of 90% following each incubation period . The cell suspensions were then centrifuged under the same conditions as above and finally resuspended in either incubation medium , for the determination of androstenedione-metabolising enzyme activity or cytochrome P450 levels , or sodium acetate buffer (section 2.19(c)) for the determination of cyclic AMP levels .

2.4 DETERMINATION OF CYCLIC AMP LEVELS .

Cyclic AMP levels were determined essentially by the radioimmunoassay technique of Brooker et al (1979) . Hepatocytes were suspended in sodium acetate buffer (section 2.19(c)) at a concentration of 1 x 10^7 cells per ml and the cellular proteins were denatured by placing samples in a boiling water bath for 10-15 minutes . The suspensions were subsequently centrifuged at 4000g for 5 minutes in a Damon IEC DPR 6000 centrifuge and the supernatant taken for cyclic AMP determination . Standard concentrations (10^{-10}M -10^{-6}M) of cyclic AMP were prepared in sodium acetate buffer and cyclic AMP content was determined in 100$_\mu$L aliquots of the standards or the unknowns . All samples (including the standards) were acetylated by the addition of 10$_\mu$L of a freshly prepared mixture of triethylamine and acetic anhydride (2:1) followed by rapid vortex mixing . To the acetylated samples were then added 150$_\mu$L of an anti-cyclic AMP antibody (raised in goats against human serum albumin conjugated to succinyl-cyclic AMP) and 100$_\mu$l (approximately 3000 c.p.m.) of

adenosine-3',5-cyclic phosphoric acid 2'-O-succinyl-3-(^{125}I)-iodotyrosine methyl ester in sodium acetate buffer . The samples were then vortex mixed and incubated at 4°C for 16 hours . Following incubation , 0.5ml of a suspension of activated charcoal in 100mM phosphate buffer (section 2.19(d)) containing 0.25% bovine serum albumin was added to each of the samples which were then immediately mixed and centrifuged at 4000g for 4minutes at 4°C in a Damon IEC DPR 6000 centrifuge . 0.6ml aliquots of the supernatant containing antibody bound cyclic AMP were taken and counted in an LKB Gamma counter . Standard displacement curves were constructed for the assay on each occasion of radioactivity bound (c.p.m.) against concentration of cyclic AMP . The amount of cyclic AMP in the unknown samples was subsequently determined and expressed as pmoles of cyclic AMP per 10^6 cells . The specificity of the antiserum was tested by measuring the ability of a range of adenine and guanine nucleotides to compete with(^{125}I)-cyclic AMP for binding to the antiserum . The antiserum was relatively specific for cyclic AMP with 10^3 fold (cyclic GMP) and 10^4 fold (AMP , ADP and ATP) higher concentrations of the other nucleotides required to produce 50% displacement of the (^{125}I)-cyclic AMP .

2.5 DETERMINATION OF ANDROSTENEDIONE METABOLISM .

The steroid 4-androstene-3,17-dione undergoes metabolism by the enzymes of the liver at the sites shown in figure 2

The activities of the enzymes responsible for the metabolism

of 4-androstene-3,17-dione were determined essentially by the method of Gustafsson and Stenberg (1974) . Approximately 10^7 hepatocytes were suspended in 3ml of incubation medium (section 2.19(b)) to which was added $500_{\mu}g$ ($0.1_{\mu}Ci$) of 4-(4-^{14}C)-androstene-3,17-dione and the suspension was incubated for 30 minutes at 37°C in a shaking water bath . The reaction was stopped by the addition of 10ml of Folch (chloroform/methanol 2:1) to each of the samples followed by the addition of 1ml of NaCl solution 0.9% to aid the extraction of the substrate and its metabolites into the organic layer . The organic layer was then removed , evaporated to dryness at 45°C under nitrogen and the residue dissolved in a small quantity of chloroform . Samples were then applied to TLC plates (Merck F254) and developed in chloroform/ethyl acetate (4:1) to separate the metabolites and unchanged substrate . The position of each of the bands on the plate were identified by autoradiography as shown in figure 6 and then the amount of ^{14}C-label in each band (metabolites and substrate) was evaluated by liquid scintillation counting in 5ml of Ecoscint (National Diagnostics , New Jersey) in a Packard Tri-Carb 2000CA counter . The amount of each metabolite , expressed as pmoles of metabolite/min/10^6 cells , was subsequently determined by the following formula :

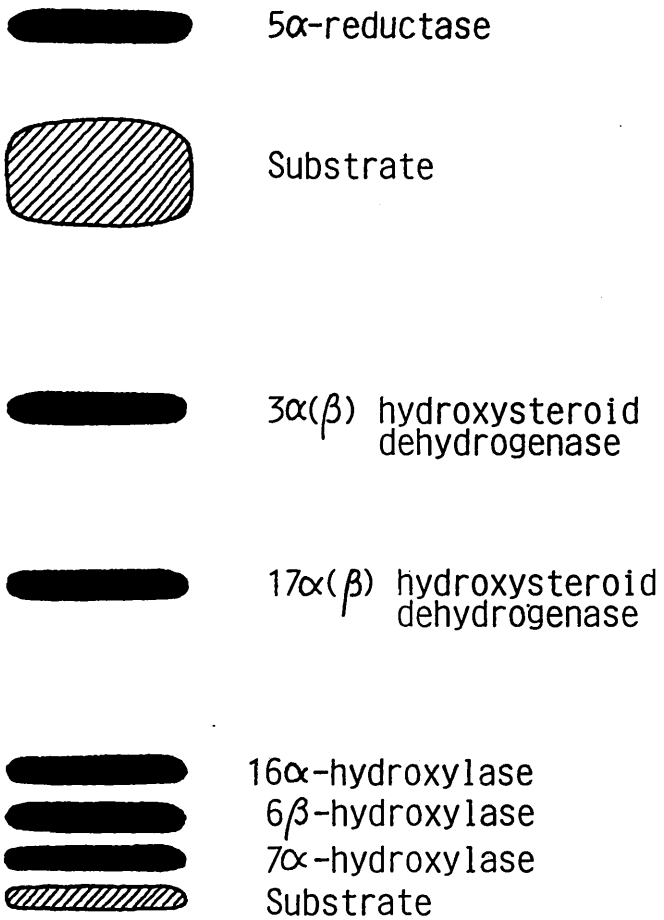

Figure 6 : Diagrammatic representation of the separation of 4-androstene-3,17-dione metabolites by one dimensional thin layer chromatography .

$$\text{pmoles metabolite/min/}10^6\text{cells} = S \times \frac{Cm}{Ct} \times \frac{1}{t} \times \frac{1}{n} \times \frac{1}{MW}$$

S = amount of substrate added (µg)

Cm = c.p.m metabolite

Ct = c.p.m. total

t = incubation time (in minutes)

n = number of cells (divided by 10^6)

MW = molecular weight of the substrate (280) .

It is apparent from figure 6 that six major metabolites of androstenedione can be separated by this method however in figure 2 we showed that 3α/β hydroxysteroid dehydrogenase does not metabolise the parent compound but metabolism by this enzyme occurs subsequent to metabolism by 5α reductase . For the purpose of this study only the activities of the enzymes metabolising the parent molecule were evaluated and therefore the amount of metabolite produced by subsequent metabolism of 5α-androstane-3,17-dione (as produced by 5α reduction of androstenedione) by 3α/β hydroxysteroid dehydrogenase was not determined separately but the amount of metabolite produced by this enzyme was added to the amount of unchanged 5α-androstane-3,17-dione to determine the total amount of metabolite produced by the activity of 5α reductase per minute per 10^6 cells .

2.6 ASSESSMENT OF THE ROLE OF COFACTORS IN CYCLIC AMP-MEDIATED ALTERATION OF ANDROSTENEDIONE METABOLISM .

To investigate if the effects of the cyclic AMP-generating agents and 8-bromo-cyclic AMP are mediated by changes in the levels or activities of the cofactors associated with cytochrome P450-linked enzymes , the metabolism of 4-androstene-3,17-dione was examined in hepatocytes which had been homogenised and supplemented with excess of the cofactors following incubation with 8-bromo-cyclic AMP . Before resuspending in incubation medium prior to the determination of 4-androstene-3,17-dione metabolism , the washed hepatocytes were resuspended in a small volume of incubation medium and homogenised using a Potter-Elvehjem homogeniser with a loose fitting teflon pestle (2500 r.p.m./5 strokes) . The volume of each of the homogenised samples was the made up to 3ml with incubation medium to give a cell density of approximately 10^7 homogenised cells in each 3ml aliquot . To each aliquot was then added 0.8mg NADP and 4mg isocitric acid in $100_{\mu}l$ of $MnCl_2$ solution (6mg/100ml) followed by $10_{\mu}l$ of isocitrate dehydrogenase to start the reaction . The samples were then preincubated for 2 minutes at 37°C in a water bath to allow for the formation of NADPH . $500_{\mu}g$ ($0.1_{\mu}Ci$) of 4-androstene-3,17-dione was then added to each of the samples (time zero) . The metabolism of androstenedione was assayed as described in section 2.5 .

2.7 DETERMINATION OF SEX DIFFERENCES IN CYCLIC AMP-MEDIATED ALTERATION OF ANDROSTENEDIONE METABOLISM .

To examine if the effects of 8-bromo-cyclic AMP upon steroid-metabolising enzymes are dependent upon the sex of the animal , the effects of this agent upon the metabolism of androstenedione were examined in hepatocytes isolated from female Wistar rats kept under the same conditions as described in section 2.1 . Hepatocytes were isolated as described in section 2.2 , preincubation with 8-bromo-cyclic AMP (10^{-4}M) for $\frac{1}{2}$ to 2 hours was carried out as described in section 2.3 and androstenedione-metabolising enzyme activity was assessed as described in section 2.5 .

2.8 DETERMINATION OF WHOLE CELL CYTOCHROME P450 CONTENT .

The total cytochrome P450 content of the hepatocytes was determined by the method of Omura and Sato (1964b) . Following incubation with the cyclic AMP-elevating agents , the cell samples were resuspended in a small quantity of incubation medium and then homogenised using a Potter-Elvehjem homogeniser with a loose fitting teflon pestle (2500 r.p.m./5 strokes) . Homogenised cell samples were then suspended in incubation medium to give a cell density of 5 x 10^7 homogenised cells per ml . The homogenates were assayed for cytochrome P450 content in 3ml aliquots of cell suspension . Samples were first reduced by the

addition of 5mg of sodium dithionite and then bubbled for 30 seconds with carbon monoxide . The reduced CO absorption spectrum was measured in a Schimadzu UV/VIS spectrophotometer between the wavelengths of 500 and 400 nm against a reference of homogenised cell suspension reduced with 5mg sodium dithionite . The absorbance relative to baseline measured at 450nm was converted to a concentration of cytochrome P450 by the use of the millimolar extinction coefficient of 91 . The cytochrome P450 concentration was subsequently expressed as nmoles of cytochrome P450 per 10^6 cells .

2.9 ELECTROPERMEABILISATION OF HEPATOCYTES .

2.9(a) Theoretical assessment of electropermeabilisation parameters .

Electropermeabilisation of freshly isolated hepatocytes was carried out essentially by the method of Knight and Baker (1982). By this method a potential difference applied across the plasma membrane of the hepatocyte punctures two pores in the membrane which allows the access of small molecules (cyclic AMP) to the interior of the cell . A diagrammatic representation of the hepatocyte subjected to an electrical field is shown in figure 7 and the potential difference across the membrane at any point (p) around the circumference of the cell (Vp) is given by the equation :

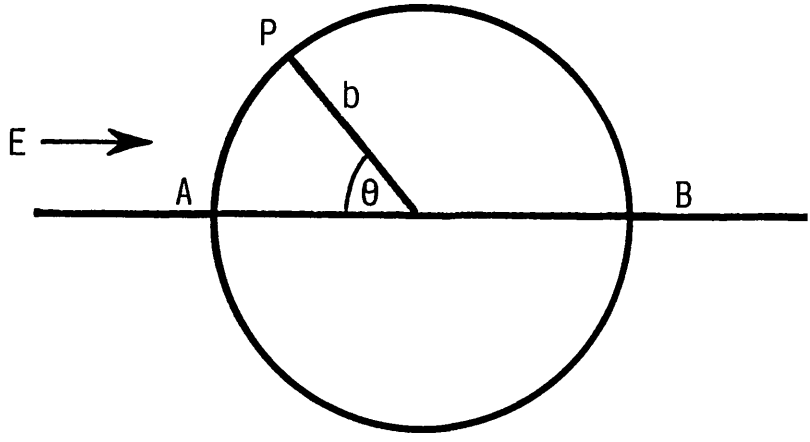

Figure 7 : Diagrammatic representation of a cell of radius b subjected to an electric field of strength E . For full explanation see section 2.9(b) (adapted from Knight and Baker 1982) .

$$Vp = CbE\cos\theta$$

Where b is the radius of the cell , E is the strength of the applied field , the angle θ is as shown in figure 7 and C is a constant dependent upon the relative conductivities of the extracellular fluid , the cytosol and the membrane with relation to cell size and membrane thickness . For simplicity the value of C is taken as 1.5 (Knight and Baker 1982) . By the equation it can be seen that the potential difference across the membrane of the hepatocyte will be maximum at points A and B as shown in figure 7 at which point the value of cos θ will be 1 therefore the equation can be simplified to :

$$Vp = 1.5bE$$

Plasma membranes have been shown to rupture at voltages in excess of 1V (Zimmerman et al 1974). Hepatocytes have a radius of approximately $10_{\mu}m$ (Drochmans et al 1975) and so it can be calculated that an applied field strength of 1000V/cm would produce a transmembrane potential of 1.5 V across the plasma membrane . The mitochondria have a radius of approximately $0.5_{\mu}m$ (Knight and Baker 1982) therefore an applied field strength of 1000V/cm would produce a transmembrane potential of 75mV . An applied field strength of 1000V/cm is theoretically sufficient , therefore , to rupture the cell membrane of the hepatocyte without

damaging other internal organelles .

2.9(b) Experimental assessment of electropermeabilisation parameters

The apparatus used to effect electropermeabilisation of the hepatocytes is shown in figure 8

To assess the efficiency of the apparatus to electropermeabilise the hepatocytes and allow access of cyclic AMP , freshly isolated hepatocytes were suspended in Ham's F10 supplemented with 0.1% bovine serum albumin with or without 10^{-4} cyclic AMP at a cell density of 3 x 10^{7} cells per ml . The cells were electropermeabilised using a range of parameters (2-10 pulses at 1000-3000 V/cm) and then centrifuged at 300g for 3 minutes in a Damon IEC DPR 6000 centrifuge . The medium was subsequently removed and the cells were resuspended in sodium acetate buffer (section 2.19(c)) and assayed for cyclic AMP content by radioimmunoassay as described in section 2.4 . The extracellular cyclic AMP carried over in the supernatant following centrifugation was determined by comparing the amount of cyclic AMP in the permeabilised cell samples (± 10^{-4}M) with nonpermeabilised cells treated in the same way . Assuming that the amount of cyclic AMP permitted access to the interior of the cell in the nonpermeabilised cell is negligible compared to the cyclic AMP in the extracellular medium and that the cyclic AMP carried over in the extracellular medium is a constant for a given number of cells centrifuged at a given g-force independent of

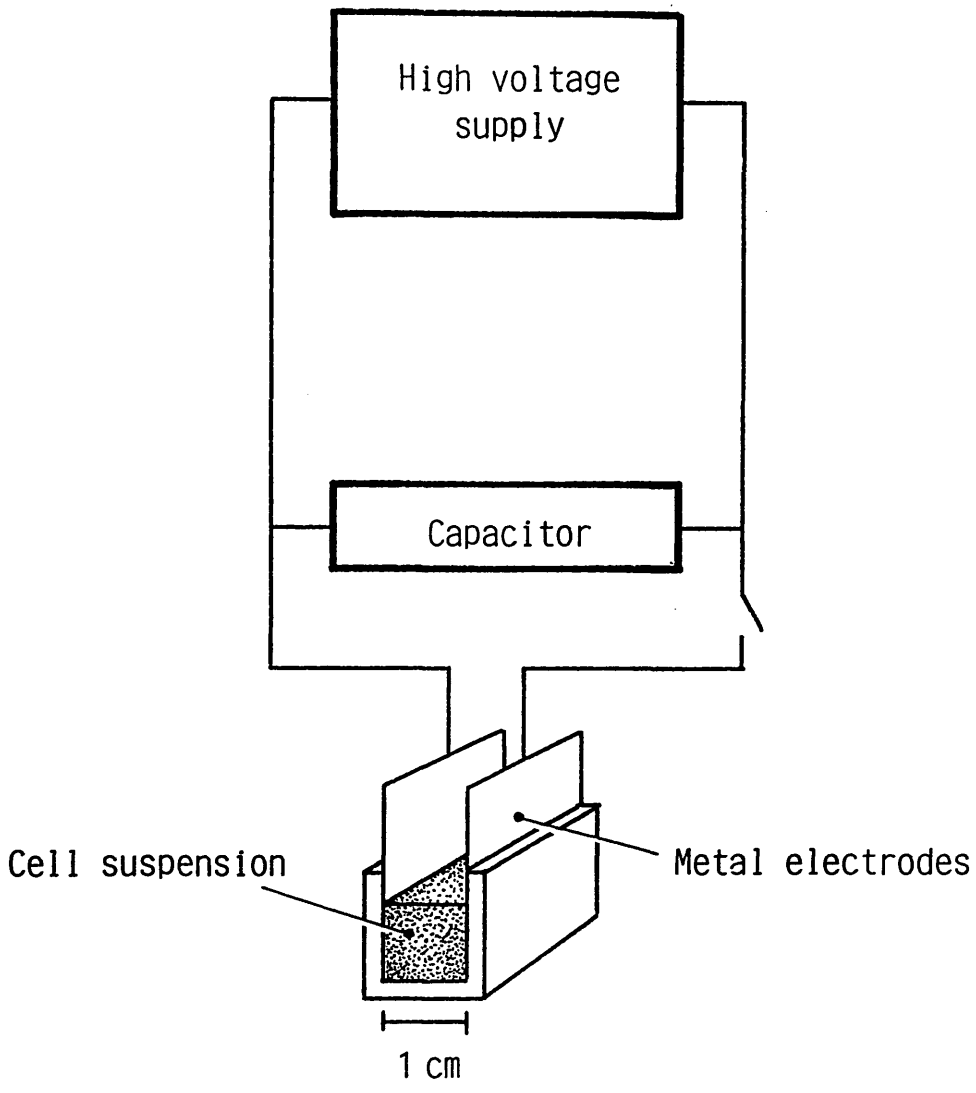

Figure 8 : Diagrammatic representation of the apparatus used to electropermeabilise hepatocyte suspensions (adapted from Knight and Baker 1982) .

permeabilisation , the amount of cyclic AMP carried over in the permeabilised test samples (Y) is equal to the difference between the intact cell cyclic AMP levels in the presence and absence of cyclic AMP . The cyclic AMP allowed access to the cell interior (X) , therefore , is given by the formula :

$$X = (perm+cAMP) - (perm-cAMP) - Y$$

$$\text{where } Y = (intact+cAMP) - (intact-cAMP)$$

Assuming that the hepatocyte is a sphere of radius 10^{-5} metres (section 2.9(a)) and that the volume of the intracellular organelles is negligible in comparison to the total intracellular volume of the cell , the intracellular volume of the cell ($4/3\pi r^3$) is 4.189×10^{-12}l . From this the intracellular concentration of cyclic AMP can be determined .

The cyclic AMP content of the intact cells (intact-cAMP) was calculated experimentally to be 0.054 ± 0.007 nmoles (n = 4) and the cyclic AMP levels of 3×10^7 cells sedimented from medium containing 10^{-4}M cyclic AMP (intact+cAMP) was calculated experimentally to be 1.95 ± 0.067 nmoles (n = 4) .

The amount of cyclic AMP carried over from the medium for 3 x 10^7 hepatocytes centrifuged at 300g (Y) was calculated thus :

$$Y = 1.95 - 0.054 \text{ nmoles}$$

therefore $Y = 1.895$ n moles

The increase in the intracellular concentration of cyclic AMP which occurs as a result of electropermeabilisation was calculated by determining the increase in the intracellular content of cyclic AMP (X) using the above formula and then dividing the content by the volume of 3 x 10^7 hepatocytes (1.26 x 10^{-4}L) .

Table 3 shows the effects of the different electropermeabilisation parameters upon the cyclic AMP content of hepatocytes and the derived intracellular concentration . Comparison of the intracellular and extracellular concentrations of cyclic AMP was taken as a measure of the degree of permeabilisation of the cell . It is apparent from table 3 that 5 pulses at 1000 V/cm results in almost 100% permeabilisation which correlates well with the theoretical assessment of permeabilisation .

Cell viability , as assessed by trypan blue exclusion , was reduced to 75-80% by the electropermeabilisation (1000 V/cm x 5) process but did not show any further reduction over a 3 hour incubation period following permeabilisation . 5 pulses at 1000 V/cm were considered to be the best compromise parameters to use for further experiments .

Parameters	Permeabilised + cAMP 10^{-4}M (nmoles)	Permeabilised cAMP-free (nmoles)	Y (nmoles	X (nmoles)	intracellular concentration
1000V x 2	8.88 ± 0.79	0.045 ± 0.007	1.896	6.939	0.55 x 10^{-4}M
1000V x 5	13.775 ± 0.48	0.036 ± 0.006	1.896	11.843	0.94 x 10^{-4}M
1000V x 10	13.475 ± 0.68	0.030 ± 0.001	1.896	11.549	0.91 x 10^{-4}M
2000V x 2	9.15 ± 0.75	0.043 ± 0.007	1.896	7.210	0.57 x 10^{-4}M
2000v x 5	12.85 ± 2.06	0.032 ± 0.009	1.896	10.922	0.86 x 10^{-4}M
2000v x 10	13.35 ± 0.85	0.026 ± 0.007	1.896	11.428	0.91 x 10^{-4}M
3000v x 2	10.89 ± 1.13	0.034 ± 0.007	1.896	8.96	0.71 x 10^{-4}M
3000v x 5	14.525 ± 0.64	0.025 ± 0.009	1.896	12.604	1.00 x 10^{-4}M
3000v x 10	13.75 ± 2.25	0.025 ± 0.010	1.896	11.829	0.94 x 10^{-4}M

Table 3 . The efficiency of a range of electropermeabilisation parameters (2-10 pulses at 1000-3000 V/cm) to effect permeabilisation of rat hepatocytes to cyclic AMP . Calculation of the intracellular concentration of cyclic AMP and subsequent determination of efficiency of permeabilisation are as discussed in the text .

2.10 INCUBATION OF PERMEABILISED HEPATOCYTES WITH CYCLIC AMP

Hepatocytes were permeabilised in the presence of varying concentrations of cyclic AMP (5×10^{-6} to 5×10^{-3}M) using the parameters chosen in 2.9(b) at a cell density of 3×10^{7} cells per ml . The cells were plated onto 10cm culture dishes (Nunclon , Denmark) with 10^{7} cells in 10ml of Ham's F10 supplemented as described previously and containing the concentrations of cyclic AMP used during permeabilisation . The cell samples were incubated as described in section 2.3 for times ranging from ½ to 3 hours . Viability of the cells was unaffected by the addition of cyclic AMP at the concentrations stated . Concentrations above 5×10^{-3}M caused progressive cell destruction and , thus , were not used .

2.11 ASSESSMENT OF CYCLIC AMP EFFECT UPON ANDROSTENEDIONE METABOLISM AND CYTOCHROME P450 LEVELS IN ELECTROPERMEABILISED HEPATOCYTES .

Following incubation with the different concentrations of cyclic AMP , the cells were removed from the culture plates , washed and resuspended in incubation medium as described in section 2.3 . Androstenedione metabolism and cytochrome P450 were assessed as described in sections 2.5 and 2.8 respectively .

2.12 ASSESSMENT OF THE ROLE OF PROTEIN SYNTHESIS IN THE ACTIONS OF CYCLIC AMP .

To assess the role of protein synthesis in the effects of (a) forskolin 10^{-5}M and (b) forskolin and IBMX 10^{-5}M/10^{-3}M in intact hepatocytes , the translation blocker , cycloheximide (10^{-5}M) (Peshka 1971) was added during the 30 preincubation period prior to the addition of the cyclic AMP elevating agents . To assess the role of protein synthesis in the effects of cyclic AMP (5 x 10^{-5}M to 5 x 10^{-3}M) in permeabilised hepatocytes , cycloheximide 10^{-5}M was added during the 30 minutes preincubation period prior to permeabilisation and then resupplemented to give a final concentration of 10^{-5}M in the culture dishes . Cell samples were incubated for times ranging from ½ to 3 hours under conditions described previously (section 2.3) . Androstenedione metabolism and cytochrome P450 levels were assessed as described in sections 2.5 and 2.8 respectively .

2.13 ASSESSMENT OF THE ROLE OF PROTEIN KINASES IN THE EFFECTS OF CYCLIC AMP .

The role of the protein kinases in the effects of cyclic AMP in permeabilised hepatocytes was assessed by using the specific protein kinases inhibitors (a) K-252a , 2 x 10^{-8}M (8R*,9S,11S*)-(-)-9-hydroxy-9-methoxycarbonyl-8-methyl-2,3,9,10 -tetrahydro-8,11-epoxy-1H,8H,11H-2,7b,11a-triazadibenzo(a,g) cycloocta (c,d,e)trinden-1-one ; inhibits all protein kinases) ; (b)

K-252b , 2 x 10^{-8}M (the 9-carboxylic acid derivative of K-252a ; a selective inhibitor of protein kinase c) ; (c) KT5720 , 6 x 10^{-8}M (the 9-hexoxy-carbonyl derivative of K-252a ; a selective inhibitor of protein kinase a) and (d) KT5822 , 2.5 x 10^{-9}M (the 9-methoxy derivative of K-252a ; a selective inhibitor of protein kinase g) (Kase et al 1986 ; Nakanishi et al 1986 ; Yasazawa et al 1986 ; Kase et al 1987). The inhibitors were added to the hepatocytes prior to permeabilisation to give the concentrations stated above . Following permeabilisation the cell cultures were resupplemented with the inhibitors to maintain the correct concentration . The inhibitors were added in solution in DMSO , control cells received DMSO only . Incubation with cyclic AMP (section 2.11) , assessment of androstenedione metabolism (section 2.5) and cytochrome P450 levels (section 2.8) were as previously described . Preliminary experiments indicated that the protein kinase inhibitors appeared not to be able to penetrate the plasma membrane in the intact cell and so the roles of the protein kinases in the actions of cyclic AMP were assessed in the permeabilised cells only .

2.14 ISOLATION OF HEPATOCYTES IN PHOSPHATE-FREE MEDIUM .

Hepatocytes were isolated by two step collagenase perfusion as described in section 2.2 . The method differed slightly , however , in that phosphate-free , calcium-free Hanks BSS (section 2.19(e)) was used throughout the isolation procedure instead of calcium-free Hanks BSS as used in section 2.2 . Viability

following isolation , as assessed by trypan-blue exclusion , was comparative to that in section 2.2 (greater than 97%) . Following isolation the hepatocytes were suspended in phosphate-free incubation medium (section 2.19(f)) supplemented with 0.1% bovine serum albumin at a cell density of 2 x 10^7 cells per ml . This medium was found not to adversely affect cell viability , as assessed by trypan-blue exclusion , over a 3 hour incubation period following its addition to the cells .

2.15 PREINCUBATION OF HEPATOCYTES WITH (^{32}P) ORTHOPHOSPHATE .

To allow for the labelling of hepatocyte ATP with (^{32}P) , hepatocyte suspensions were incubated for 1 hour at 37°C with (^{32}P)-orthophosphate at a concentration of 10µCi per 10^6 cells . Incubations were conducted in 25ml conical flasks placed in a water bath maintained at 37°C and the atmosphere above the cell suspension was maintained at O_2/CO_2 , 95%:5% by using a closed system connected to a gas cylinder .

2.16 ELECTROPERMEABILISATION AND INCUBATION WITH CYCLIC AMP .

Due to the changes in the levels of whole cell cytochrome P450 mediated by high concentrations of cyclic AMP (5 x 10^{-3}M) (results : section 3.6(b)) the effects of high concentrations of cyclic AMP upon phosphorylation of cytochrome P450 were not determined as comparison between control and test cell samples

would have been complicated by the cyclic AMP-mediated alteration in cytochrome P450 as determined in section 2.8 due to the limitations of this assay procedure .

Following preincubation with (^{32}P)-orthophosphate , the hepatocytes were centrifuged at 300g for 3 minutes in a Damon-IEC DPR 6000 centrifuge , the supernatant was removed and the cells washed with fresh phosphate-free incubation medium supplemented with 0.1% bovine serum albumin . This was repeated three times to ensure removal of excess (^{32}P) orthophosphate from the cells . The hepatocytes were then suspended in phosphate-free incubation medium supplemented with 0.1% bovine serum albumin at a cell density of 3 x 10^7 cells/ml and electropermeabilised as described previously (section 2.9(b)) . Viability , as assessed by trypan-blue exclusion was comparable to that following electropermeabilisation of the hepatocytes in the presence of Hams F10 supplemented with 0.1% bovine serum albumin and 100IU penicillin/streptomycin (75-80%) indicating that electropermeabilising the cell in the presence of phosphate-free incubation medium supplemented with 0.1% bovine serum albumin did not have any additional adverse effects upon cell viability other than those considered to be acceptable in section 2.9(b) .

For assessment of the cyclic AMP-mediated phosphorylation of whole cell proteins , the electropermeabilised hepatocytes were suspended in phosphate-free incubation medium supplemented with 0.1% bovine serum albumin at a cell density of 5 x 10^6cells/ml and incubations were conducted in 100µl aliquots of this cell suspension (5 x 10^5 cells) .

For assessment of cyclic AMP-mediated phosphorylation of specific cytochrome P450 isozymes . the electropermeabilised hepatocytes were suspended in phosphate-free incubation medium supplemented with 0.1% bovine serum albumin at a cell density of 10^7cells/ml and incubations were carried out in 1ml aliquots of this cell suspension (10^7 cells) .

Cyclic AMP was added to each of the test cell samples to give a final concentration in the aliquot of 5×10^{-5}M . Cyclic AMP was added to the 100µl aliquots of cell suspension in 5µl of distilled water and to the 1ml aliquots of cell suspension in 50µl of distilled water . Control cells samples received the equivalent volume of vehicle only . Incubations were conducted in a water bath maintained at 37°C for times ranging from 15 to 60 minutes .

2.17 PREPARATION OF SOLUBILISED CELL SAMPLES .

2.17(a) Whole cell proteins .

Following incubation with 5×10^{-5} cyclic AMP , 400µl of sample solvent (bromophenol blue 0.01%$^{w}/_{v}$, sodium dodecyl sulphate 4.6%$^{w}/_{v}$, "Tris" buffer 20%$^{w}/_{v}$, mercaptoethanol 10%$^{v}/_{v}$ and glycerol 15%$^{v}/_{v}$ in distilled water pH 6.8) was added to each of the 100µl aliquots of cell suspension and then samples were placed in a boiling water bath for 3 minutes to ensure solubilisation of cellular proteins . The solubilised samples were then allowed to cool to room temperature before proceeding .

2.17(b) Specific cytochrome P450 isozymes .

Following incubation with 5 x 10^{-5}M cyclic AMP , the hepatocyte cell samples were centrifuged at 300g for 3 minutes in a Damon-IEC DPR 6000 centrifuge and the supernatant was removed . A small volume (1-2ml) of sodium cholate solution 1.8%$^{w}/_{v}$ was added to each of the cell samples and homogenisation of cells was ensured by drawing each of the samples into a syringe fitted with a 26g needle . The volume of each of the samples was made up to 10ml with sodium cholate solution 1.8%$^{w}/_{v}$ and samples were centrifuged at 105,000g for 1 hour at 4°C in a Beckman L8-M ultracentrifuge . 500µl of a 1:1000 dilution of specific cytochrome P450 PB-2a antibody , cytochrome P450 PB-3a antibody or cytochrome P450 MC-1b antibody (nomenclature of Wolf : table 1) in phosphate-free incubation medium supplemented with 0.1% bovine serum albumin was added to 1ml aliquots of the supernatant (10^{6} cells) . Antibodies had been raised in rabbits against isolated rat cytochrome P450 isozymes . Cytochrome P450 PB-2a antibody was found to be highly specific for cytochrome P450 PB-2a but showed very slight cross reactivity with three other unspecified isozymes. Cytochrome P450 PB-3a antibody was found to be relatively specific for cytochrome P450 PB-3a , the major phenobarbitone inducible isozyme (table 1) but it also showed some cross reactivity with cytochrome P450e which is the second phenobarbitone inducible cytochrome P450 isozyme . Cytochrome P450 MC-1b antibody was found to be relatively specific for cytochrome P450 MC-1b , the major 3-methylcholanthrene-inducible isozyme

(table 1) but it also showed very slight cross reactivity with cytochrome P450d which is the second 3-methylcholanthrene-inducible isozyme. Following the addition of the specific antibodies , cell samples were placed on ice for 30 minutes and then centrifuged at 25,000g for 20 minutes at 4°C in a Beckman L8-M ultracentrifuge . The supernatant was removed from each of the samples and the immunoprecipitate was washed and dissolved in 50µl of sample solvent (section 2.17(a)) . Samples were then boiled as described in section 2.17(a) and then allowed to cool to room temperature before proceeding .

2.18 SODIUM DODECYL SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE) OF SOLUBILISED CELL SAMPLES AND DETECTION OF PHOSPHOPROTEINS .

Samples were resolved into their constituent proteins on 7.5% polyacrylamide slab gels as described by Smith (1984) . For samples containing whole cell proteins , 20µl (2 x 10^4 cells) were added to each sample well . For samples containing immunoprecipitated cytochrome P450 isozymes , the whole of the sample was added to the well (extract from 10^6 cells) . The samples were subjected to electrophoresis towards the anode at a constant voltage of 150V for about 3 hours until the bromophenol blue marker in the sample solvent (section 2.17(a)) was at the bottom of the gel and then the gels were fixed and stained for 1-2 hours at room temperature in 0.1% $^{w}/_{v}$ Coomassie Brilliant Blue R-250 in methanol/glacial acetic acid/water (5:1:1) . Gels were

destained for approximately 24 hours at room temperature in methanol/glacial acetic acid/water (8:1:1) with several changes of the solution . The gel was then placed on a piece of absorbant paper , covered in non porous plastic film (Cling Film) and then dried under vacuum at a temperature of 60°C on a Bio-Rad Model 224 Gel Slab Dryer for 3 hours . Dried gels were then autoradiographed at -70°C for up to four weeks . The incorporation of (^{32}P) into whole cell proteins or immunoprecipitated cytochrome P450 isozymes was assessed by visual examination of the X-ray film after developing .

2.19 BUFFERS AND PHYSIOLOGICAL SOLUTIONS .

(a) Calcium-free Hanks Basic salt solution .

KCl : 200mg

KH_2PO_4 : 30mg

NaCl : 4000mg (4g)

Na_2HPO_4 : 24mg

$NaHCO_3$: 1050mg (1.05g)

In glass distilled water to 1 litre .

(b) Incubation medium .

Glucose : 1000mg (1g)

$MgSO_4$: 100mg

$MgCl_2$: 100mg

$CaCl_2$: 185mg

In calcium-free Hanks basic salt solution to 1 litre .

(c) Sodium acetate buffer (50mM) .

Sodium acetate : 4.1g

Glass distilled water : 500ml

Adjusted to pH 5 with glacial acetic acid

Glass distilled water to 1 litre .

(d) Potassium phosphate buffer (100mM)

K_2HPO_4 : 4.61g

KH_2PO_4 : 10g

Glass distilled water : 500ml

Adjusted to pH 6.4 with HCl .

Glass distilled water to 1 litre .

(e) Calcium-free , Phosphate-free Hanks basic salt solution .

KCl : 200mg

K_2SO_4 : 30mg

NaCl : 4000mg (4g)

Na_2SO_4 : 24mg

$NaHCO_3$: 1050mg (1.05g)

In glass distilled water to 1 litre

(f) Phosphate-free incubation medium .

As in 2.19(b) above but made up to 1 litre in calcium-free phosphate-free Hanks basic salt solution .

2.20 CHEMICALS .

Chemicals and reagents are listed according to manufacturer .

Sigma Chemicals Ltd. , Poole , Dorset , U.K.

8-Bromo-cyclic AMP

Adrenaline

Isobutylmethylxanthine

Forskolin

Cyclic AMP

4-Androstene-3,17-dione

Cycloheximide

Bromophenol Blue

Coomassie Brilliant Blue R-250

Sodium dodecyl sulphate

"Tris" buffer

bis-Acrylamide

Acrylamide

BCL Ltd. , Lewes , East Sussex , U.K.

Collagenase

Bovine serum albumin (Fraktion V)

NADP

Isocitric acid

Isocitrate dehydrogenase

Amersham International plc , Aylesbury , Bucks. U.K.

Adenosine-3'5'-cyclic phosphoric acid 2'-O-succinyl

3-(^{125}I)iodotyrosine methyl ester

4-(4-^{14}C)Androstene-3,17-dione

Gibco BRL Ltd. , Paisley , Scotland , U.K.

Hams F10

Penicillin/streptomycin solution

Kodak , France

X ray film (X-Omat S)

Developer (D-19)

Fixer (FX-40)

Miscellaneous

Prazocin was supplied by Pfizer , Sandwich , Kent , U.K.

(^{32}P)Orthophosphate was supplied by the Western Infirmary ,

Glasgow , Scotland , U.K.

K-252a , K-252b , KT5720 , KT5822 : Were kind gifts from Dr H. Kase , Kyowa Hakko Kogyo Co. Ltd. , Tokyo , Japan

Cytochrome P450 antibodies specific for cytochromes P450 PB-2a , PB-3a and MC-1b , were a kind gift from Dr C.R. Wolf , ICRF , Edinburgh , Scotland , U.K.

All other chemicals used were of the highest analytical grade commercially available .

2.21 STATISTICS .

Results were expressed as percentage of relevant control ± standard deviation . Statistical analysis was performed using Student's t-test and statistical significance was set at $p<0.05$ in all cases .

RESULTS

3 RESULTS

3.1 METABOLISM OF ANDROSTENEDIONE IN HEPATOCYTES ISOLATED FROM MALE RATS AND CULTURED FOR 24 HOURS .

Using the substrate , androstenedione , at least five different enzyme activities can be calculated : 7α-, 6β- and 16α-hydroxylase , 17α/β hydroxysteroid dehydrogenase (17-OHSD) and 5α reductase . Although not dealt with in this thesis , it has previously been established in this laboratory that the metabolism of androstenedione proceeds linearly with time (up to 70 minutes) and cell number (up to 1.2 x 10^7 cells) (Hussin 1988) . Using an identical concentration of androstenedione (500$_\mu$g in 3ml of cell suspension) in this present study , therefore , the cell number of 10^7 cells and incubation period of 30 minutes are within the limits of linearity established above .

Table 4 shows the activities of these five enzymes in hepatocytes isolated from male rats and maintained in Hams F10 supplemented with 0.1% bovine serum albumin and 100IU penicillin/streptomycin . It is apparent from the table that although the activities of the enzymes decline progressively over the 24 hour incubation period under these culture conditions , the ratios of the enzyme which results in the overall enzyme profile does not vary significantly over this time period.

time	7α-OH	6β-OH	16α-OH	17-OHSD	5α-Red
½hr	26.2 ± 4.1	74.8 ± 14.7	196.0 ± 21.2	118.1 ± 15.4	304.4 ± 91.9
1hr	24.0 ± 3.1	78.0 ± 6.8	166.7 ± 18.4	93.8 ± 14.8	202.5 ± 7.6
2hr	14.8 ± 2.8	47.2 ± 11.6	87.3 ± 24.1	72.0 ± 2.1	173.3 ± 42.0
24hr	12.8 ± 4.0	15.4 ± 3.6	19.0 ± 3.2	14.4 ± 4.2	80.3 ± 10.3

Table 4 : The activities of 7α-hydroxylase (7α-OH) , 6β-hydroxylase (6β-OH) , 16α-hydroxylase (16α-OH) , 17α/β-hydroxysteroid dehydrogenase (17-OHSD) and 5α-reductase (5α-Red) in isolated rat hepatocytes over a 24 hour period of culture in Hams F10 supplemented with 0.1% bovine serum albumin and 100IU penicillin/streptomycin . Results are expressed as pmoles of metabolite formed per minute per 10^6 cells ± S.D. for 12 different cell samples .

3.2 METABOLISM OF ANDROSTENEDIONE IN HEPATOCYTES ISOLATED FROM MALE AND FEMALE RATS .

Table 5 shows the activities of each of the five enzymes which metabolise androstenedione in untreated hepatocytes freshly isolated from either male or female rats . It can be seen that the well documented , sexually differentiated enzymes profiles (table 2) are expressed in the isolated hepatocyte . Thus :

7α-hydroxylase	female = male
6β-hydroxylase	female < male
16α-hydroxylase	female << male
17-OHSD	female ≤ male
5α-reductase	female >> male

In the results shown in table 5 female 16α-hydroxylase activity is 25.9% of male 16α-hydroxylase activity , female 6β-hydroxylase activity is 40.6% of male 6β-hydroxylase activity and female 5α-reductase activity is 233.6% of male 5α-reductase activity . The non sexually differentiated enzymes involved in the metabolism of 4-androstene-3,17-dione ; 7α-hydroxylase and 17-OHSD, have activities in the female rat of 83.3% and 91.9% , respectively , of corresponding male activities .

	7α-OH	6β-OH	16α-OH	17-OHSD	5α-Red
Male	47.7 ± 4.4	76.1 ± 2.4	117.5 ± 5.0	87.9 ± 6.1	320.6 ± 17.5
Female	39.8 ± 3.0	30.7 ± 3.5	30.4 ± 2.0	80.8 ± 3.1	748.9 ± 93.9

Table 5 . The activities of 7α-hydroxylase (7α-OH) , 6β-hydroxylase (6β-OH) , 16α-hydroxylase (16α-OH) , 17α/β-hydroxysteroid-dehydrogenase (17-OHSD) and 5α-reductase (5α-Red) in hepatocytes freshly isolated from male and female rats . Results are expressed in pmoles of metabolite formed per minute per 10^6 cells ± S.D. for four different cell samples .

3.3 EFFECT OF ELEVATING CYCLIC AMP UPON THE METABOLISM OF ANDROSTENEDIONE IN ISOLATED RAT HEPATOCYTES .

3.3(a) Effects of 8-bromo-cyclic AMP .

(i) Effects of 8-bromo-cyclic AMP in male rat hepatocytes

As illustrated in table 5 , the control activity of each of the enzymes is dependent upon the particular enzyme being studied, to illustrate the effects of cyclic AMP upon the activities of the enzymes , therefore , each of the activities is expressed as percentage of the relevant control activity .

The effects of 8-bromo-cyclic AMP (10^{-4}M) upon each of the enzyme activities following a 2 hour incubation period are shown in table 6 . It is apparent that 8-bromo-cyclic AMP causes a reduction in the activities of all of the enzymes in a non-selective manner and produces no change in the sexually differentiated male profile of the enzyme activities . The activities of 6β-hydroxylase and 16α-hydroxylase which are inhibited to 43.76% and 41.94% , respectively , of corresponding control activities would be consistent with a feminising effect of cyclic AMP however the inhibition of the activity of 5α-reductase to 31.45% of corresponding control activity , is inconsistent with regards to a feminising effect of cyclic AMP . The time course of the effect of 8-bromo-cyclic AMP upon the activities of the sexually differentiated enzymes ; 6β-hydroxylase , 16α-hydroxylase and 5α-reductase is shown in figure 9 . No inhibition of enzyme

	7α-OH	6β-OH	16α-OH	17-OHSD	5α-Red
Control	47.8 ± 4.4	76.1 ± 2.4	117.5 ± 5.0	87.9 ± 6.1	320.6 ± 17.5
8Br-cAMP	25.8 ± 1.5*	33.3 ± 2.0*	49.3 ± 5.7*	50.4 ± 3.6*	100.8 ± 29.0*
	(54.0 ± 3.3)	(43.8 ± 2.7)	(41.9 ± 4.9)	(57.3 ± 4.1)	(31.5 ± 9.1)

Table 6 . The effects of 8-bromo-cyclic AMP (10^{-4}M) upon the activities of 7α-hydroxylase (7α-OH) , 6β-hydroxylase (6β-OH) , 16α-hydroxylase (16α-OH) , 17α/β hydroxysteroid dehydrogenase (17-OHSD) and 5α-reductase (5α-Red) in isolated rat hepatocytes following an incubation period of 2 hours . Results are expressed as pmole of metabolite formed per minute per 10^6 cells ± S.D. or (in brackets) as percentage of control ± S.D. for 4 different cell samples . (* : $p<0.05$)

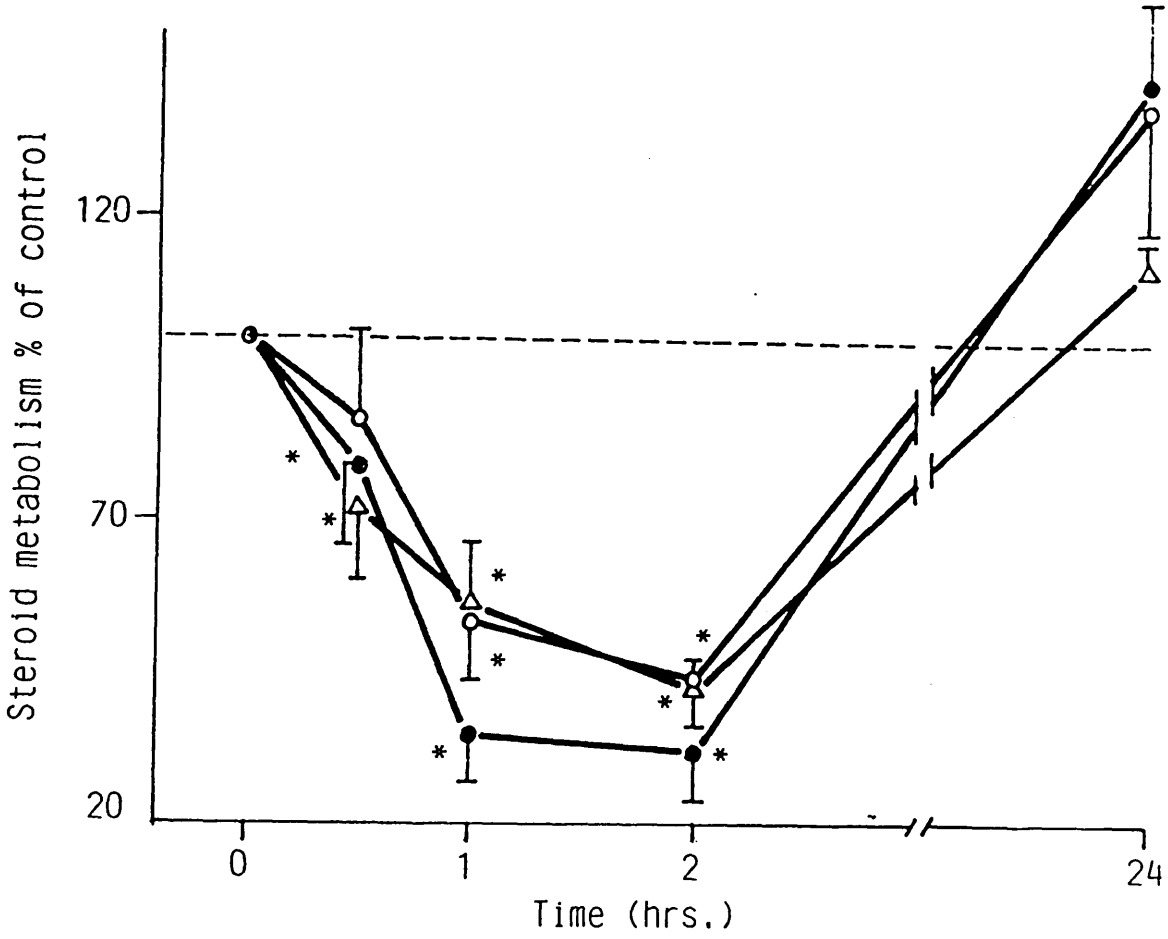

Figure 9 : Change in 6β- (-o-) (6β-OH) and 16α-hydroxylase (-△-) (16α-OH) and 5α-reductase (-●-) (5α-Red) activities with time in hepatocytes isolated from male rats , following administration of 8-bromo-cyclic AMP (10^{-4}M) . Results are expressed as percentage of relevant control ± S.D. of at least 4 different cell samples (* : $p<0.05$) . Control activities expressed as pmoles of metabolite formed per minute per 10^6 cells at each time were as follows :

	6β-OH	16α-OH	5α-Red
½ hour	50.1 ± 12.8	72.9 ± 13.5	249.0 ± 71.5
1 hour	67.7 ± 22.4	99.9 ± 25.2	299.1 ± 36.3
2 hour	76.1 ± 2.4	117.5 ± 5.0	320.6 ± 17.5
24 hour	29.1 ± 2.4	45.4 ± 2.2	169.5 ± 28.3

activities occurs following the short incubation periods (2-20 minutes) (as shown in table 7) but inhibition is apparent following an incubation period of ½ hour and maximal inhibition (30-50% of control) occurs at 2 hours following addition . The activities have returned to control levels by 24 hours .

(ii) Effects of 8-bromo-cyclic AMP in female rat hepatocytes

The effects of 8-bromo-cyclic AMP upon the activities of 6β-hydroxylase , 16α-hydroxylase and 5α-reductase in the female rat are shown in figure 10 . As was apparent in the male rat , non-specific inhibition of enzyme activity (40-60% of control) occurs between ½ hour and 2 hours following addition and the activities have returned to control levels by 24 hours .

The nonspecific inhibitory effect of 8-bromo-cyclic AMP which was of the same magnitude in rats of both sexes , indicates that the effects of elevating cyclic AMP using this agent are not sex dependent and do not affect sexual differentiation of enzyme activity . This was the case for all of the cyclic AMP elevating agents studied in the intact hepatocytes and for cyclic AMP in electropermeabilised hepatocytes . To simplify the discussion , therefore , only the activities of the cytochrome P450 containing enzymes ; 6β-hydroxylase , 16α-hydroxylase and 7α-hydroxylase , will be shown and discussed in subsequent experiments as being representative of all of the enzyme activities .

	7α-OH	6β-OH	5α Red
2 min	105.3 ± 16.1	115.6 ± 0.2	106.5 ± 13.9
5 min	111.6 ± 12.2	99.3 ± 14.8	107.9 ± 13.7
10 min	97.9 ± 11.3	92.1 ± 11.0	104.6 ± 13.9
20 min	102.5 ± 25.8	99.9 ± 14.6	112.0 ± 20.2

Table 7 : Activities of 7α-(7α-OH) and 6β-hydroxylase (6β-OH) and 5α-reductase (5α-Red) in isolated hepatocytes , 2 to 20 minutes following the administration of 8-bromo-cyclic AMP (10^{-4}M) . Results are expressed as percentage of relevant control activities ± S.D. for at least 3 different cell samples . Control activities , expressed in pmole of metabolite per minute per 10^6 cells , were as follows :

	7α-OH	6β-OH	16α-OH
2 min	19.0 ± 4.5	19.6 ± 2.7	151.8 ± 7.6
5 min	22.6 ± 1.5	18.7 ± 4.7	160.2 ± 45.8
10 min	18.7 ± 1.9	22.5 ± 3.3	148.3 ± 44.2
20 min	21.1 ± 3.5	22.7 ± 3.3	166.1 ± 25.1

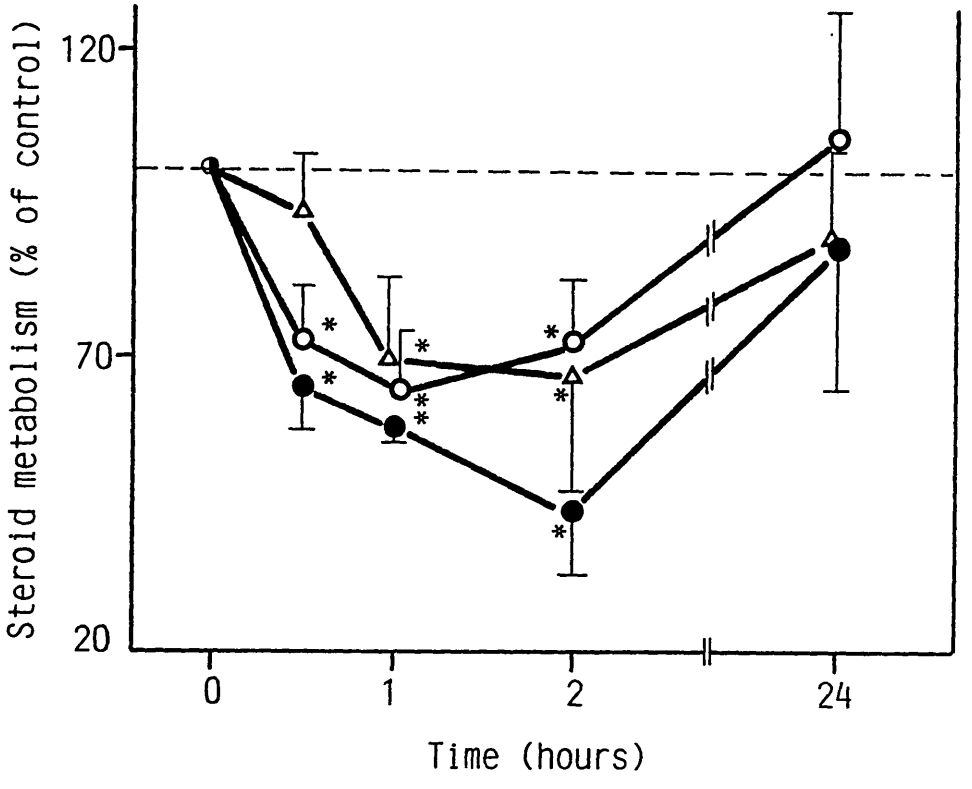

Figure 10 : Change in 6β- (-O-) (6β-OH) , 16α-hydroxylase (-Δ-) (16α-OH) and 5α-reductase (-●-) (5α-Red) activities with time in hepatocyte isolated from female rats , following administration of 8-bromo-cyclic AMP (10^{-4}M) . Results are expressed as percentage of relevant control ± S.D. of at least 4 different cell samples (* : $p<0.05$) . Control activities expressed as pmoles of metabolite formed per minute per 10^6 cells at each time are as follows :

	6β-OH	16α-OH	5α-Red
½ hour	30.9 ± 4.0	30.4 ± 2.3	749.9 ± 111.7
1 hour	45.2 ± 3.6	55.7 ± 10.8	865.8 ± 186.9
2 hour	24.5 ± 4.6	34.8 ± 0.7	449.6 ± 76.1
24 hour	28.6 ± 4.2	42.3 ± 6.9	327.0 ± 78.5

(iii) Effects of homogenisation and readdition of excess cofactors on the actions of 8-bromo-cyclic AMP in male rat hepatocytes .

The time course of the effect of 8-bromo-cyclic AMP in hepatocytes homogenised and resupplemented with excess cofactors prior to assessment of 4-androstene-3,17-dione metabolism is shown in figure 11 . As was apparent in intact hepatocytes , non-specific inhibition (50-60%) of all of the enzyme activities occurred between ½ an hour and 2 hours following the addition of the 8-bromo-cyclic AMP . This indicates that the effects of elevating cyclic AMP are not mediated by depletion of cofactors .

3.3(b) Effects of adrenaline and correlation with cyclic AMP levels .

(i) Adrenaline alone .

Figure 12 shows the time course of the effect of adrenaline (10^{-7}M) upon cyclic AMP levels and 4-androstene-3,17-dione metabolism . The basal control level of cyclic AMP in freshly isolated hepatocytes from male rats was approximately 1 nanomole per 10^6 cells , this level was found not to vary significantly over the early time periods studied (2 minutes to 1 hour) although cyclic AMP levels increased to approximately 1.5 nanomoles per 10^6 cells after 2 hours in culture and to 3 nanomoles per 10^6 cells by 24 hours . This may indicate some

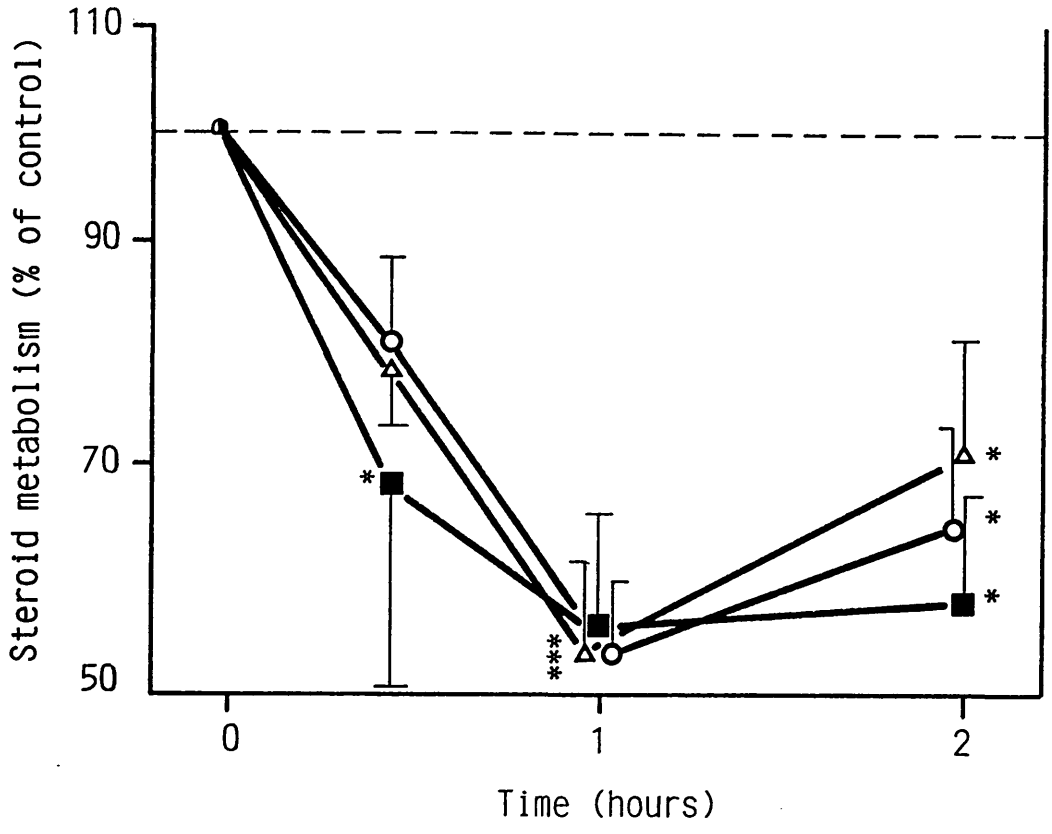

Figure 11 : Change in 7α-(-■-) (7α-OH) , 6β-(-○-) (6β-OH) and 16α-hydroxylase (-△-) (16α-OH) activities with time in hepatocytes isolated from male rats following administration of 8-bromo-cyclic AMP (10^{-4}M) and subsequent homogenisation and readdition of excess cofactors . Results are expressed as percentage of relevant control ± S.D. of at least 4 different cell samples (* : $p<0.05$) . Control activities expressed in pmoles of metabolite formed per minute per 10^6 cells were as follows :

	7α-OH	6β-OH	16α-OH
½ hour	94.1 ± 13.8	133.1 ± 15.7	180.9 ± 14.4
1 hour	87.4 ± 3.2	112.4 ± 6.7	127.7 ± 12.3
2 hour	83.4 ± 5.4	96.2 ± 5.3	131.9 ± 4.3

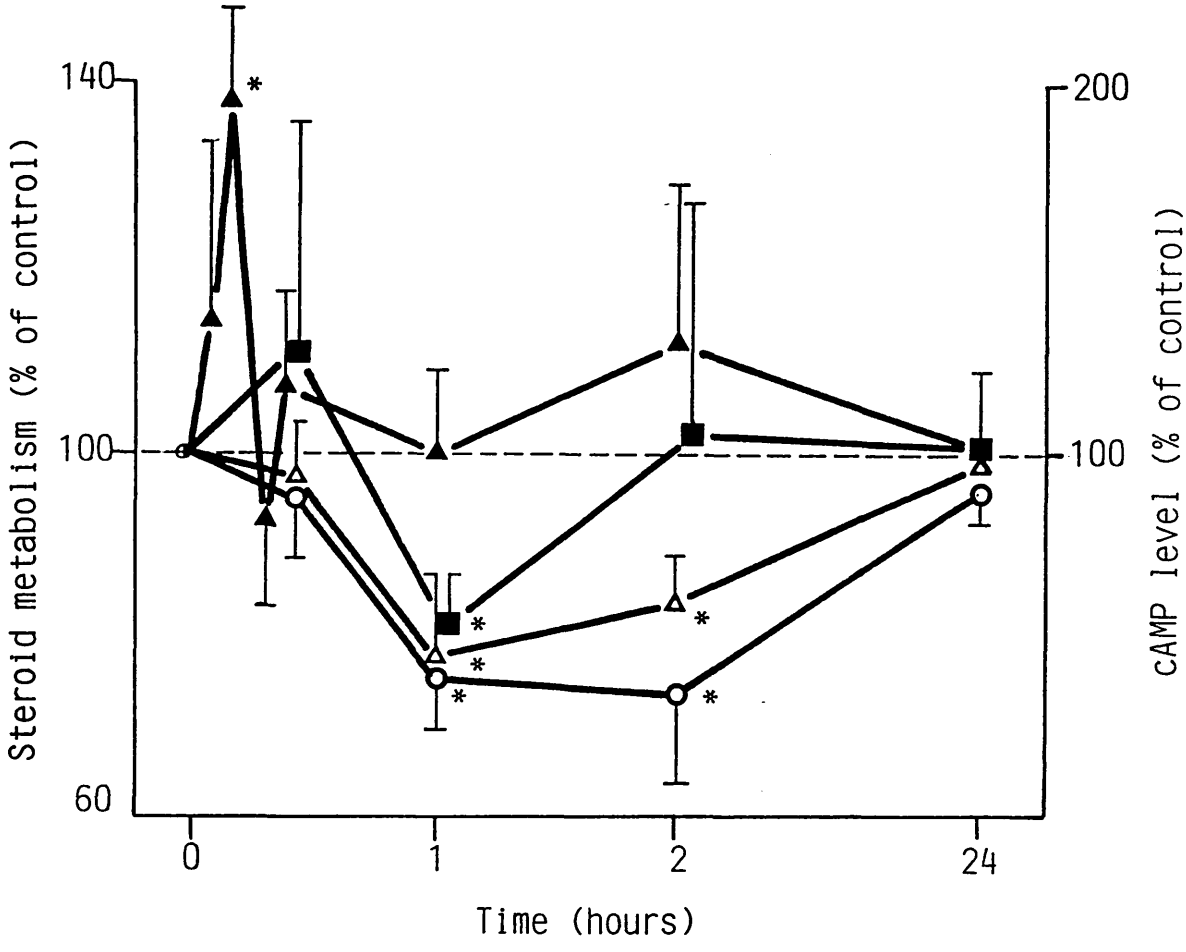

Figure 12 : Change in cyclic AMP content (▲) , 7α-(■) (7α-OH) , 6β-(○) and 16α-hydroxylase (△) (16α-OH) with time in isolated hepatocytes following addition of adrenaline (10^{-7}M) . Results are expressed as percentage of relevant control ± S.D. for at least 4 different cell samples (* : $<p0.05$) . Control activities expressed in pmoles of metabolite formed per minute per 10^6 cells and cyclic AMP content expressed in pmoles per 10^6 cells at each time were as follows :

	7α-OH	6β-OH	16α-OH	cyclic AMP
2 min	-	-	-	1.08 ± 0.16
5 min	-	-	-	1.13 ± 0.29
10 min	-	-	-	1.02 ± 0.36
20 min	-	-	-	1.05 ± 0.18
½ hour	70.6 ± 0.9	93.9 ± 1.0	118.2 ± 6.6	1.03 ± 0.17
1 hour	67.5 ± 10.9	92.3 ± 14.4	113.8 ± 14.5	1.06 ± 0.33
2 hour	69.7 ± 9.8	101.1 ± 14.3	125.8 ± 22.7	1.47 ± 0.39
24 hour	19.4 ± 5.7	14.6 ± 1.2	31.9 ± 4.3	3.22 ± 0.11

influence of the tartrate ion because control cyclic AMP levels in hepatocytes cultured in the absence of the tartrate ion tended to decrease over this 24 hour period (0.3 to 0.4 nanomoles per 10^6 cells) . The addition of adrenaline produced a rise in the cyclic AMP levels to 1.89 ± 0.39 nanomoles per 10^6 cells 10 minutes following its addition . This represents a rise to 185% of corresponding control levels . After 20 minutes cyclic AMP content had returned to control level and did not differ significantly from control over the rest of time period studied (24 hours) . Enzyme activity was inhibited following an incubation period of 30 minutes but no inhibition was apparent before this time . Inhibition was maximal (60-75% of control) between 1-2 hours following addition and at 24 hours did not differ significantly from control activities . It appears , therefore , that there is a time lag between the elevation of cyclic AMP levels and the subsequent inhibition of enzyme activity .

(ii) Adrenaline in the presence of propranolol and prazocin .

The effects of adrenaline (10^{-7}M) in combination with propranolol (10^{-5}M) (panel A) or prazocin (10^{-5}M) (panel B) upon the metabolism of 4-androstene-3,17-dione are show in figure 13 . The actions of adrenaline were unaffected by the presence of prazocin (an α_1-blocker) . In the presence of prazocin inhibition of enzyme activities was of a similar magnitude (50-70%) and followed a comparable time course to that seen in the absence of prazocin (see section 3.5) . The actions of

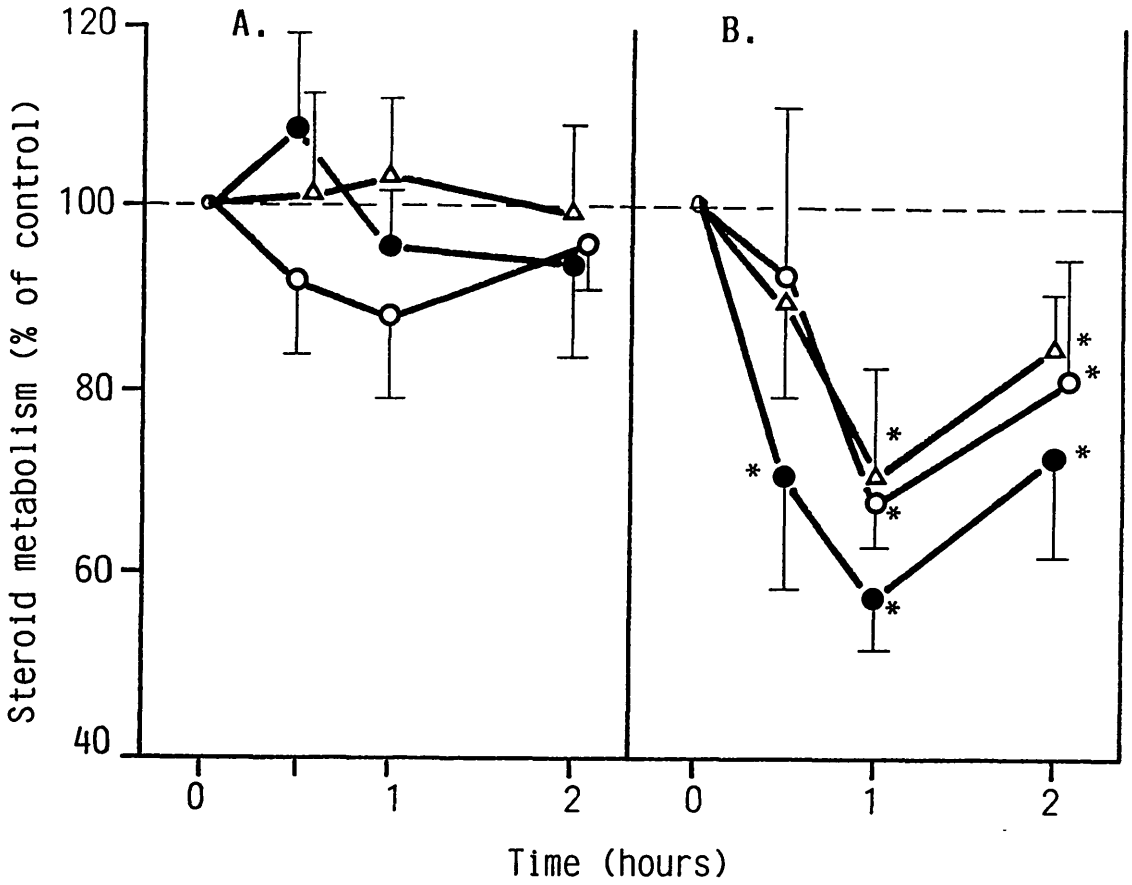

Figure 13 : Change in 7α-(●-) (7α-OH) , 6β-(-o-) (6β-OH) and 16α-hydroxylase (-△-) (16α-OH) with time in isolated hepatocytes following administration of adrenaline (10^{-7}M) in the presence of propranolol (10^{-5}M) (panel A) or prazocin (10^{-5}M) (panel B) . Results are expressed as percentage of relevant control ± S.D. for at least 4 different cell samples (* : $p<0.05$) . Control activities , expressed in pmoles of product formed per minute per 10^6 cells , were as follows :

	7α-OH	6β-OH	16α-OH
½ hour	17.2 ± 4.5	31.5 ± 8.2	42.3 ± 10.6
1 hour	28.2 ± 5.3	45.0 ± 6.3	64.1 ± 5.7
2 hour	24.3 ± 2.2	37.1 ± 3.4	49.6 ± 5.6

adrenaline , however , were blocked by the presence of propranolol (a β-blocker) . In the presence of propranolol enzyme activity did not differ significantly from control over the time course studied (2 minutes to 2 hours) . This indicates that the effects of adrenaline upon enzyme activity are probably mediated only by the elevation of cyclic AMP (via stimulation of β_2 receptors) and are not influenced by phosphatidylinositol turnover and calcium mobilisation (via stimulation of α_1 receptors) .

3.3(c) Effects of isobutylmethylxanthine (IBMX) and correlation with cyclic AMP levels .

The effects of the phosphodiesterase inhibitor , IBMX (10^{-3}M), upon cyclic AMP levels and the metabolism of 4-androstene-3,17-dione are shown in figure 14 . This compound inhibits the breakdown of cyclic AMP in the cell and so , therefore , would be expected to produce a rise in cyclic AMP . The cyclic AMP levels rose to 186% of control 2 minutes after addition of IBMX but rapidly dropped back to control levels . This initial transient rise in cyclic AMP levels was followed by a secondary sustained rise which occurred after 30 minutes and levels of cyclic AMP were maximally elevated to 259% of control levels at 24 hours following addition . The effects upon androstenedione metabolism showed a similar pattern of inhibition to that seen with 8-bromo-cyclic AMP and adrenaline in that maximum inhibition (60-75% of control) occurred at 1-2 hours . Results obtained with adrenaline indicate that the inhibitory effect of IBMX upon androstenedione metabolism

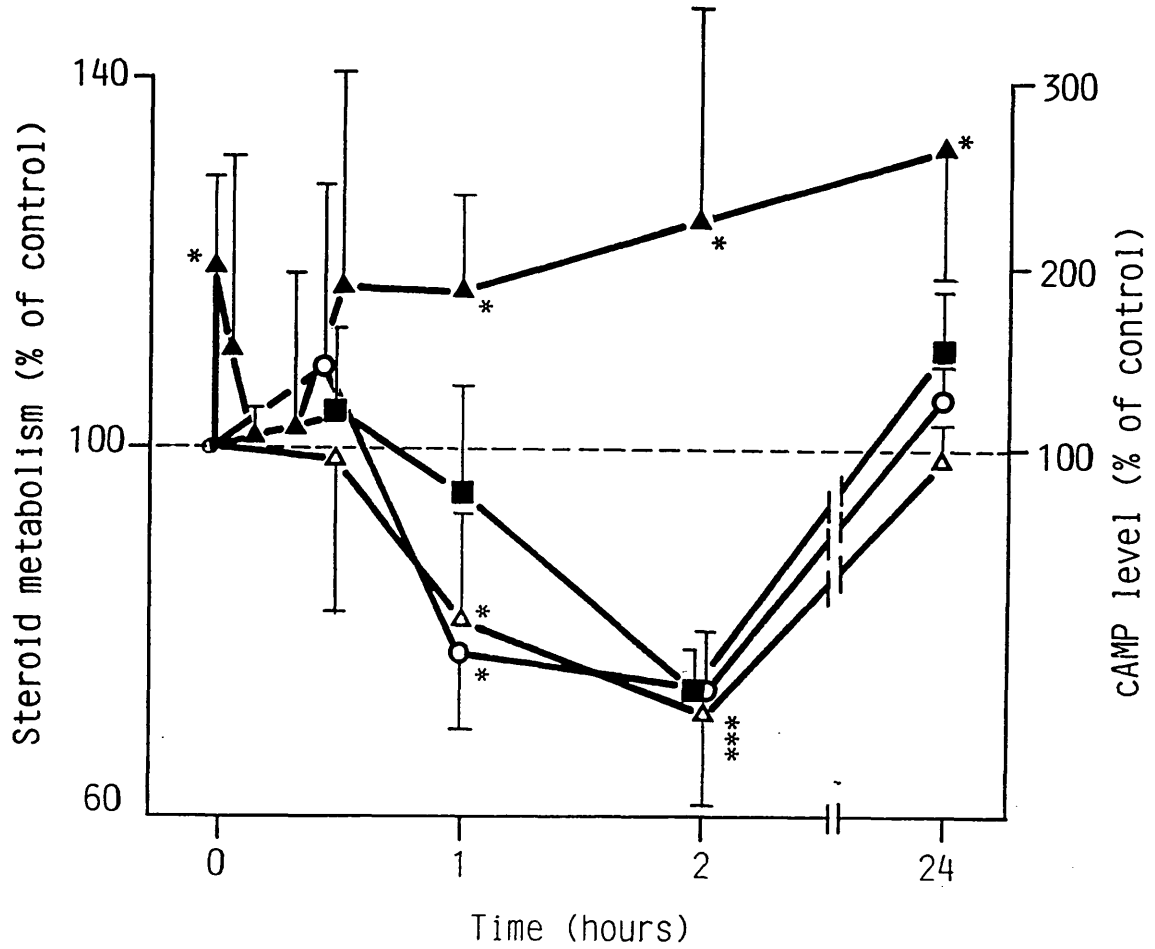

Figure 14 : Change in cyclic AMP content (▲) , 7α-(■) (7α-OH) , 6β-(○) (6β-OH) and 16α-hydroxylase (△) (16α-OH) activities with time in isolated hepatocytes following administration of isobutylmethyl-xanthine (10^{-3}M) . Results are expressed as percentage of relevant control ± S.D. for at least 4 different cell samples (* : $p<0.05$) . Control activities , expressed in pmoles of metabolite formed per minute per 10^6 cells , and cyclic AMP content , expressed in pmoles per 10^6 cells , were as follows :

	7α-OH	6β-OH	16α-OH	cyclic AMP
2 min	-	-	-	2.77 ± 1.72
5 min	-	-	-	1.37 ± 0.68
10 min	-	-	-	1.29 ± 0.47
20 min	-	-	-	0.76 ± 0.36
½ hour	23.7 ± 3.6	50.1 ± 12.8	72.9 ± 13.5	1.59 ± 0.86
1 hour	37.1 ± 4.6	67.7 ± 22.4	99.9 ± 25.2	1.09 ± 0.52
2 hour	47.8 ± 4.5	76.1 ± 2.4	117.5 ± 5.0	1.40 ± 0.75
24 hour	35.8 ± 1.5	29.1 ± 2.4	45.4 ± 2.2	0.42 ± 0.24

is likely to be related to the initial increase in cyclic AMP levels rather than later sustained rise . Good correlation exists between the magnitude of the initial rise in cyclic AMP levels produced by adrenaline (185% of control) and IBMX (186% of control) and the subsequent inhibition of steroid metabolism (60-75% of control) by these agents .

3.3(d) Effects of a combination of adrenaline and IBMX and correlation with cyclic AMP levels .

The combination of adrenaline (10^{-7}M) and IBMX (10^{-3}M) , as shown in figure 15 , produced a much greater increase in intracellular cyclic AMP levels than either of the two agents used alone , rising to 266% of control after 10 minutes incubation time . This sharp rise was followed by a slower decline in cyclic AMP levels than produced by adrenaline alone . The combination of these two agents resulted in marked inhibition of the metabolism of 4-androstene-3,17-dione (to 20-40% of corresponding control activities after 1-2 hours) . The magnitude of the enzyme inhibition was greater than that seen when using either adrenaline or IBMX alone . This indicates that the degree of enzyme inhibition is related to the height of the cyclic AMP peak in the first ten minutes .

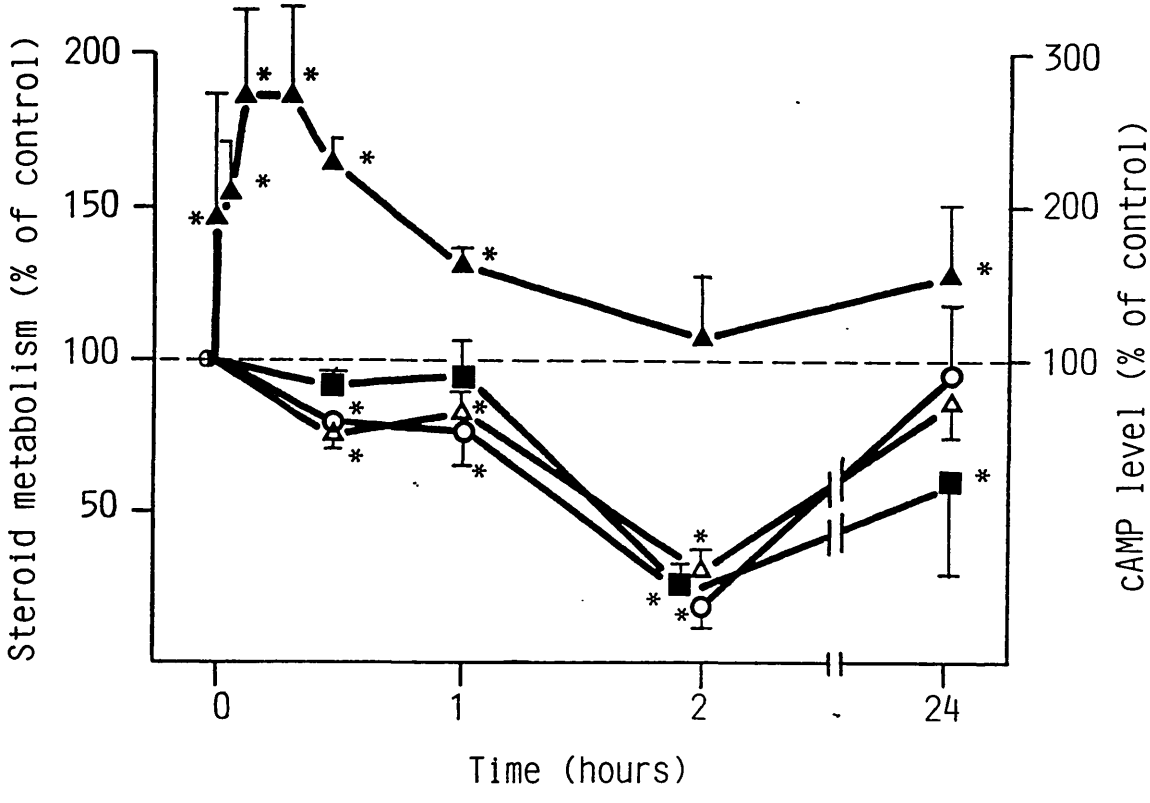

Figure 15 : Change in cyclic AMP content (▲) , 7α-(■) (7α-OH) , 6β-(○)(6β-OH) and 16α-hydroxylase (△) (16α-OH) activities with time in isolated hepatocytes following administration of adrenaline (10^{-7}M) and isobutylmethylxanthine (10^{-3}M) . Results are expressed as percentage of relevant control ± S.D. (* : $p<0.05$) . Control activities , expressed in pmoles of metabolite per minute per 10^6 cells , and cyclic AMP content , expressed in pmoles per 10^6 cells , were as follows :

	7α-OH	6β-OH	16α-OH	cyclic AMP
2 min	-	-	-	1.08 ± 0.16
5 min	-	-	-	1.13 ± 0.29
10 min	-	-	-	1.02 ± 0.36
20 min	-	-	-	1.05 ± 0.18
½ hour	70.6 ± 0.9	93.9 ± 1.0	118.2 ± 6.6	1.03 ± 0.17
1 hour	67.5 ± 10.9	92.3 ± 14.4	113.9 ± 14.5	1.06 ± 0.33
2 hour	69.7 ± 9.8	101.1 ± 14.3	125.8 ± 22.7	1.47 ± 0.39
24 hour	19.4 ± 5.7	14.6 ± 1.2	31.9 ± 4.3	3.22 ± 0.11

3.3(e) Effects of forskolin and correlation with cyclic AMP levels .

Figure 16 shows the effects of directly stimulating adenylate cyclase with forskolin (10^{-5}M) upon cyclic AMP and 4-androstene-3,17-dione metabolism . This compound bypasses the need for receptor activation and was seen to greatly increase the cyclic AMP levels of the hepatocytes (to 607% of control) 5 minutes after addition . Thereafter levels declined but remained elevated 2 hours after addition . Inhibition of androstenedione metabolism subsequent to this rise in cyclic AMP was , however , less marked than that observed with the combination of adrenaline and IBMX (section 3.3(d)) (65-75% of control activities) . This unexpected result may indicate that forskolin has additional actions (other than stimulation of adenylate cyclase) . In a different batch of hepatocytes (figure 17) , however , cyclic AMP was maximally elevated to 488% of control by the addition of forskolin and the subsequent inhibition of enzyme activity (45% of corresponding control activity at 2 hours) was of a similar magnitude to that observed with the combination of adrenaline and IBMX . This suggests that the loss of enzyme inhibition illustrated in figure 16 is due to the excessive cyclic AMP levels produced rather than any additional action of forskolin .

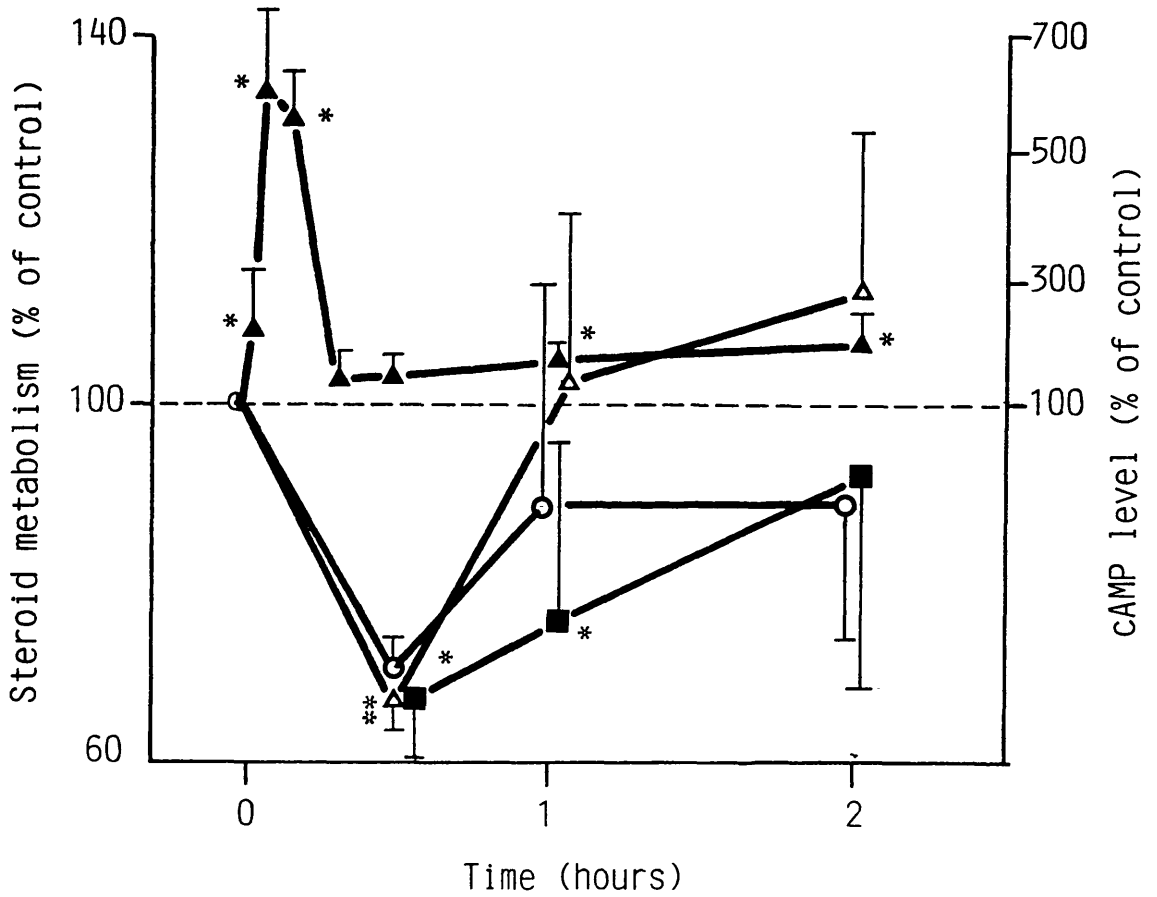

Figure 16 : Change in cyclic AMP content (▲) , 7α-(■) (7α-OH) , 6β-(○) (6β-OH) and 16α-hydroxylase (△) (16α-OH) activities with time in isolated hepatocytes following administration of forskolin (10^{-5}M). Results are expressed as percentage of relevant control ± S.D. for at least 4 different cell samples (* : $p<0.05$) . Control activities , expressed in pmoles of metabolite per min per 10^6 cells , and cyclic AMP content , expressed in pmoles per 10^6 cells , were as follows :

	7α-OH	6β-OH	16α-OH	cyclic AMP
2 min	-	-	-	1.07 ± 0.40
5 min	-	-	-	1.09 ± 0.14
10 min	-	-	-	1.11 ± 0.13
20 min	-	-	-	1.04 ± 0.17
½ hour	60.1 ± 18.9	56.4 ± 7.5	86.7 ± 21.1	1.12 ± 0.29
1 hour	49.3 ± 16.1	47.7 ± 15.6	48.4 ± 6.8	1.01 ± 0.24
2 hour	47.2 ± 11.4	54.1 ± 9.6	51.9 ± 5.8	0.81 ± 0.24

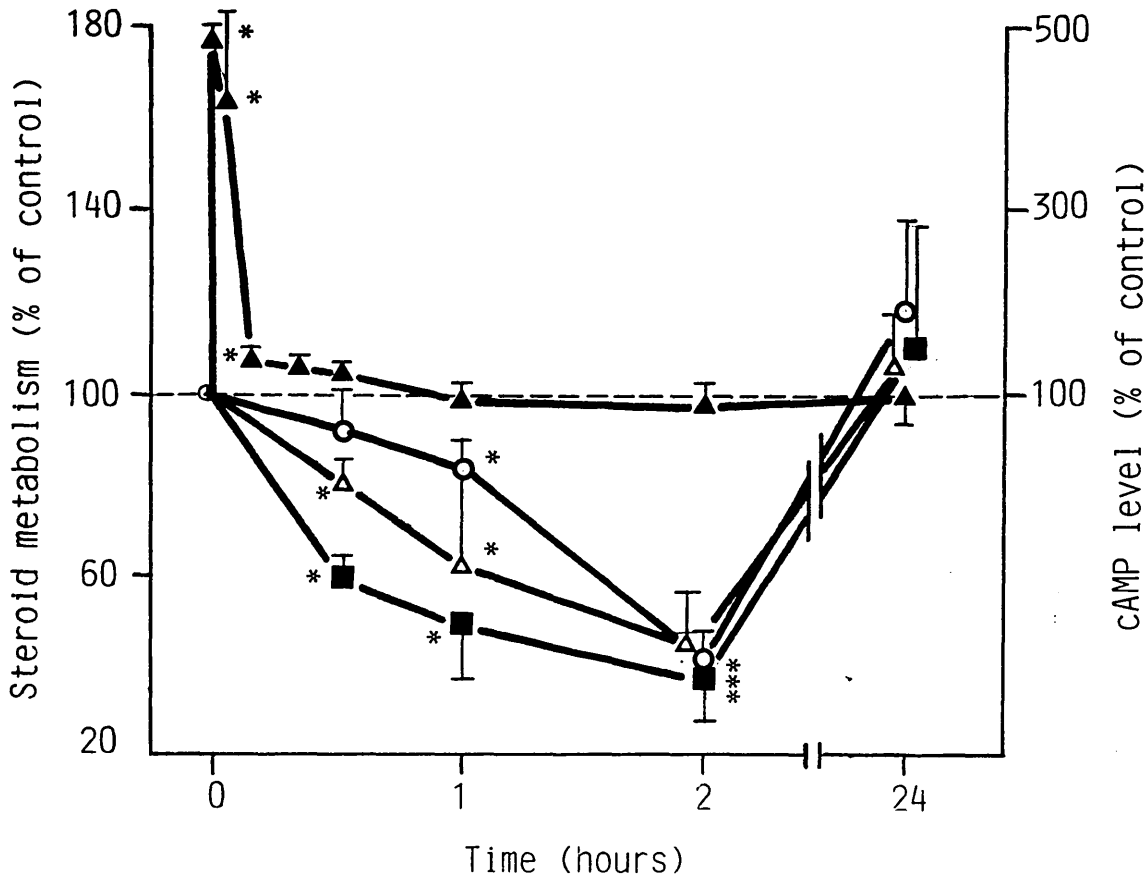

Figure 17 : Change in cyclic AMP content (▲) , 7α-(■) (7α-OH) , 6β-(○)(6β-OH) and 16α-hydroxylase (△) (16α-OH) activities with time in isolated hepatocytes following administration of forskolin (10^{-5}M) . Results are expressed as percentage of relevant control for at least 4 different cell samples (* : $p<0.05$) . Control activities , expressed in pmoles of metabolite per minute per 10^6 cells , and cyclic AMP content , expressed in pmoles per 10^6 cells , were as follows :

	7α-OH	6β-OH	16α-OH	cyclic AMP
2 min	-	-	-	1.08 ± 0.16
5 min	-	-	-	1.13 ± 0.29
10 min	-	-	-	1.04 ± 0.48
20 min	-	-	-	1.24 ± 0.25
½ hour	143.9 ± 30.8	108.0 ± 22.0	171.9 ± 44.7	1.08 ± 0.34
1 hour	93.7 ± 9.3	113.5 ± 12.0	148.2 ± 18.5	1.30 ± 0.20
2 hour	111.4 ± 16.1	123.6 ± 15.0	158.8 ± 23.5	1.22 ± 0.15
24 hour	69.3 ± 22.5	67.8 ± 16.3	132.8 ± 34.3	0.27 ± 0.03

3.3(f) Effects of a combination of forskolin and IBMX and correlation with cyclic AMP levels .

The combination of forskolin (10^{-5}M) and IBMX (10^{-3}M) was used to examine further the effects of excessive cyclic AMP levels upon the metabolism of 4-androstene-3,17-dione . Figure 18 shows the results obtained for hepatocyte samples in which addition of forskolin + IBMX resulted in increase in cyclic AMP levels to 1256% of control 5 minutes after addition . Thereafter levels declined but remained elevated for the duration of the study . Androstenedione metabolism was slightly inhibited at ½ hour but this was followed by significant stimulation of enzyme activity at 1 hour (130-160% of control) . The enzyme activities had returned to control levels by 2 hours . As no secondary stimulation of enzyme activity was seen after the addition of IBMX which also caused a marked rise in cyclic AMP levels at later time periods (section 3.3(c)) , it appears that both the inhibition and stimulation of enzyme activity are related to the initial rise in cyclic AMP levels produced in the first ten minutes . In a different batch of hepatocytes (figure 19) cyclic AMP levels were elevated to 1912% of control by the addition of forskolin + IBMX . At this greater elevation of cyclic AMP no inhibition of enzyme activity is apparent and only the (secondary) stimulatory effect (140-160% of control)is observed .

It appears , therefore , that the effects of elevating cyclic AMP upon hepatic steroid metabolism can be separated into two phases ; at lower , physiological levels (2-3 fold stimulation),

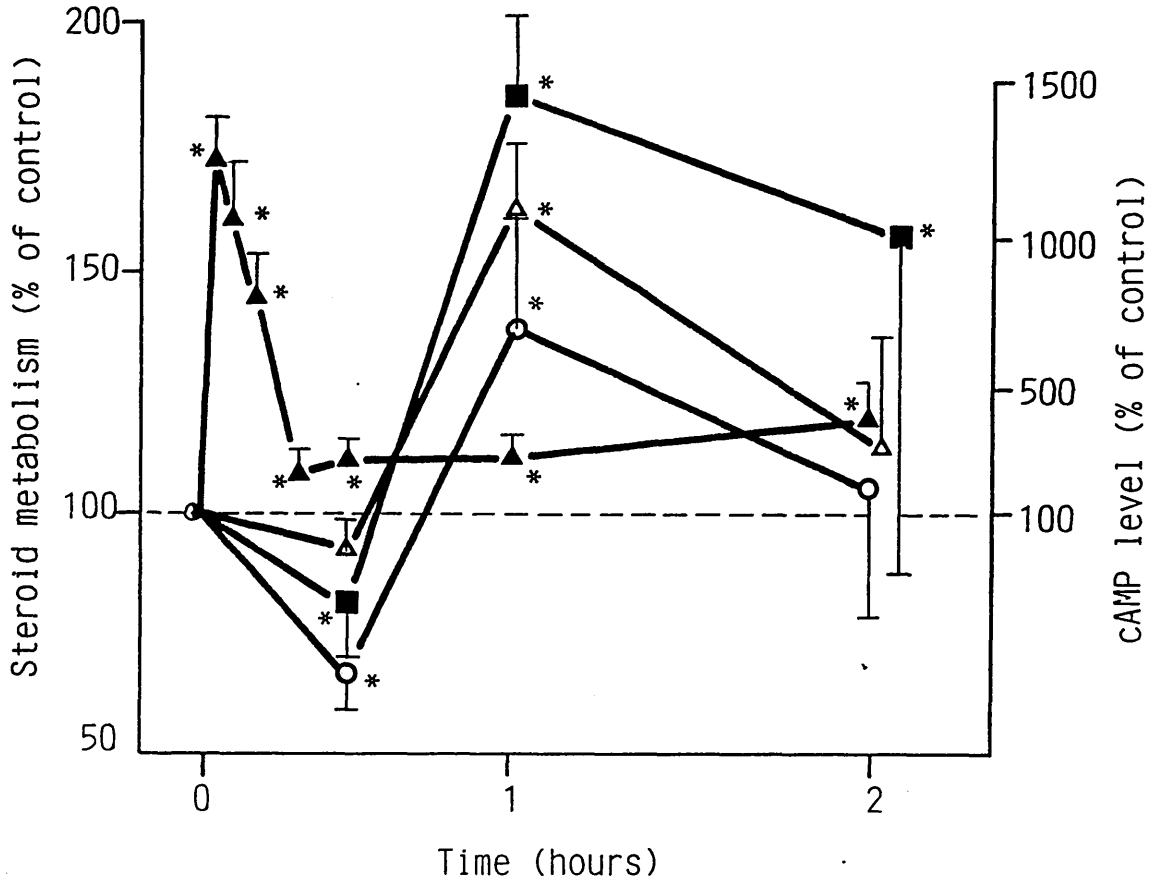

Figure 18 : Change in cyclic AMP content (▲) , 7α-(■) (7α-OH) , 6β-(-○-)(6β-OH) and 16α-hydroxylase (△) (16α-OH) activities with time in isolated hepatocytes following the administration of forskolin (10^{-5}M) and isobutylmethylxanthine (10^{-3}M) . Results are expressed as percentage of relevant control ± S.D. for at least 4 different cell samples (* : $p<0.05$). Control activities , expressed in pmoles of metabolite per minute per 10^6 cells , and cyclic AMP content , expressed in pmoles per 10_6 cells , were as follows :

	7α-OH	6β-OH	16α-OH	cyclic AMP
2 min	-	-	-	1.16 ± 0.16
5 min	-	-	-	1.11 ± 0.47
10 min	-	-	-	1.03 ± 0.38
20 min	-	-	-	1.09 ± 0.12
½ hour	74.1 ± 18.3	69.0 ± 15.3	115.0 ± 26.2	1.44 ± 0.47
1 hour	93.2 ± 37.9	90.6 ± 14.9	129.6 ± 40 4	1.10 ± 0.24
2 hour	93.8 ± 37.9	94.7 ± 24.5	119.6 ± 33.1	0.95 ± 0.23

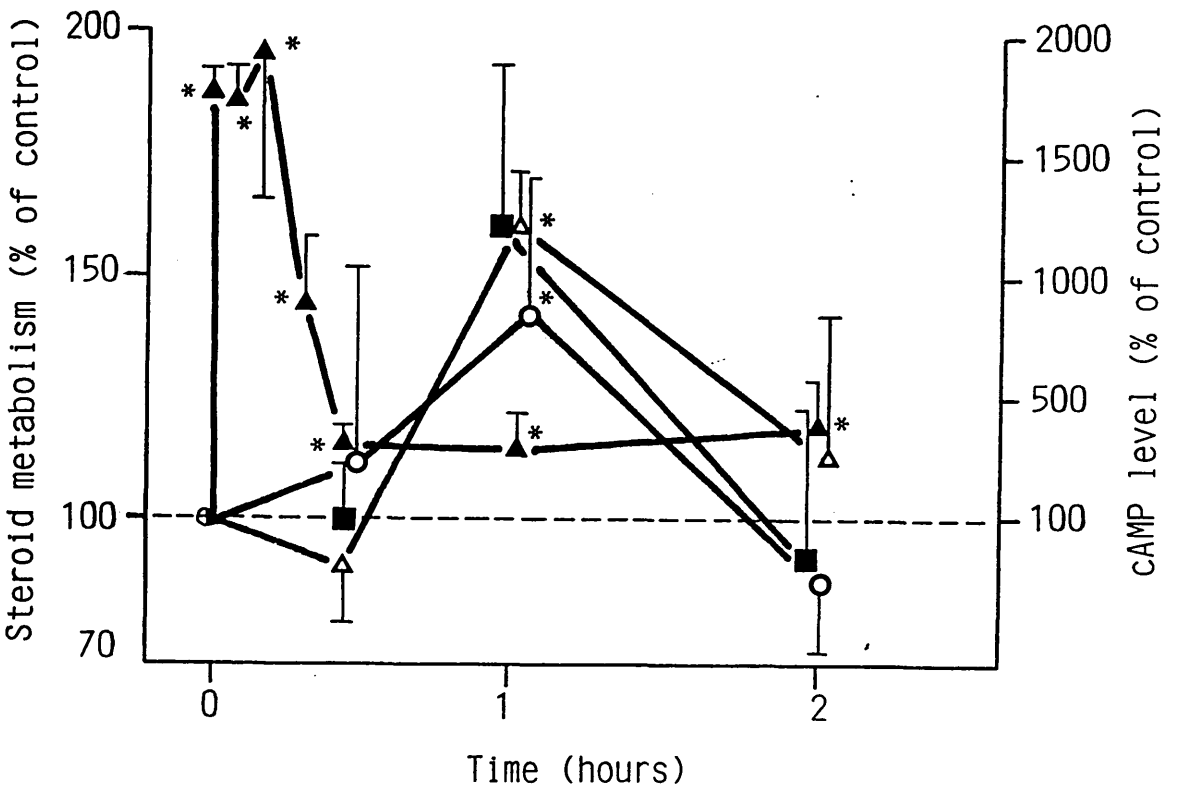

Figure 19 : Change in cyclic AMP content (▲) , 7α-(■) (7α-OH) , 6β-(○)(6β-OH) and 16α-hydroxylase (△) (16α-OH) activities with time in isolated hepatocytes following the administration of forskolin (10^{-5}M) and isobutylmethylxanthine (10^{-3}M) . Results are expressed as percentage of relevant control ± S.D. (* : $p<0.05$) . Control activities , expressed in pmoles of metabolite per minute per 10^6 cells , and cyclic AMP content , expressed in pmoles per 10^6 cells , were as follows :

	7α-OH	6β-OH	16α-OH	cyclic AMP
2 min	-	-	-	1.07 ± 0.40
5 min	-	-	-	1.09 ± 0.14
10 min	-	-	-	1.11 ± 0.13
20 min	-	-	-	1.04 ± 0.17
½ hour	60.1 ± 18.9	56.4 ± 7.5	86.7 ± 21.1	1.12 ± 0.29
1 hour	49.3 ± 16.1	47.7 ± 15.6	54.1 ± 9.6	1.01 ± 0.24
2 hour	47.2 ± 11.4	54.1 ± 9.6	51.9 ± 5.8	0.81 ± 0.24

an inhibition is seen whereas at higher levels (>10 fold stimulation) a stimulatory effect is seen at a later time point than the inhibition .

3.3(g) Effects of different concentrations of cyclic AMP in electropermeabilised hepatocytes .

The effects of different concentrations of cyclic AMP (5 x 10^{-5}M - 5 x 10^{-3}M) upon the metabolism of 4-androstene-3,17-dione metabolism in electropermeabilised rat hepatocytes are shown in figure 20 . Panel A shows the time course of the effect of 5 x 10^{-5}M cyclic AMP (concentrations of cyclic AMP below this level were found to be ineffective in modifying androstenedione metabolism) . Enzyme activities were inhibited to 65-75% of control at 1 hour after addition but recovered rapidly to control levels between 2 and 3 hours . The effects of 5 x 10^{-4}M cyclic AMP are shown in panel B . Effects were similar to those seen with 5 x 10^{-5}M except that the magnitude of inhibition was less marked (80-85% of control) and there was a tendency towards stimulation at 2 hours . The effect of 5 x 10^{-3}M (panel C) , however , was markedly different , exhibiting little inhibition at the earlier time periods but marked stimulation of enzyme activity (130-137% of control activities) at 2 hours . This stimulation was transient and had virtually disappeared at 3 hours following addition .

These results correlate with those obtained in intact hepatocytes and appear to indicate that the two opposing effects

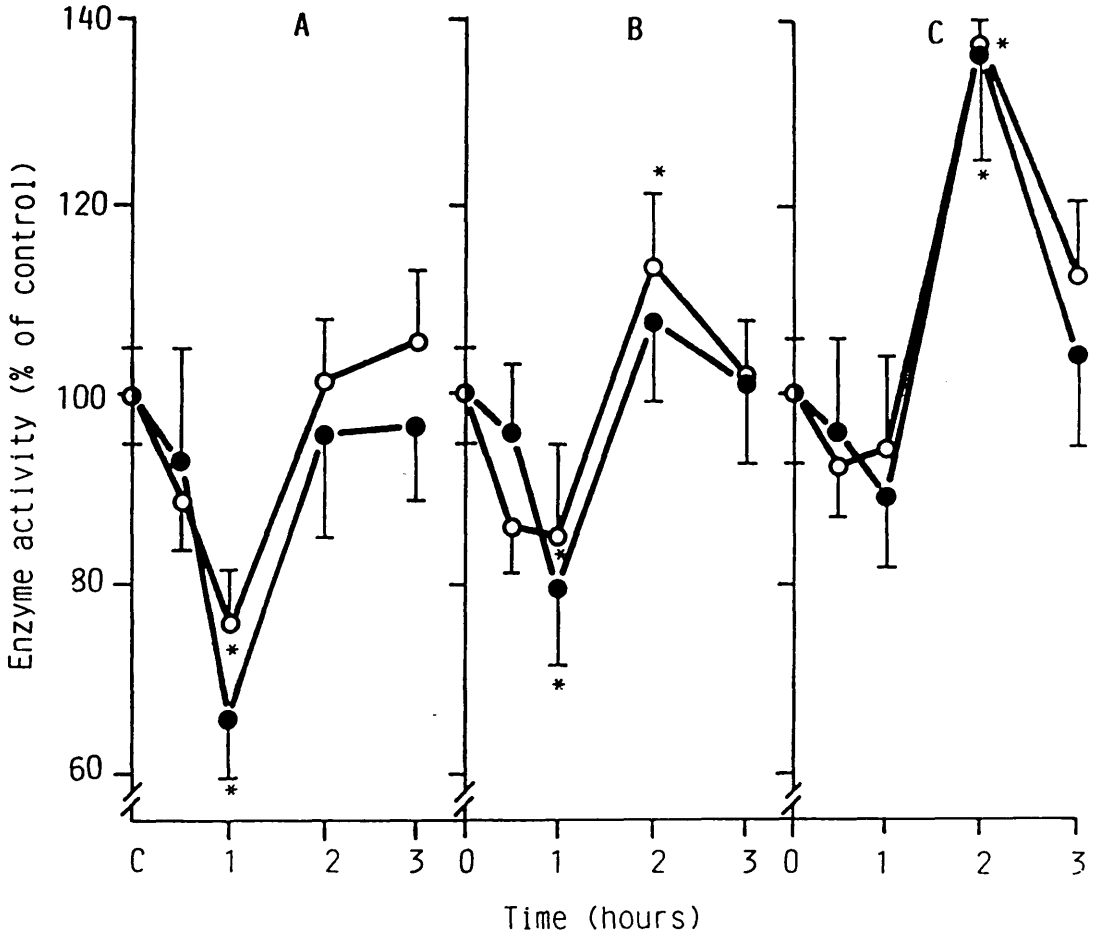

Figure 20 : Change in 7α-(●-) (7α-OH) , and 6β-hydroxylase (-o-) (6β-OH) activities with time in electropermeabilised hepatocytes following administration of 5 x 10^{-5}M cyclic AMP (panel A) , 5 x 10^{-4}M cyclic AMP (panel B) and 5 x 10^{-3}M cyclic AMP (panel C) . Results are expressed as percentage of relevant control ± S.D. for at least 4 different cell samples (* : $p<0.05$) . Control activities , expressed in pmoles of metabolite per minute per 10^6 cells , were as follows :

	½ hour	1 hour	2 hour	3 hour
7α-OH	55.3 ± 9.2	97.6 ± 8.8	66.9 ± 12.0	58.5 ± 5.4
6β-OH	85.7 ± 6.5	94.9 ± 9.6	69.1 ± 6.1	77.3 ± 10.9

seen are due to cyclic AMP elevation rather than any alternative mode of action . Low concentrations of cyclic AMP (5 x 10^{-5}M) produce effects upon enzyme activity which correspond to those produced by 8-bromo-cyclic AMP , adrenaline , IBMX , and adrenaline + IBMX i.e. primary inhibition . Intermediate concentrations (5 x 10^{-4}M) produce effects which correspond to those produced by forskolin (figure 15) i.e. loss of the primary inhibitory effect and a tendency towards secondary stimulation . High concentrations of cyclic AMP (5 x 10^{-3}M) produce effects which correspond to those produced by forskolin + IBMX i.e. secondary stimulatory effect only .

3.4 THE EFFECTS OF CYCLOHEXIMIDE UPON THE ACTIONS OF CYCLIC AMP IN ALTERATION OF ANDROSTENEDIONE METABOLISM .

3.4(a) Effects of cycloheximide upon the actions of a combination of forskolin and IBMX in intact hepatocytes .

The effects of forskolin (10^{-5}M) in combination with IBMX (10^{-3}M) upon the metabolism of 4-androstene-3,17-dione in the presence of cycloheximide (10^{-5}M) are shown in figure 21 . Cycloheximide is an inhibitor of messenger RNA translation at the level of the ribosomes (Peshka 1971) and was used to evaluate the role of protein synthesis in the actions of the combination of forskolin and IBMX . When protein synthesis was blocked by cycloheximide , the secondary stimulatory response was abolished

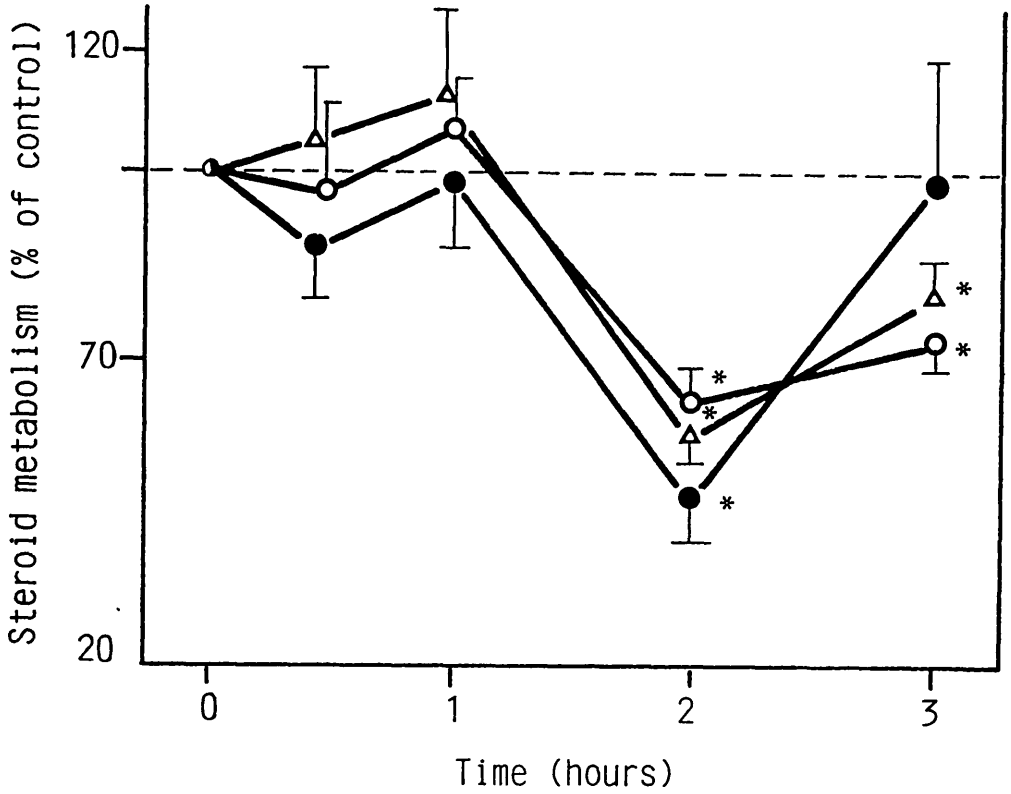

Figure 21 : Change in 7α-(●) (7α-OH) , 6β-(○) (6β-OH) and 16α-hydroxylase (△) (16α-OH) activities with time in isolated hepatocytes following the administration of forskolin (10^{-5}M) in the presence of cycloheximide (10^{-5}M) . Results are expressed as percentage of relevant control ± S.D. for at least 4 different cell samples (* : $p<0.05$) . Control activities , expressed as pmoles of metabolite per minute per 10^6 cells , were as follows :

	½ hour	1 hour	2 hour	3 hour
7α-OH	23.3 ± 5.2	30.5 ± 7.8	28.6 ± 3.4	22.4 ± 1.2
6β-OH	36.6 ± 10.0	75.2 ± 12.1	74.7 ± 5.2	55.0 ± 5.2
16α-OH	42.8 ± 10.0	81.9 ± 12.7	97.3 ± 5.7	62.1 ± 5.1

and the magnitude of the inhibitory effect of this large concentration of cyclic AMP was enhanced (40-60% at 2 hours) .

From these results it is apparent that the secondary stimulatory effect of high concentrations of cyclic AMP , as produced by the combination of forskolin and IBMX , is dependent upon protein synthesis but the initial inhibition occurs independently of protein synthesis . It is also apparent that the loss of the inhibitory effects of cyclic AMP at high concentrations is also due to protein synthesis presumably because inhibition is masked by the greater magnitude of the stimulatory effect .

It can be seen in figure 21 that each of the enzyme activities shown is affected in a similar way by preincubation with cycloheximide indicating that each of the enzymes is under identical control with regards to the mechanism of action of cyclic AMP . In sections 3.4(b) and 3.5 , therefore , only the activity of 7α hydroxylase will be illustrated in the figures and discussed , to allow comparison with control time courses . In each case all of the enzymes were affected in an identical manner by the addition of the agents .

3.4(b) Effects of cycloheximide upon the actions of cyclic AMP in electropermeabilised hepatocytes .

Figure 22 shows the effects of preincubation with cycloheximide (10^{-5}M) on the actions of cyclic AMP 5 x 10^{-5}M (panel A) , 5 x 10^{-4}M (panel B) and 5 x 10^{-3}M (panel C) upon

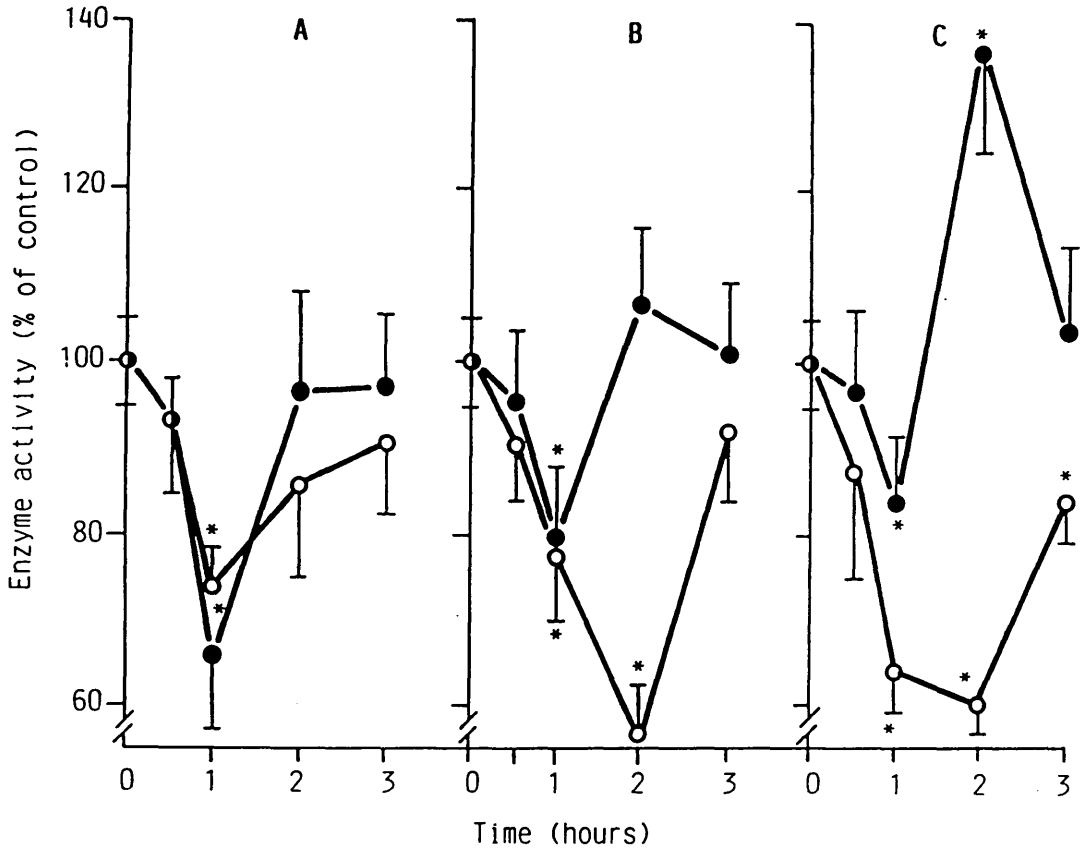

Figure 22 : Change in 7α-hydroxylase activity in electropermeabilised hepatocytes with time following the administration of 5 x 10^{-5}M cyclic AMP (panel A) , 5 x 10^{-4}M cyclic AMP (panel B) and 5 x 10^{-3}M cyclic AMP (panel C) in the presence (-O-) or absence (-●-) of cycloheximide (10^{-5}M). Results are expressed as percentage of relevant control ± S.D. for at least 4 different cell samples (* : $p<0.05$) . Control 7α-hydroxylase activities, expressed in pmoles of metabolite per minute per 10^6 cells , were as follows:

	½ hour	1 hour	2 hour	3 hour
control	55.3 ± 9.2	97.6 ± 8.8	66.9 ± 12.0	58.5 ± 5.4
control (cycloheximide)	80.6 ± 6.6	86.4 ± 8.8	83.7 ± 5.9	60.6 ± 6.7

the metabolism of 4-androstene-3,17-dione . It can be seen that when protein synthesis was blocked by cycloheximide the initial inhibitory effect of 5 x 10^{-5}M cyclic AMP was unaffected but recovery from inhibition was somewhat slower (not complete by 2 hours) . In the presence of cycloheximide , 5 x 10^{-4}M cyclic AMP produced much greater inhibition (57% of corresponding control activity) than in the cells treated with cyclic AMP alone (80% of corresponding control activity) and the inhibition was slower to reverse (not recovered by 3 hours) . When cells were treated with 5 x 10^{-3}M cyclic AMP following preincubation with cycloheximide , the stimulation of enzyme activity was abolished and marked inhibition of enzyme activity occurred (maximum of 60% of corresponding control at 2 hours) over the time course of ½ to 3 hours .

These effects correlate with those seen in 3.4(a) above i.e. the stimulatory effect but not the inhibitory effect is dependent upon protein synthesis and the loss of the inhibitory effect at high concentrations of cyclic AMP is due to it being masked by the greater magnitude of the stimulatory effect . It is also apparent from the lower concentrations of cyclic AMP (5 x 10^{-5}M) that recovery from inhibition is also dependent upon protein synthesis and that cyclic AMP stimulated- protein synthesis occurs even at the lower concentrations but is too weak to produce the secondary stimulation of enzyme activity .

3.5 THE EFFECTS OF PROTEIN KINASE INHIBITORS UPON THE ACTIONS OF CYCLIC AMP IN ALTERATION OF ANDROSTENEDIONE METABOLISM IN ELECTROPERMEABILISED HEPATOCYTES .

3.5(a) Effects of K-252a (general protein kinase inhibitor)

Figure 23 shows the effects of preincubating with K-252a (an inhibitor of all protein kinases) (2×10^{-8}M) upon the time course of the effect of 5×10^{-5}M cyclic AMP (panel A) and 5×10^{-3}M cyclic AMP (panel B) on 7α hydroxylase activity . Both the primary inhibitory effect produced by 5×10^{-5}M cyclic AMP and the secondary stimulation produced by 5×10^{-3}M cyclic AMP were abolished by preincubation with K-252a indicating that both of these effects are produced by activation of a protein kinase by cyclic AMP .

3.5(b) Effects of KT5720 (inhibitor of cyclic AMP-dependent protein kinase) .

The effects of preincubating with KT5720 (a specific inhibitor of PKa) (6×10^{-8}M) upon the actions of 5×10^{-5}M cyclic AMP (panel A) and 5×10^{-3}M cyclic AMP (panel B) are shown in figure 24 . As was seen following preincubation with the general protein kinase inhibitor , both the stimulatory and inhibitory effects of cyclic AMP were abolished in the presence of KT5720 indicating both these effects were due to the activation of

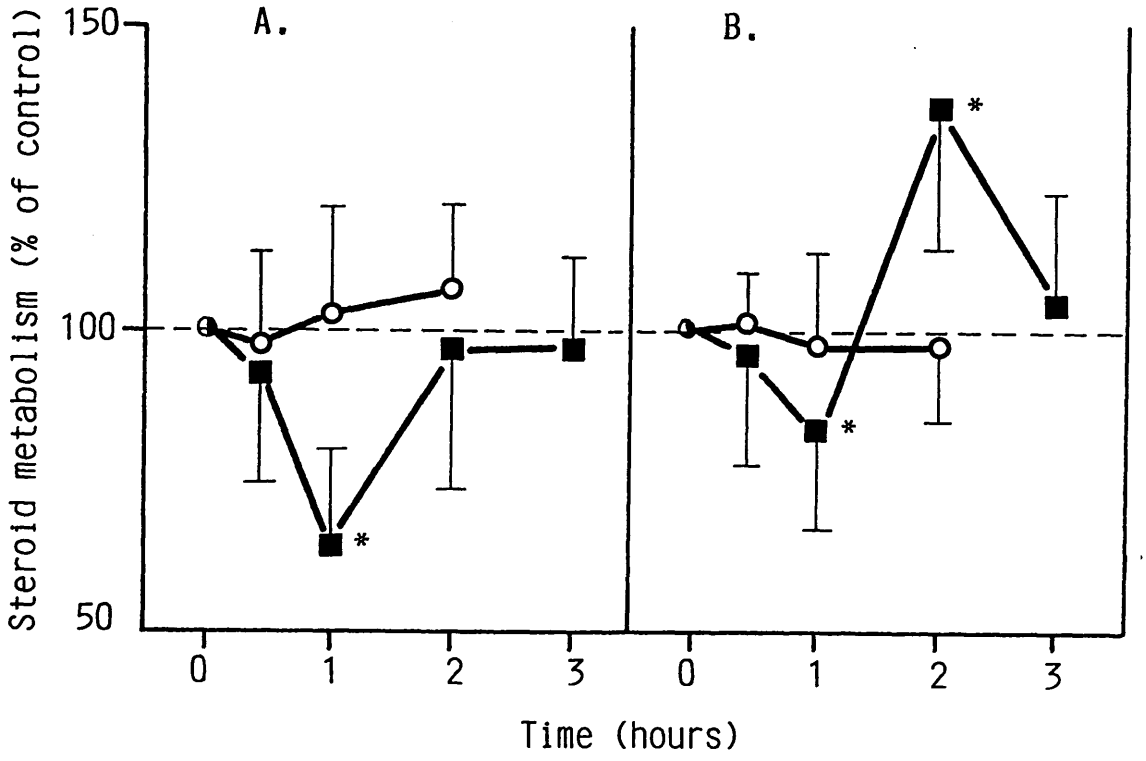

Figure 23 : Change in 7α-hydroxylase activity in electropermeabilised hepatocytes with time following the administration of 5 x 10^{-5}M cyclic AMP (panel A) and 5 x 10^{-3}M cyclic AMP (panel B) in the presence (-o-) and absence (-■-) of K-252a (2 x 10^{-8}M) . Results are expressed as percentage of relevant control ± S.D. for at least 4 different cell samples.(* : $p<0.05$). Control 7α-hydroxylase activities , expressed in pmole of metabolite per minute per 10^6 cells , were as follows :

	½ hour	1 hour	2 hour
control	55.3 ± 9.2	97.6 ± 8.8	66.9 ± 12.0
control (K-252a)	52.2 ± 18.0	44.8 ± 13.1	41.5 ± 6.3

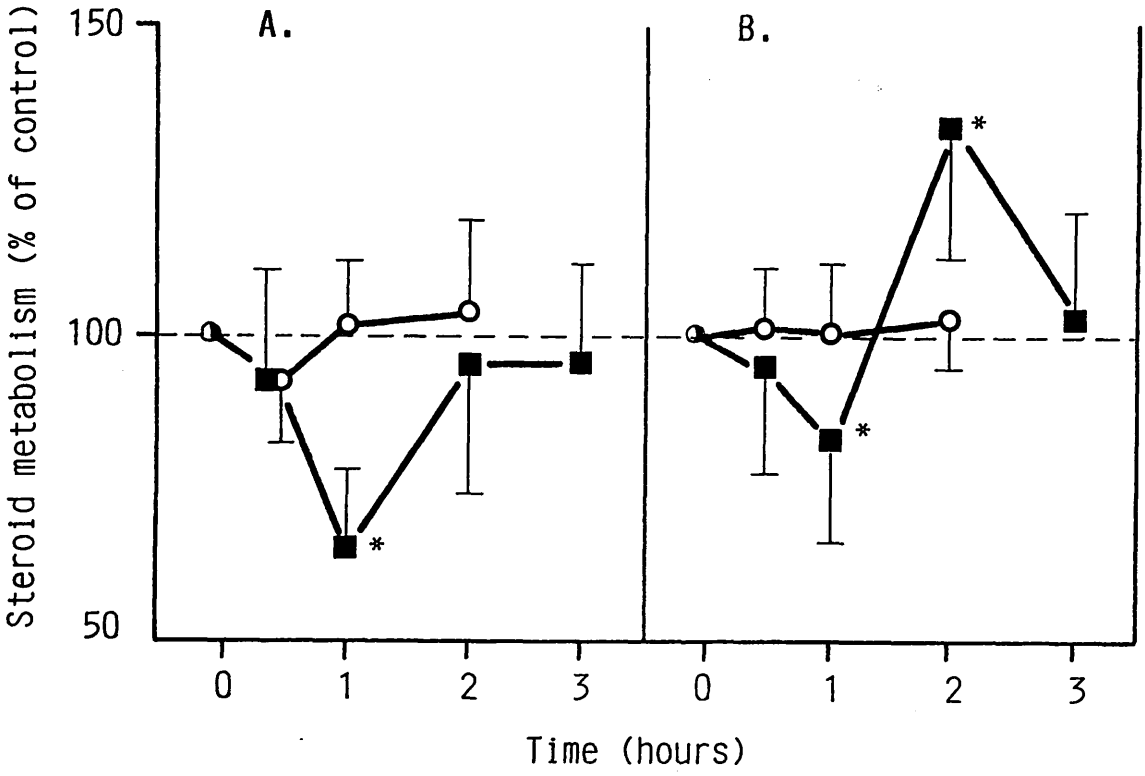

Figure 24 : Change in 7α-hydroxylase activity in electropermeabilised hepatocytes with time following the administration of 5 x 10^{-5}M cyclic AMP (panel A) and 5 x 10^{-3}M (panel B) in the presence (-o-) and absence (-■-) of KT5720 (6 x 10^{-8}M) . Results are expressed as percentage of relevant control ± S.D. for at least 4 different cell samples (* : $p<0.05$). Control 7α-hydroxylase activities , expressed as pmole of metabolite per minute per 10^6 cells , were as follows :

	½ hour	1 hour	2 hour
control	55.3 ± 9.2	97.6 ± 8.8	66.9 ± 12.0
control (KT5720)	58.3 ± 18.0	47.3 ± 13.4	41.5 ± 12.4

PKa .

3.5(c) Effects of K-252b (inhibitor of protein kinase c)

The effects of preincubating with K-252b (a specific inhibitor of PKc) (2×10^{-8}M) upon the actions of 5×10^{-5}M cyclic AMP (panel A) and 5×10^{-3}M cyclic AMP (panel B) are shown in figure 25 . When PKc is inhibited by the presence of K-252b the primary inhibitory effect attributed to the initial activation of PKa by 5×10^{-5}M cyclic AMP (section 3.5(b)) was abolished and the presence of K-252b revealed a slight stimulatory effect (110% of corresponding control activity) which may otherwise have been masked by the predominant inhibition . The secondary stimulatory effects of 5×10^{-3}M cyclic AMP in the presence of K-252b also occurred at an earlier time than in the corresponding cyclic AMP time course in untreated cells and showed a dose-dependent increase in magnitude above the stimulation seen with 5×10^{-5}M cyclic AMP (to 136% of corresponding control activity) . This stimulation , however , was not maintained throughout the time course . At 2 hours the stimulation was less than that seen in the corresponding cyclic AMP time course in untreated cells .

It would appear that in the absence of PKc , therefore , cyclic AMP has no inhibitory effects at any concentration and dose dependent stimulation occurs at an earlier time period than seen in the presence of PKc .

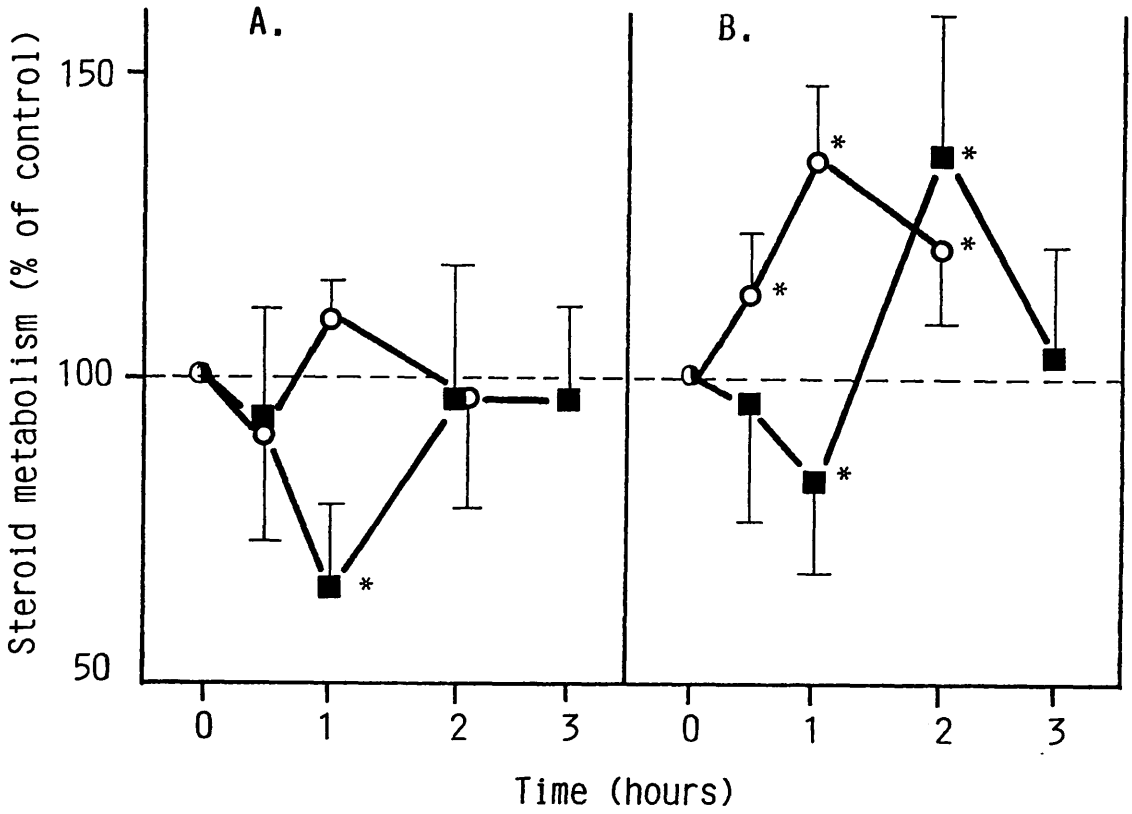

Figure 25 : Change in 7α-hydroxylase activity with time in electro-permeabilised hepatocytes following administration of 5 x 10^{-5}M cyclic AMP (panel A) and 5 X 10^{-3}M cyclic AMP (panel B) in the presence (-o-) or absence (-■-) of K-252b (2 X 10^{-8}M) . Results are expressed as percentage of relevant control ± S.D. for at least 4 different cell samples (* : $p<0.05$). Control 7α-hydroxylase activities , expressed in pmole of metabolite per minute per 10^6 cells , were as follows :

	½ hour	1 hour	2 hour
control	55.3 ± 9.2	97.6 ± 8.8	66.9 ± 12.0
control (K-252b)	44.7 ± 10.3	35.7 ± 7.7	55.8 ± 10.6

3.5(d) Effects of a combination of K-252b and cycloheximide .

The effects of cycloheximide upon the cyclic AMP mediated early stimulation of steroid metabolism in the presence of K-252b, were examined to ascertain if the stimulation produced by PKa activation in the absence of PKc was due to protein synthesis . The results are shown in figure 26 for 5 x 10^{-5}M cyclic AMP , K-252b (2 x 10^{-8}M) and cycloheximide (10^{-5}M) (panel A) and 5 x 10^{-3}M cyclic AMP , K-252b (2 x 10^{-8}M) and cycloheximide (10^{-5}M) (panel B) . Effects in the presence of K-252b and cycloheximide alone are also shown for comparison . In the presence of cycloheximide the slight stimulation of enzyme activity produced by 5 x 10^{-5}M cyclic AMP and K-252b alone was abolished and activity did not differ significantly from corresponding control activity . The greater stimulation of enzyme activity produced by 5 x 10^{-3}M cyclic AMP and K-252b was also inhibited by the presence of cycloheximide but unlike the lower concentration of cyclic AMP, slight inhibition of enzyme activity occurred (80% of corresponding control activity at 1 hour) .

It would appear that the inhibitory effects of physiological concentrations of cyclic AMP (5 x 10^{-5}M) are produced only by activation of PKc and that PKa activation has no direct inhibitory effects upon enzyme activity . At pharmacological concentrations of cyclic AMP (5 x 10^{-3}M) PKa activation does have a direct inhibitory action upon enzyme activity however the degree of inhibition produced by 5 x 10^{-3}M cyclic AMP in the presence of K-252b and cycloheximide was less marked than in the presence of

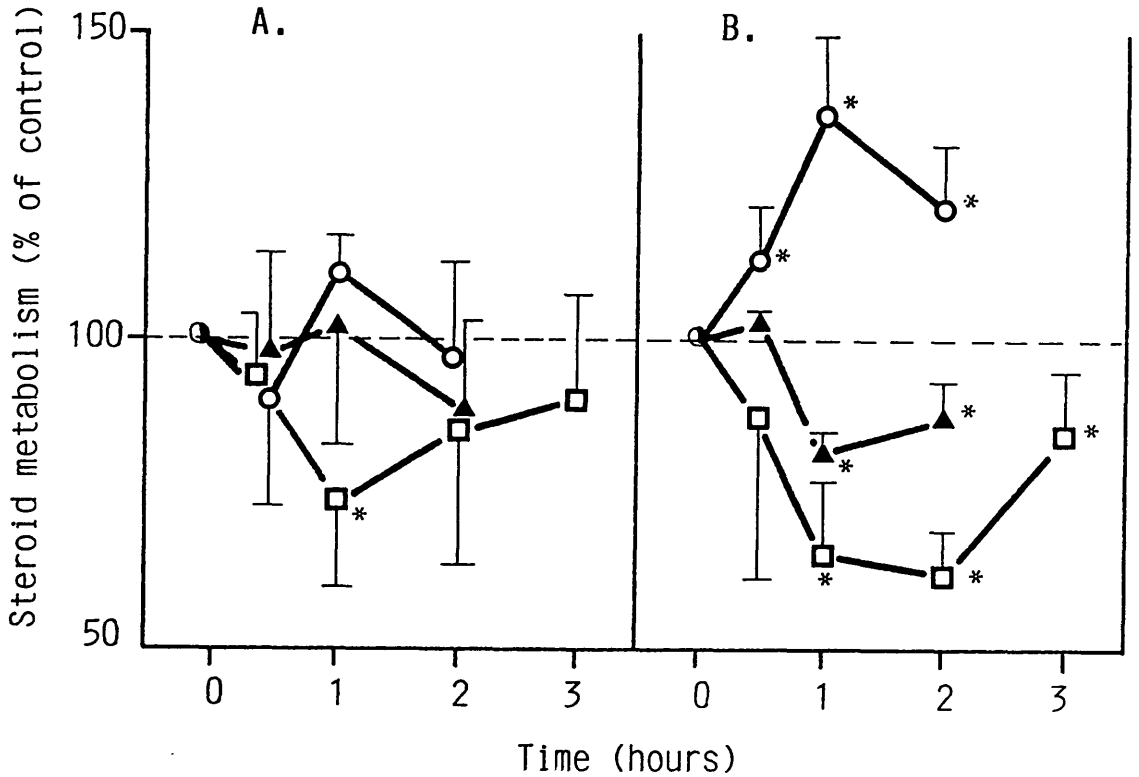

Figure 26 : Change in 7α-hydroxylase activity in electropermeabilised hepatocytes with time following the administration of 5 x 10^{-5}M cyclic AMP (panel A) and 5 X 10^{-3}M cyclic AMP (panel B) in the presence of K-252b (2 X 10^{-8}M) (-○-) , cycloheximide (10^{-5}M) (-□-) and a combination of cycloheximide and K-252b (-▲-) . Results are expressed as percentage of relevant control ± S.D. for at least 4 different cell samples (* : $p<0.05$). Control 7α-hydroxylase activities , expressed in pmole of metabolite per minute per 10^6 cells , were as follows :

	½ hour	1 hour	2 hour
control (K-252b)	44.7 ± 10.3	35.7 ± 7.7	55.8 ± 10.6
control (cycloheximide)	80.6 ± 6.6	86.4 ± 8.8	83.7 ± 5.9
control (cycloheximide + K-252b)	43.9 ± 5.0	40.6 ± 1.6	41.9 ± 10.4

cycloheximide alone (section 3.4(b)) which may indicate that both PKa and PKc activation are required for the full inhibitory effects of 5 x 10^{-3}M cyclic AMP . The early stimulatory effects of PKa activation in the absence of PKc are blocked by the presence of cycloheximide and so are mediated by an increase in protein synthesis .

3.5(e) Effects of KT5822 (inhibitor of cyclic GMP-dependent protein kinase) .

The effects of preincubating with KT5822 (a specific inhibitor of PKg) (2.5 X 10^{-9}M) upon the actions of 5 x 10^{-5}M cyclic AMP (panel A) and 5 x 10^{-3}M cyclic AMP (panel B) are shown in figure 27 . No significant difference was apparent between the effects of cyclic AMP upon steroid metabolism in the presence or in the absence of PKg indicating that activation of this protein kinase has no role to play in the actions of cyclic AMP upon hepatic steroid metabolism .

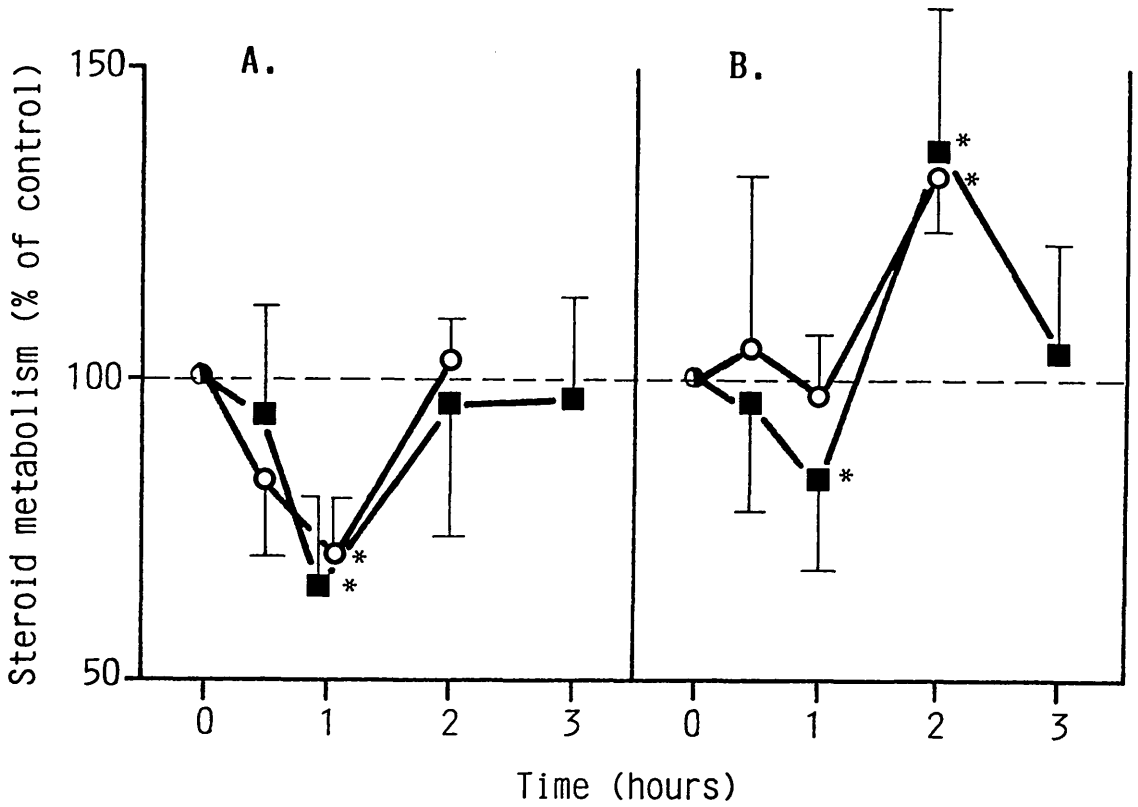

Figure 27 : Change in 7α-hydroxylase activity with time in electro-permeabilised hepatocytes following the administration of 5 X 10^{-5}M cyclic AMP (panel A) and 5 X 10^{-3}M cyclic AMP (panel B) in the presence (-o-) and absence (-■-) of KT5822 (2.5 X 10^{-9}M) . Results are expressed as percentage of relevant control ± S.D. for at least 4 different cell samples (* : $p<0.05$) . Control 7α-hydroxylase activities , expressed as pmole of metabolite per minute per 10^6 cells , were as follows :

	½ hour	1 hour	2 hour
control	55.3 ± 9.2	97.6 ± 8.8	66.9 ± 12.0
control (KT5822)	42.7 ± 5.7	50.8 ± 10.9	46.6 ± 11.0

3.6 EFFECTS OF ELEVATING CYCLIC AMP UPON CYTOCHROME P450 LEVELS IN ISOLATED RAT HEPATOCYTES .

3.6(a) Effects of 8-bromo-cyclic AMP , adrenaline , adrenaline and IBMX , forskolin and forskolin and IBMX in intact hepatocytes .

Basal levels of cytochrome P450 in freshly isolated , untreated hepatocytes were calculated to be approximately 50 pmoles per 10^6 cells . Levels showed a high degree of biological variation between animals and decreased by up to 33% over the 2 hour incubation periods studied . Because of these variations in control levels , all cytochrome P450 measurements made in the treated cells were converted to percentage of mean control to allow comparison between the two groups .

Table 8 shows the effects of (a) 8-bromo-cyclic AMP 10^{-4}M ; (b) adrenaline 10^{-7}M ; (c) adrenaline 10^{-7}M and IBMX 10^{-3}M ; (d) forskolin 10^{-5}M and (e) forskolin 10^{-5}M and IBMX 10^{-3}M , upon the levels of cytochrome P450 in hepatocytes for up to 2 hours following the addition of the agents . Elevation of cyclic AMP by agents (a)-(d) produced no significant variation between the levels of cytochrome P450 in treated cells and corresponding controls . Elevating cyclic AMP to a higher concentration with Forskolin and IBMX , however , increased cytochrome P450 levels at ½ to 1 hour incubation times (maximum of 137% of corresponding control following 1 hour incubation) . This effect was transient and following 2 hour incubation cytochrome P450 levels did not

Cyclic AMP-elevating agent	½ hour	1 hour	2 hour
8-bromo-cyclic AMP	99.88 ± 6.85	99.63 ± 8.86	93.61 ± 1.29
adrenaline	95.59 ± 10.65	100.55 ± 7.30	94.92 ± 3.44
adrenaline + IBMX	107.17 ± 7.49	114.82 ± 4.75	100.66 ± 20.34
forskolin	104.65 ± 3.19	103.64 ± 10.32	98.38 ± 4.50
forskolin + IBMX	120.24 ± 6.18 *	137.42 ± 9.64 *	104.65 ± 5.59

Table 8 . The effects of 8-bromo-cyclic AMP (10^{-4}M) , adrenaline (10^{-7}M), isobutylmethylxanthine (IBMX)(10^{-3}M), adrenaline and IBMX (10^{-7}M/10^{-3}M), forskolin (10^{-5}M) and forskolin and IBMX (10^{-5}M/10^{-3}M) upon the levels of whole cell cytochrome P450 in isolated rat hepatocytes over a 2hr period following the administration of the agent . Results are expressed as percentage of corresponding control cytochrome P450 levels ± S.D. for at least 3 different cell samples . (* : $p<0.05$) . Control cytochrome P450 levels expressed as pmoles per 10^6 cells at each time were as follows :

½ hour	1 hour	2 hour
44.8 ± 4.0	30.2 ± 2.3	30.8 ± 1.2

differ significantly from corresponding controls . The time course of the effect of forskolin and IBMX upon cytochrome P450 levels is shown in figure 28 .

3.6(b) Effects of cyclic AMP in electropermeabilised hepatocytes .

Table 9 shows the effects of 5 x 10^{-5}M and 5 x 10^{-3}M cyclic AMP upon the levels of cytochrome P450 in permeabilised hepatocytes for 2 hours following the addition of the cyclic AMP. The addition of 5 x 10^{-5}M had no significant effect upon cytochrome P450 levels which correlates well with the effects seen in the intact cells with agents which produce low concentrations of cyclic AMP . As was apparent with the combination of forskolin and IBMX , 5 x 10^{-3}M cyclic AMP transiently elevated cytochrome P450 levels to 120% of corresponding control at 1 hour and after 2 hours levels had returned to control . The time course of the effects of 5 x 10^{-3}M cyclic AMP upon cytochrome P450 levels is shown in figure 29 .

3.7 EFFECTS OF 5 X 10^{-5}M CYCLIC AMP UPON CYTOCHROME P450 LEVELS IN HEPATOCYTES PRETREATED WITH CYCLOHEXIMIDE OR K-252b .

The effects of 5 x 10^{-5}M cyclic AMP upon cytochrome P450 levels in cells pretreated with (a) cycloheximide and (b) K-252b are shown in table 10 . Neither of the pretreatments had any

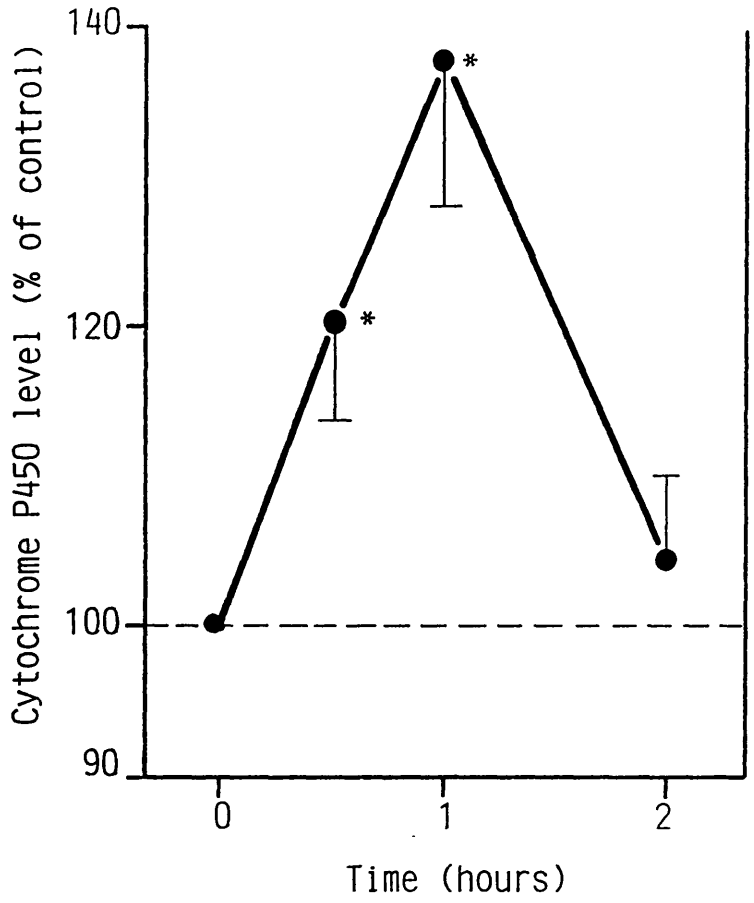

Figure 28 : Change in cytochrome P450 content of isolated hepatocytes with time following the administration of forskolin (10^{-5}M) and isobutylmethylxanthine (10^{-3}M) . Results are expressed as percentage of relevant control ± S.D. for at least 4 different cell samples (* : $p<0.05$). Control cytochrome P450 levels , expressed in pmole per 10^6 cells , were as follows :

½ hour	1 hour	2 hour
45.7 ± 7.1	38.3 ± 12.9	30.8 ± 1.2

Conc.of cyclic AMP	½ hour	1 hour	2 hour
5 x 10^{-5}M	101.33 ± 10.18	106.30 ± 7.48	100.34 ± 2.64
5 x 10^{-3}M	100.99 ± 3.37	120.16 ± 3.34 *	102.43 ± 7.84

Table 9 . The effects of 5 x 10^{-5}M cyclic AMP and 5 x 10^{-3}M cyclic AMP upon the levels of whole cell cytochrome P450 in isolated rat hepatocytes over a 2 hour period following the administration of cyclic AMP . Results are expressed as percentage of relevant cytochrome P450 levels ± S.D. for at least 4 different cell samples . (* : $p<0.05$) . Control cytochrome P450 levels expressed in pmoles per 10^6 cells at each time were as follows:

½ hour	1 hour	2 hour
45.7 ± 7.1	38.3 ± 12.9	39.6 ± 10.2

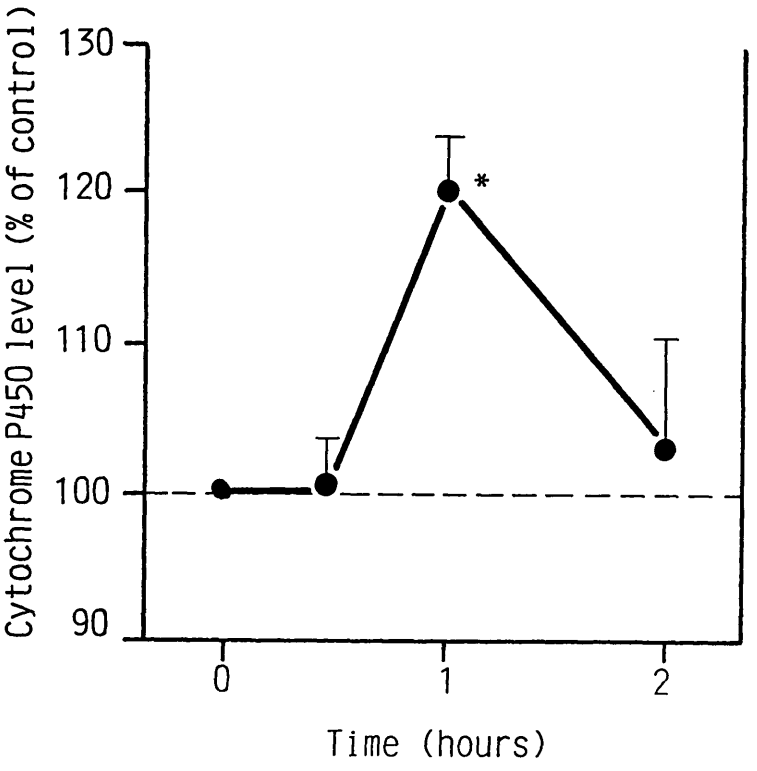

Figure 29 : Change in cytochrome P450 content of electropermeabilised hepatocytes with time following the administration of 5 X 10^{-3}M cyclic AMP. Results are expressed as percentage of relevant control ± S.D. for at least 4 different cell samples (* : $p<0.05$) . Control cytochrome P450 levels , expressed as pmole per 10^6 cells , were as follows :

½ hour	1 hour	2 hour
45.7 ± 7.1	38.3 ± 12.9	39.6 ± 10.2

	½ hour	1 hour	2 hour
c-AMP	101.33 ± 10.18	106.30 ± 7.48	100.34 ± 2.64
c-AMP + cyclohex	102.13 ± 5.60	101.72 ± 6.36	94.56 ± 1.31
c-AMP + K-252b	108.18 ± 4.17	96.02 ± 5.39	105.68 ± 7.12

Table 10 . The effects of 5 x 10^{-5}M cyclic AMP in non-pretreated rat hepatocytes and in rat hepatocytes pretreated with cycloheximide (10^{-5}M) or K-252b (2 x 10^{-8}M) , upon the levels of whole cell cytochrome P450 over a 2 hour time period following the addition of cyclic AMP . Results are expressed as percentage of corresponding control cytochrome P450 levels ± S.D. for at least 4 different cell samples . Control cytochrome P450 levels expressed in pmoles per 10^6 cells at each time were as follows :

	½ hour	1 hour	2 hour
control	45.7 ± 7.1	38.3 ± 12.9	39.6 ± 10.2
control (cyclohex)	47.0 ± 12.6	38.6 ± 4.3	36.8 ± 6.2
control (K-252b)	43.8 ± 9.7	38.1 ± 12.3	36.3 ± 8.4

effect upon cytochrome P450 levels .

3.8 EFFECTS OF CYCLOHEXIMIDE UPON THE ACTIONS OF 5 X 10^{-3}M CYCLIC AMP IN ALTERATION OF CYTOCHROME P450 LEVELS .

Figure 30 shows the effects of 5 x 10^{-3}M cyclic AMP upon cytochrome P450 levels in cells which had been preincubated with cycloheximide to block protein synthesis . The increase in cytochrome P450 mediated by cyclic AMP in untreated cells was completely blocked in the presence of cycloheximide and instead marked decrease in the levels of cytochrome P450 occurred . This loss of cytochrome P450 was maintained throughout the time course (½ to 2 hours) and was maximally suppressed to 72% of corresponding control (cycloheximide only) levels . High concentrations of cyclic AMP , therefore , appear to have two effects upon cytochrome P450 levels (a) protein synthesis independent degradation and (b) protein synthesis dependent synthesis of de novo cytochrome P450 . In non-pretreated cells cyclic AMP-mediated induction is the more marked of the two effects over the time period and thus an overall increase in cytochrome P450 levels occurs .

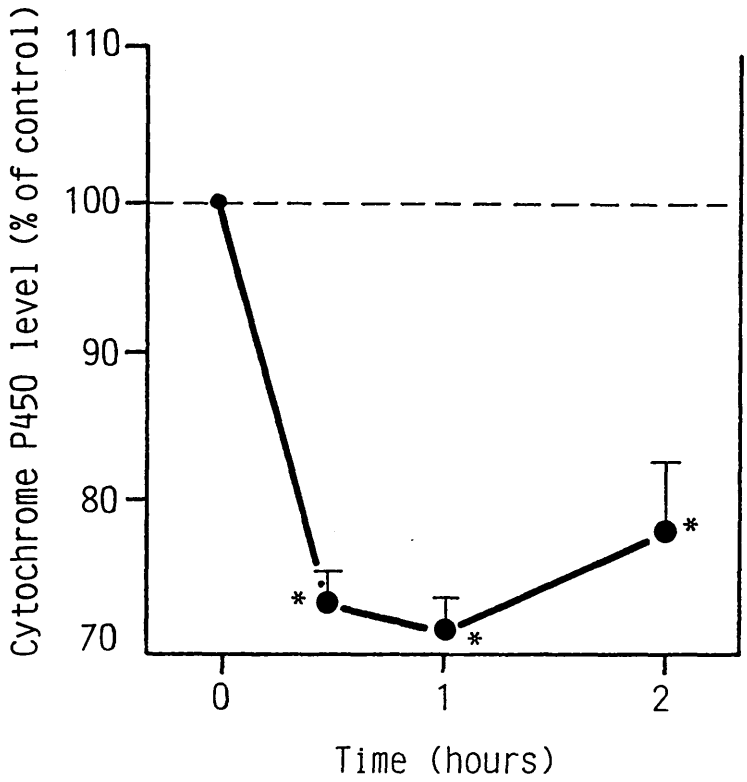

Figure 30 : Change in cytochrome P450 content of electropermeabilised hepatocytes with time following the administration of 5 X 10^{-3}M cyclic AMP in the presence of cycloheximide (10^{-5}M) . Results are expressed as percentage of relevant control ± S.D. for at least 4 different cell samples (* : $p<0.05$) . Control cytochrome P450 levels , expressed as pmole of metabolite per 10^6 cells , were as follows :

	½ hour	1 hour	2 hour
control	45.7 ± 7.1	38.3 ± 12.9	39.6 ± 10.2
control (cycloheximide)	47.0 ± 12.6	38.6 ± 4.3	36.8 ± 6.2

3.9 EFFECTS OF PROTEIN KINASE INHIBITORS UPON THE ACTIONS OF CYCLIC AMP IN ALTERATION OF CYTOCHROME P450 LEVELS .

3.9(a) Effects of KT5720 .

The effects of cyclic AMP in the presence of KT5720 (a specific inhibitor of PKa) upon cytochrome P450 levels are shown in table 11 . The increase in cytochrome P450 levels mediated by cyclic AMP in the untreated cells was blocked by the presence of KT5720 and levels did not differ significantly from control levels throughout the duration of the study . This indicates that the increase in cytochrome P450 content was due to cyclic AMP activation of PKa .

3.9(b) Effects of K-252b alone and in combination with cycloheximide .

Figure 31 shows the time course of the effect of 5×10^{-3}M cyclic AMP in hepatocytes pretreated with (a) K-252b (a specific inhibitor of PKc) or (b) K-252b and cycloheximide . In the presence of K-252b and cycloheximide the blocking of de novo protein synthesis with cycloheximide unmasked PKa mediated degradation of cytochrome P450 (82% of corresponding control levels at 1 hour) . The loss of cytochrome P450 , however , was less marked than that observed following pretreatment with cycloheximide alone (section 5.4) indicating that cyclic AMP mediated degradation is due , in part , to PKc activation .

	½ hour	1 hour	2 hour
c-AMP	100.99 ± 3.37	120.16 ± 3.34 *	102.43 ± 7.84
c-AMP + KT5720	97.03 ± 1.98	105.77 ± 5.69	95.46 ± 0.72

Table 11 . The effects of 5 x 10^{-3}M cyclic AMP alone and in the presence of KT5720 (6 x 10^{-8}M) upon the whole cell levels of cytochrome P450 in isolated rat hepatocytes . Results are expressed as percentage of control cytochrome P450 levels ± S.D. for 4 different cell samples . Control cytochrome P450 levels expressed as pmoles per 10^6 cells.

	½ hour	1 hour	2 hour
control	45.7 ± 7.1	38.3 ± 12.9	39.6 ± 10.2
control (KT5720)	42.9 ± 4.3	42.9 ± 2.7	43.5 ± 1.9

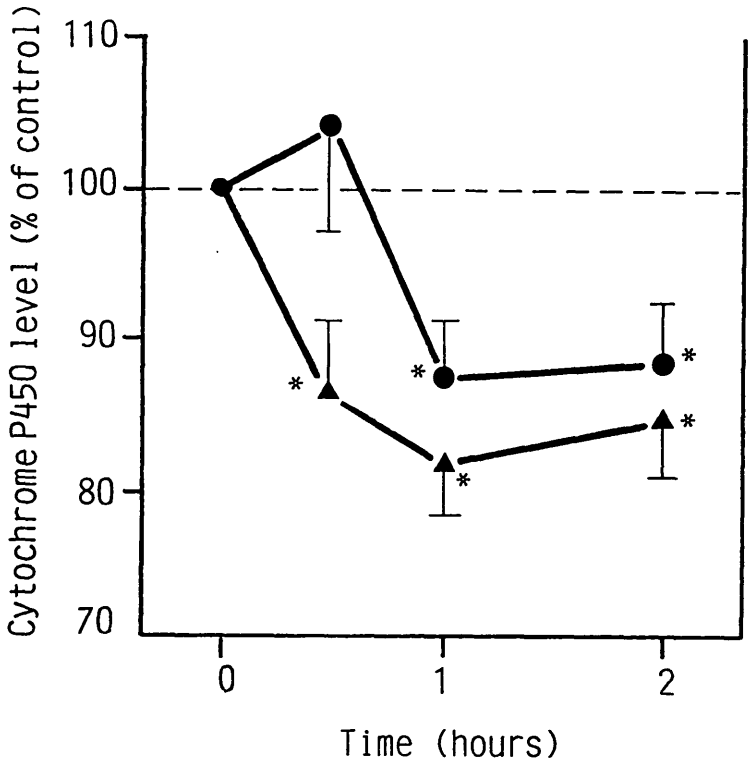

Figure 31 : Change in cytochrome P450 content of electropermeabilised hepatocytes with time following the administration of 5 X 10^{-3}M cyclic AMP in the presence of K-252b (2 X 10^{-8}M) (●–) or a combination of K-252b and cycloheximide (10^{-5}M) (▲) . Results are expressed as percentage of relevant control ± S.D. for at least 4 different cell samples (* : $p<0.05$). Control cytochrome P450 levels , expressed in pmole per 10^6 cells , were as follows :

	½ hour	1 hour	2 hour
control (K-252b)	43.8 ± 9.7	38.1 ± 12.3	36.3 ± 8.4
control (K-252b + cycloheximide)	46.3 ± 0.8	35.9 ± 1.6	28.3 ± 1.7

This implies that both PKc and PKa mediate protein synthesis -independent degradation of cytochrome P450 and that cyclic AMP mediated degradation is due to a combination of PKa (direct activation by cyclic AMP) and PKc (indirect activation by cyclic AMP secondary to activation of PKa) .

When K252b was used alone , degradation of cytochrome P450 was of a similar magnitude to that seen in the presence of K-252b and cycloheximide but the effect was delayed , and at ½ hour , levels did not differ significantly from control levels whereas in the K252b/cycloheximide treated cells levels were already suppressed to 86% of corresponding control levels at this time . This delay in the drop of cytochrome P450 levels may be attributed to stimulation of protein synthesis by PKa , independent of PKc , which counteracts its degrading effects upon cytochrome P450 . This stimulation of protein synthesis appears to be transient in nature and so cytochrome P450 levels decline to the reduced level corresponding to that observed in the K-252b/cycloheximide pretreated cells at 1-2 hours .

3.10 EFFECTS OF 5 x 10^{-5}M CYCLIC AMP UPON THE PHOSPHORYLATION OF PROTEINS IN ELECTROPERMEABILISED HEPATOCYTES .

3.10(a) Whole cell proteins .

Figure 32 shows the effects of 5 x 10^{-5}M cyclic AMP upon the phosphorylation of whole cell proteins following an incubation period of 30 minutes . Lane A shows molecular weight markers .

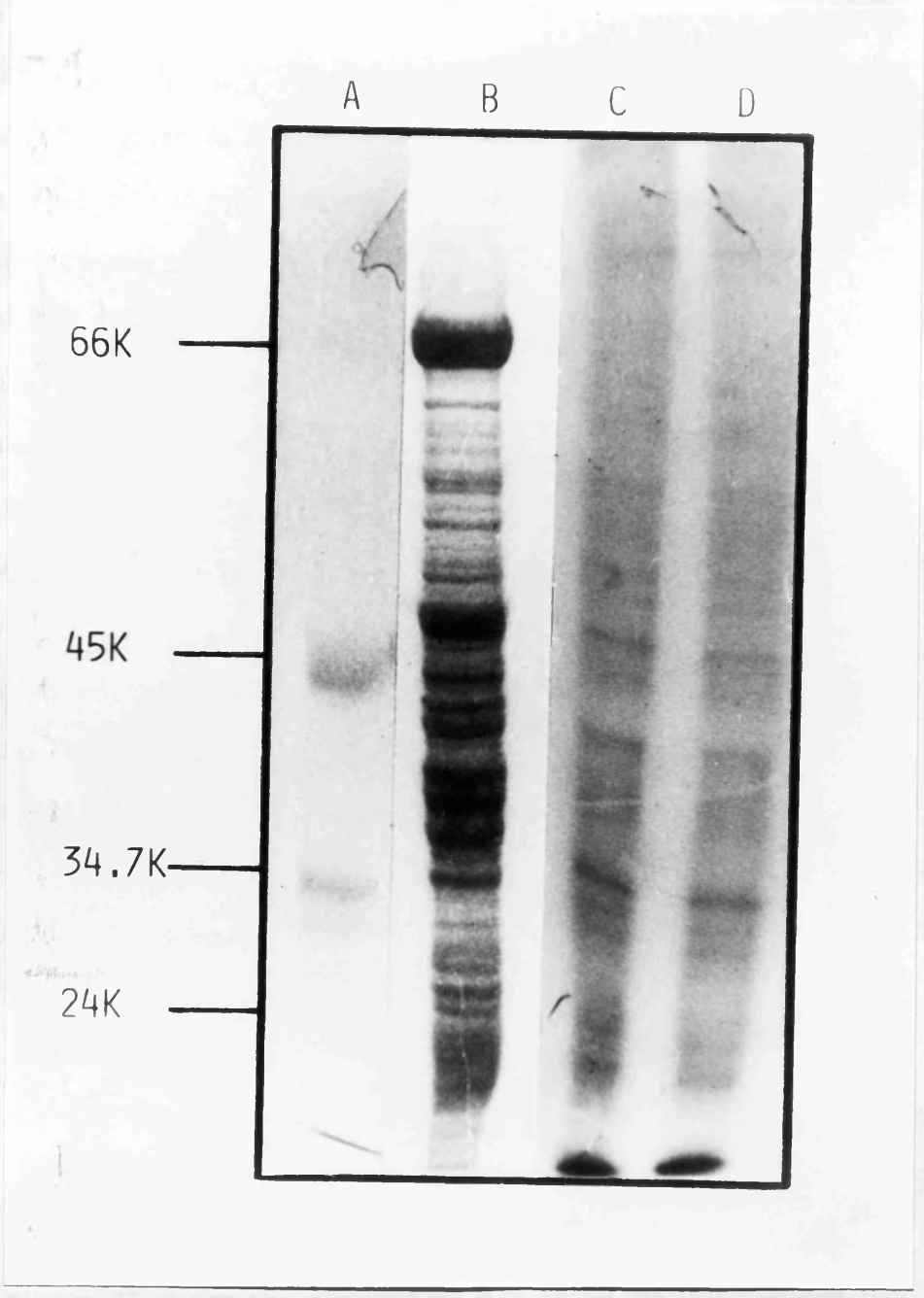

Figure 32 : SDS-PAGE and autoradiogram of homogenised electro-permeabilised hepatocytes prelabelled with (^{32}P) and incubated for 30 minutes in the presence or absence (control) of 5 X 10^{-5}M cyclic AMP prior to homogenisation . Lane A shows the SDS-PAGE of molecular weight markers . Lane B shows the SDS-PAGE of homogenised hepatocyte proteins (identical in control and cyclic AMP-treated cells) . Lane C shows the autoradiogram of the SDS-PAGE of control hepatocytes . Lane D shows the autoradiogram of the SDS-PAGE of cyclic AMP-treated cells .

Lane B shows the separation of whole cell proteins by SDS-PAGE following staining of the protein bands with Coomassie Blue R-250. Lane C shows the autoradiogram of control hepatocytes and lane D shows the autoradiogram of cyclic AMP-treated hepatocytes . It is apparent that by the method used we are able to detect the labelling on cellular proteins in the whole cell extract . We were unable , however , to detect any difference between control and treated groups in the degree of labelling with ^{32}P .

Figure 32 shows the results obtained following an incubation period of 30 minutes (which correlates with the inhibitory effect of 5 x 10^{-5}M cyclic AMP upon the metabolism of androstenedione) . Hepatocytes incubated for 15 minutes , 45 minutes and 60 minutes in the presence or absence of 5 x 10^{-5}M cyclic AMP yielded identical results to those shown in figure 32 in that ^{32}P-labelling of proteins was detectable by the method used but we could detect no stimulation of phosphorylation by 5 x 10^{-5}M cyclic AMP .

3.10(b) Immunoprecipitated cytochromes P450 PB-2a , PB-3a and MC-1b .

Figure 33 shows the effects of 5 x 10^{-5}M cyclic AMP upon the ^{32}P-labelling of immunoprecipitated cytochromes P450 PB-2a , PB-3a and MC-1b following an incubation period of 30 minutes . It is apparent from figure 33 that although we were able to detect large amounts of protein of approximate Mr 45,000-50,000 which is assumed to correspond to the immunoprecipitated cytochrome P450

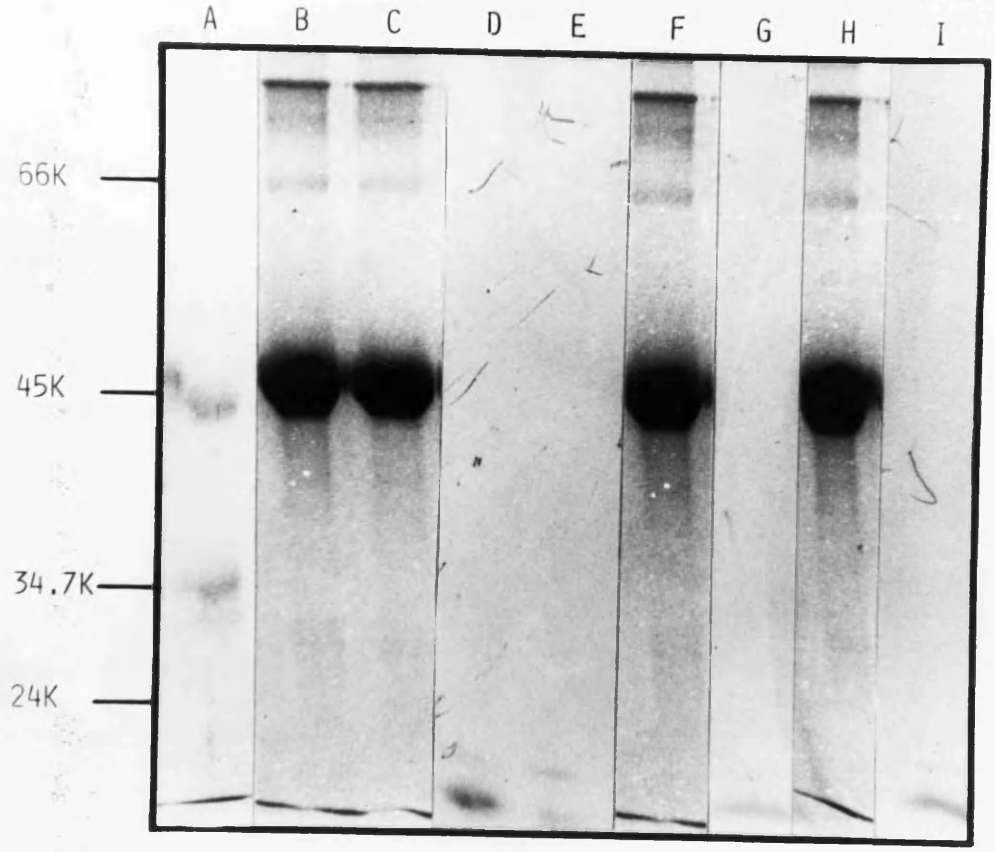

Figure 33 : SDS-PAGE and autoradiogram of cytochromes P450 PB-2a , PB-3a and MC-1b immunoprecipitated from electropermeabilised hepatocytes pre-labelled with (^{32}P) and incubated for 30 minutes in the presence or absence (control) of 5 X 10^{-5} cyclic AMP prior to immunoprecipitation . Lane A shows the SDS-PAGE of molecular weight markers . Lanes B and C show the SDS-PAGE of immunoprecipitated cytochrome P450 PB-2a from control (lane B) and cyclic AMP treated (lane C) hepatocytes . Lanes D and E show the auto-radiogram of lane B (control cells) and lane C (cyclic AMP-treated cells) respectively . Lanes F and G show the SDS-PAGE (lane F) and autoradiogram (lane G) of immunoprecipitated cytochrome P450 PB-3a from cyclic AMP treated hepatocytes . Lanes H and I show the SDS-PAGE (lane H) and auto-radiogram (lane I) of immunoprecipitated cytochrome P450 MC-1b from cyclic AMP-treated hepatocytes .

isozymes , following the immunoprecipitation of PB-2a from control cells (lane B) , PB-2a from cyclic AMP-treated cells (lane C) PB-3a from cyclic AMP-treated cells (lane F) and MC-1b from cyclic AMP-treated cells (lane H) , we were unable to detect any ^{32}P-labelling of PB-2a from control cells (lane D) or any stimulation of ^{32}P-labelling of PB-2a (lane E) , PB-3a (lane G) or MC-1b (lane I) . Although not illustrated in figure 33 , cytochromes P450 PB-3a and MC-1b immunoprecipitated from control cells also showed no incorporation of ^{32}P following an incubation period of 30 minutes which correlates with the results illustrated in figure 33 for PB-2a .

Figure 33 shows only results obtained following an incubation period of 30 minutes (which correlates with the inhibitory effects of 5 x 10^{-5}M cyclic AMP upon androstenedione metabolism). Hepatocytes incubated for 15 minutes , 45 minutes and 60 minutes in the presence and absence of 5 x 10^{-5}M cyclic AMP yielded identical results to those illustrated in figure 33 in that large amounts of protein of Mr 45,000-50,000 were precipitated by the specific antibodies but no ^{32}P-labelling was detectable either in control or cyclic AMP-mediated groups .

These results suggest that stimulation of phosphorylation of cytochrome P450 is not the mechanism by which 5 x 10^{-5}M cyclic AMP mediates inhibitory effects upon the metabolism of androstenedione.

DISCUSSION

4 DISCUSSION

4.1 CHOICE OF SUBSTRATE .

Much of the previous work concerning the mechanisms of action and the regulation of the hepatic monooxygenase system has utilised xenobiotic substrates . Kinetic studies have shown , however , that the apparent Km values for drug-metabolising enzymes e.g. 300-1400 μM for ethylmorphine-N-demethylase (Castro and Gillette 1967) are generally higher than the corresponding values reported for steroid-metabolising enzymes (Skett 1978) . This indicates that the hepatic monooxygenases exhibit low affinity for xenobiotic substrates . In this present study , therefore , we used the endogenous steroid , 4-androstene-3,17-dione , as the substrate .

This steroid is widely available in both its unlabelled and (^{14}C) labelled forms (4(4-^{14}C)androstene-3,17-dione) and is widely used in this laboratory . The metabolism of this steroid is well characterised (section 1.5) . The metabolites have been separated by thin layer chromatography (Gustafsson and Stenberg 1974a) (section 2.5) and also by radio-gas chromatography and gas chromatography-mass spectrophotometry (Gustafsson and Stenberg 1974a) .

Using thin layer chromatography in this present study we have been able to assess the activities of at least five different enzymes , three of which contain cytochrome P450 as an integral component , namely , 6β hydroxylase (Waxman et al 1985) , 7α

hydroxylase (Waxman et al 1987 ; Nagata et al 1986) and 16α hydroxylase (Waxman 1984) (section 1.5) , and two of which do not contain cytochrome P450 as a component , namely , 17α/β hydroxysteroid dehydrogenase and 5α reductase (Golf and Graef 1978) (section 1.5) . The activities of some of these enzymes are sexually differentiated (Gustafsson and Stenberg 1974a) (table 2) .

The use of 4-androstene-3,17-dione as the substrate , therefore , allows the assessment of differential changes of enzyme activity such as occur during feminisation or masculinisation of hepatic enzyme profiles and also does not restrict assessment to cytochrome P450-containing monooxygenases .

4.2 CHOICE OF EXPERIMENTAL MODEL .

Many of the early studies conducted to evaluate hormonal regulation of the hepatic monooxygenase system utilised "in vivo" models and involved injection of the hormone into the whole animal. The major drawback of such models is that additional actions of the hormones at sites other than directly at the level of the liver cannot be ruled out and it is well documented that many interactions exist between the components of the endocrine system . Many examples of such interactions have been discussed in the Introduction (section 1.11) for each of the hormones reported to mediate effects upon hepatic monooxygenases . It would be erroneous to assume , therefore , that all of the effects of these hormones were being produced directly at the level of the

liver rather than by the alteration of the synthesis , release and action of other hormones .

Other studies have been conducted "in vitro" utilising microsomes or reconstituted membrane systems however , although these models overcome problems of interactions , they cannot be used to study extracellular effects or the effects of intracellular pathways and their validity in the study of physiological processes is questionable .

We overcame these problems in this present study by using the isolated rat hepatocyte as our model and thereby overcame problems of hormone interaction whilst maintaining many of the physiological features of the system .

One of the major arguments against the use of isolated rat hepatocytes as a physiological model is that the highly structured arrangement of cells in intact liver is absent in homogenous suspensions or cultures of hepatocytes .

The intact liver contains three different cell types , namely, parenchymal cells (hepatocytes) , reticuloendothelial cells and Kupffer cells whereas modern techniques for the isolation of hepatocytes yield suspensions which are virtually free from contamination by these other cell types . In the intact liver , however , cooperation between the different cell types is known to occur e.g. cooperation between reticuloendothelial cells and parenchymal cells in the catabolism of haem by haem oxygenase (Maines and Anders 1973a) . This effect will be lost when hepatocytes are isolated .

In addition , the parenchymal cells themselves are

heterologous with regards to their content of cytochrome P450 and the ratios of the different cytochrome P450 isozymes . The variation in the content of the different cytochrome P450 isozymes is dependent upon the position of the parenchymal cells within the liver lobule e.g. phenobarbitone-inducible isozymes are located mainly in the centrilobular region of the lobule whereas 3-methylcholanthrene-inducible isozymes are located mainly in the periportal region (Ioannides and Park 1987) . The content of other enzymes also varies depending upon the location of the parenchymal cell populations . e.g. phase 2 conjugating enzymes are present at much higher concentrations in the centrilobular than in the periportal regions of the lobule (Ioannides and Park 1987) . In addition , different regions of the liver exhibit differences in glycolysis/gluconeogenesis in that hepatocytes of the perivenous region are involved mainly in glycolysis whereas the hepatocytes of the periportal region are involved mainly in gluconeogenesis . This heterologous distribution of parenchymal cells will be lost when the cells are isolated .

The loss of the structured arrangement of cells which occurs when hepatocytes are isolated , therefore , should be remembered when extrapolating data obtained in isolated rat hepatocytes to the physiological situation .

Another major problem that has previously been encountered when using isolated rat hepatocytes for the study of the hepatic monooxygenase system is that rapid loss of cytochrome P450 occurs from hepatocytes during culture (Stewart et al 1985) and during longer periods of culture loss of differentiated function occurs

due to cell proliferation (Bissell and Guzelian 1980) . The content of cytochrome P450 has been reported to fall to as little as 25% of the cytochrome P450 content of freshly isolated hepatocytes following 24 hours in culture (Stewart et al 1985) and many attempts have been made to develop culture media which prevent this marked loss of cytochrome P450 .

Most cell lines require serum for growth in culture and it has been reported that the presence of fetal calf serum promotes hepatocyte attachment to the culture dish , spreading and maintenance of viability over several days in culture . Stenberg et al (1978) , however , reported that the presence of fetal calf serum resulted in marked loss of androstenedione-16α-hydroxylase and androstenedione-5α-reductase activities and it is now accepted that serum enriched culture media are unsuitable for the maintenance of isolated rat hepatocytes in the study of hepatic monooxygenases . Supplementing the medium with a variety of substances e.g. dexamethasone (Michalopoulos and Pitot 1975) , adrenocorticosteroids (Michalopoulos et al 1976) , ascorbic acid (Bissell and Guzelian 1979) , 5-aminolaevulinic acid (Guzelian and Bissell 1976) , nicotinamide (Paine et al 1979) or metyrapone (Lake and Paine 1982) , has been reported to maintain the levels of cytochrome P450 over short periods of culture . The drawback to maintenance of cytochrome P450 levels by these agents , however , is that some cytochrome P450 isozymes are more susceptible to in vitro degradation than others (Stewart et al 1985) and so the overall profile of the hepatocyte monooxygenases changes . This is particularly important when

studying the hormonal regulation of steroid-metabolising enzymes which exhibit characteristic profiles in the male and female rat liver in vivo . Another drawback of using dexamethasone , adrenocorticosteroids or any other hormone supplement when examining the regulation of hepatic monooxygenases is that these hormones may themselves produce effects upon enzyme activities (section 1.11) .

In this laboratory , Hussin and Skett (1986) have characterised a system for primary culture of adult rat hepatocytes maintained in Hams F10 supplemented with 0.1% bovine serum albumin and 100IU penicillin/streptomycin which overcomes some of these problems .

The absence of hormones in the medium allows the examination of the hormonal regulation of hepatic monooxygenases at both an extracellular and an intracellular level .

Hepatocytes were cultured at a cell density of 1×10^5 cells per cm^2 whereas it has been reported that proliferation of hepatocytes in culture occurs at low cell densities but is inhibited at these high cell densities (Nakamura et al 1983a) . It has been postulated that a growth-inhibiting factor is produced by the plasma membrane of mature rat hepatocytes which inhibits growth and promotes differentiation of the cells (Nakamura et al 1983b) . This postulated factor , therefore , may prevent growth and promote differentiation of the hepatocytes when cultured at the high cell densities used in this present study .

In this present study we still noted a 33% loss of total cytochrome P450 content over a 3 hour period of culture (section

3.20) . At 24 hours the activities of all of the steroid-metabolising enzymes were markedly decreased in comparison to freshly isolated rat hepatocytes (table 4) and this presumably represents corresponding decreases in the levels of both cytochrome P450-dependent and cytochrome P450-independent enzymes. Culture of the hepatocytes under these conditions did not result in significant change in the ratios of steroid-metabolising enzymes (table 4) and sexual differentiation of these enzymes was maintained (table 5) .

We therefore consider that the isolated rat hepatocyte maintained in Hams F10 supplemented with 0.1% bovine serum albumin and 100IU penicillin/streptomycin is a valid , although not perfect , model for the study of the hormonal and intracellular regulation of hepatic steroid-metabolising monooxygenases over the time periods used in this present study .

4.3 EFFECTS OF ELEVATING CYCLIC AMP UPON STEROID-METABOLISING ENZYMES .

The major drawback of examining the physiological regulation of any intracellular effect by cyclic AMP is that it does not readily penetrate the cell membrane and any that does gain access will be rapidly degraded by cyclic AMP phosphodiesterase for which it is a very good substrate (Beebe et al 1986) . We overcame this problem in two ways .

In the first part of the study we examined the effects upon

steroid metabolism of a number of agents which elevate cyclic AMP indirectly in intact hepatocytes i.e. 8-bromo-cyclic AMP (an analogue of cyclic AMP which readily penetrates the cell membrane), adrenaline (receptor-mediated elevation of cyclic AMP), isobutylmethylxanthine (elevates cyclic AMP by inhibiting its degradation) and forskolin (elevates cyclic AMP by direct stimulation of adenylate cyclase) .

In the second part of the study we overcame problems of cyclic AMP access to the interior of the cell by electropermeabilising the hepatocyte cell membrane . This allowed assessment of the effects of direct addition of different concentrations of cyclic AMP upon steroid-metabolising enzymes .

4.3(a) Effects of elevating cyclic AMP (indirect) in intact hepatocytes .

(i) Effects upon steroid-metabolising enzymes .

Incubation of isolated rat hepatocytes with 8-bromo-cyclic AMP had a marked inhibitory effect upon the metabolism of 4-androstene-3,17-dione in hepatocytes isolated from male rats (figure 9) . This was not a feminising effect as all enzymes were inhibited to a similar degree whereas feminisation would have resulted in suppression of 16α- and 6β- hydroxylase activities , little effect upon the activities of 7α hydroxylase and 17α/β hydroxysteroid dehydrogenase and stimulation of 5α reductase

activity . This effect was also not sex-dependent as enzymes were inhibited to a similar degree in hepatocytes isolated from female rats (figure 10) .

The inability of 8-bromo-cyclic AMP to feminise the male profile of steroid-metabolising enzyme activity may indicate that alteration of cyclic AMP levels has no role to play in mediating "in vivo" feminisation of hepatic monooxygenases . In addition , the identical effects of cyclic AMP in both male and female rats differs markedly from the effects of hormones postulated to mediate "in vivo" inhibition of hepatic monooxygenases by elevation of cyclic AMP levels . In these in vivo studies female hepatic monooxygenases were reported to be refractory to the inhibitory effects of thyroxine (Kato and Gillette 1965a ; Kato et al 1970)) (section 1.11(h)) ; diabetes (Kato and Gillette 1965a ; Kato et al 1971) (section 1.11(i)) ; adrenalectomy (Kato and Gillette 1965a ; Kato et al 1971) (section 1.11(j)) and adrenaline (Kato and Gillette 1965a) . Similarly the inhibitory effects of "in vivo" administration of dibutyryl cyclic AMP upon hepatic monooxygenase activity was also reported to occur in the male rat but not in the female rat (Ross et al 1973) .

Postulated mechanisms for both the hormonal maintenance of sexually differentiated enzyme profiles and for mediating the production of "in vivo" refractoriness of female hepatic monooxygenases will be discussed in section 4.10(c) .

The inhibitory effect of 8-bromo-cyclic AMP , however , does indicate that the "in vivo" inhibition of hexobarbitone metabolism mediated by administration of dibutyryl-cyclic AMP to male rats

(Weiner et al 1972a ; Ross et al 1973) is probably produced directly at the level of the liver rather than by the alteration of hormone release from components of the endocrine system . This correlates with the finding that the inhibitory effects of dibutyryl-cyclic AMP also occur in the isolated perfused liver and in liver slices (Weiner et al 1972b) .

Incubation of hepatocytes with adrenaline resulted in rapid , transient elevation of cyclic AMP levels which , after 10 minutes, had returned to the levels of corresponding control hepatocytes. This rapid rise and decline of cyclic AMP levels correlates with the findings of Exton et al (1971) in the perfused rat liver . The rapid rate of decline may be due to desensitisation of the adrenergic β_2 receptor (Bouvier et al 1987 ; Refnes et al 1987) or to activation of cyclic AMP phosphodiesterase (Corbin et al 1985) (section 1.14(a)) .

As shown in figure 12 , the appearance of cyclic AMP-mediated inhibition of the metabolism of 4-androstene-3,17-dione occurred 1 to 2 hours following administration of adrenaline . The time lag between the effects of adrenaline upon cyclic AMP levels and its effects upon steroid-metabolising enzyme activity indicates that cyclic AMP probably produces its effects by activation of a cascade process . In 1971 , Exton et al postulated that the effects of adrenaline upon phosphorylase activation , an important step in the stimulation of glycogenolysis , were mediated by a similar cyclic AMP cascade process . The effects of adrenaline upon phosphorylase activation , however , are now thought to be

mediated via the actions of adrenaline at the α_1 receptor which is linked to inositol phospholipid hydrolysis and mobilisation of calcium whereas β_2-mediated generation of cyclic AMP is thought to be functionally unimportant for the activation of phosphorylase in the male rat (Morgan et al 1983b ; Studer and Borle 1982 ; Birnbaum and Fain 1977 ; Studer et al 1984) .

The effects of adrenaline upon steroid-metabolising enzymes in the male rat , however , differ markedly from its effects upon phosphorylase in that the inhibitory effects of adrenaline appear to be mediated only via β_2 receptors and generation of cyclic AMP (as blocked by propranolol) and the effects of adrenaline at α_1 receptors (as blocked by prazocin) do not appear to mediate its inhibitory effects upon steroid-metabolising enzymes (figure 13).

These findings are unusual in that α_1 receptors on the plasma membrane of male rat hepatocytes greatly outnumber β_2 receptors. Some other intracellular events , however , such as adrenaline-mediated induction of ornithine decarboxylase and its associated messenger RNA (Evoniuk et al 1985) and adrenaline-stimulated phosphorylation and subsequent activation of fructose 1,6-biphosphatase (Nilsson-Ekdahl and Ekman 1987) have also been reported to be due to elevation of cyclic AMP and not due to calcium mobilisation . The results of this present study also correlate with the findings of Hussin et al (1988) who reported that incubation of male rat hepatocytes with the calcium ionophore, A23187 , had no effect upon the activity of the steroid-metabolising enzymes in hepatocytes isolated from male rats .

Incubation of hepatocytes with isobutylmethylxanthine (IBMX) resulted in a two phase elevation of cyclic AMP (figure 14) . Levels were elevated within 2 minutes and had returned to control levels after 5 minutes but this was followed by a secondary sustained rise in cyclic AMP levels which occurred after 30 minutes .

IBMX was included in the study as a phosphodiesterase inhibitor and , therefore , would be expected to elevate cyclic AMP as a result of reduced cellular degradation of the cyclic nucleotide . The initial peak in cyclic AMP levels , however , is probably of too transient a duration to be accounted for by the phosphodiesterase-blocking action of the agent . IBMX is also known to mediate antagonistic effects at adenosine receptors as another of its actions (Daly 1982) and this is thought to partially account for the stimulatory effects of this and other methylxanthines upon the central nervous system . This is unlikely to be the underlying mechanisms mediating the initial rise in cyclic AMP levels , however , because adenosine receptors present on the plasma membrane of hepatocytes have been reported to be A_2, linked via Ns to the stimulation of adenylate cyclase , as opposed to A_1 , linked via Ni to the inhibition of adenylate cyclase (Daly 1982) . The presence of IBMX , therefore , would be expected to decrease and not to increase cyclic AMP levels . In addition , the antagonistic activity of IBMX at A_1 or A_2 receptors would be unlikely to result in any alteration in cyclic AMP levels in hepatocytes but would augment the effects of other hormones upon cyclic AMP generation in vivo . The precise mechanism by

which IBMX mediates this initial , transient elevation of cyclic AMP , therefore , is unknown .

The second gradual rise in cyclic AMP levels which occurs after 30 minutes has characteristics and time of onset which would be expected of phosphodiesterase-blocking activity .

As shown in figure 14 , incubation of hepatocytes with IBMX resulted in inhibition of the metabolism of 4-androstene-3,17-dione which occurred over a similar time course and was of a similar magnitude to that produced by adrenaline and in an analogous manner the increase in cyclic AMP levels produced by IBMX within 2 minutes also correlated with that produced by adrenaline .

It appears , therefore , that it is the initial transient elevation of cyclic AMP and not the secondary sustained rise attributed to phosphodiesterase-blocking activity , which mediates the inhibitory effects of IBMX upon steroid-metabolising enzyme activity .

Incubation of hepatocytes with the combination of adrenaline and IBMX resulted in much greater generation of cyclic AMP than mediated by either adrenaline or IBMX alone . Cyclic AMP levels were maximally elevated at 10 minutes and levels declined at a slower rate than those produced by adrenaline alone presumably due to the phosphodiesterase-blocking activity of IBMX . The combination of the two agents also had a correspondingly greater inhibitory effect upon the metabolism of 4-androstene-3,17-dione (figure 15) .

It is apparent , therefore , that the concentrations of cyclic AMP generated by adrenaline , IBMX and a combination of adrenaline and IBMX , mediate concentration-dependent inhibition of steroid-metabolising enzyme activity .

The concentrations of cyclic AMP generated by these agents in this present study may correlate with the concentrations of cyclic AMP which are generated by hormones in vivo . Elevation of cyclic AMP to these levels , therefore , may be one mechanism by which some hormones e.g. thyroxine (Müller and Seitz 1987) (section 1.16(g))) , adrenaline (Exton et al 1971) (1.16(b)) and glucagon (Exton et al 1971) (1.16(c)) , mediate inhibitory effects upon hepatic monooxygenase (see section 1.11 under separate hormones) . Comparison between the inhibitory effects of elevating cyclic AMP in this present study , however , and the inhibitory effects of these hormones upon hepatic monooxygenase activity in vivo , is difficult because cyclic AMP levels were not measured in these in vivo studies . Subcutaneous injection of glucagon into rat pups , however , was reported to result in 4 fold elevation of hepatic cyclic AMP levels 2.5 minutes following administration (Evoniuk et al 1985) . In the same study , Evoniuk and co-workers reported that isoproterol (a specific β-adrenergic agonist) , administered in an identical manner , also produced a 3 to 4 fold elevation of hepatic cyclic AMP levels 5 minutes after injection . These levels of cyclic AMP correlate with those generated by adrenaline , IBMX and a combination of adrenaline and IBMX in this present study . In addition both glucagon (Weiner et al 1972a) and adrenaline (Fouts 1962 ; Kato

and Gillette 1965a) have been reported to have an inhibitory effect upon hepatic monooxygenase activity in vivo .

More direct evidence that correlation between the elevation of cyclic AMP (2 to 3 fold) and inhibition of hepatic monooxygenase activity may be a physiological effect has been provided in a study by Olson and Weiner (1980) . These workers reported that the toxohormones secreted by the Walker 256 carcinsarcoma had an inhibitory effect upon the in vivo metabolism of p-chloro-N-methylaniline in male rats and this inhibitory effect was accompanied by a rise in hepatic cyclic AMP levels to 154% of sham operated controls . It was concluded by these workers that the inhibitory effects of the toxohormones upon hepatic monooxygenase activity may be mediated by alterations in the levels of hepatic cyclic AMP and this proposal correlates well with the findings of this present study .

The physiological implications of these findings will be discussed in more detail in section 4.10(a) .

Forskolin has been reported to elevate intracellular cyclic AMP levels by directly activating the catalytic subunit of adenylate cyclase (Ruiz et al 1986) . Incubation of hepatocytes with forskolin alone (figure 16) or a combination of forskolin and IBMX (figures 18 and 19) generated concentrations of cyclic AMP which were far in excess of concentrations which are likely to be produced by in vivo hormonal stimulation .

At these higher concentrations of cyclic AMP a later , stimulatory effect upon the metabolism of 4-androstene-3,17-dione

was apparent and led to progressive reduction in the degree of inhibition . As the cyclic AMP levels were increased further , the inhibitory effect was abolished and instead marked stimulation of enzyme activity occurred at this later time .

It is widely accepted that forskolin acts specifically upon adenylate cyclase to elevate cyclic AMP (Seamon et al 1981 , Ruiz et al 1986 , Shi et al 1986 , Ho et al 1986) and its effects upon other intracellular systems have been little studied . It is conceivable , however , that the later stimulatory effects of forskolin , alone and in combination with IBMX , upon steroid metabolism occur independently of cyclic AMP generation but may be due to some previously undocumented effect of forskolin upon other intracellular second messengers or alternatively due to a direct action of forskolin itself . These alternatives are unlikely because forskolin mediated-elevation of cyclic AMP levels to 488% of corresponding control levels (figure 17) resulted in subsequent inhibition of steroid metabolism corresponding to that mediated by a combination of adrenaline and IBMX .

It is likely , therefore , that the loss of the inhibitory effects and the appearance of later stimulatory effects are due to the high concentrations of cyclic AMP generated by forskolin rather than any alternative mode of action . This may indicate that cyclic AMP has a second effect upon hepatic monooxygenase activity at these higher concentrations i.e. stimulation .

It is unlikely that concentrations of cyclic AMP correlating with those generated by forskolin and a combination of forskolin

and IBMX in this present study can be generated by physiological concentrations of hormones . It is possible , however , that these high concentrations of cyclic AMP correlate with those which have been proposed to mediate the inductive effects of phenobarbitone , 3-methylcholanthrene (Byus et al 1976) and Arochlor-1254 (Costa et al 1976) upon cytochrome P450 isozymes in vivo (section 1.17(b)) . Byus et al (1976) , however , reported that following parental administration of phenobarbitone or 3-methylcholanthrene , hepatic cyclic AMP levels were maximally elevated to 200% of corresponding control levels by 3-methylcholanthrene and to 150% of corresponding control levels by phenobarbitone . These elevations of cyclic AMP were correlated to subsequent induction of hepatic monooxygenases which occurred 24 hours following the administration of the agents .

These concentrations of cyclic AMP differ markedly from the high concentrations of cyclic AMP which we have found to be necessary to mediate stimulatory effects in this present study . In addition , the time between maximum elevation of cyclic AMP and induction of hepatic monooxygenase activity mediated by in vivo administration of phenobarbitone and 3-methylcholanthrene appears to be much longer than that for cyclic AMP in this present study although comparison between the two studies is difficult because we did not examine the effects of forskolin or a combination of forskolin and IBMX following an incubation period of 24 hours .

A possible role for cyclic AMP in the effects of these inducing agents will be discussed in more detail in section 4.11 .

Cyclic AMP , therefore appears to have a concentration- and time-dependent biphasic effect upon steroid metabolism which appears to reflect the relative magnitudes of two opposing effects i.e. inhibition which predominates at low concentrations and stimulation which predominates at high concentrations .

The mechanism by which cyclic AMP mediates these two opposing effects will be discussed in more detail in sections 4.4 to 4.8 .

(ii) Drawbacks of indirectly elevating cyclic AMP .

One of the drawbacks of using indirect mechanisms for elevating cyclic AMP is the possibility that the agents are producing effects by additional actions not related to cyclic AMP generation .

It is likely that the inhibitory effects of adrenaline are due to cyclic AMP generation and not to elevation of cytosolic calcium levels because the effects of adrenaline were inhibited by propranolol (a β blocker) but not by prazocin (an α_1 blocker).

Methylxanthines have been reported to mediate relaxation of smooth muscle by elevation of intracellular cyclic GMP levels (Kukovetz et al 1980) . Elevation of hepatic cyclic GMP levels , therefore , may be an alternative mechanism by which IBMX mediates inhibition of steroid metabolism . Methylxanthines have also been reported to stimulate the release of calcium from the endoplasmic reticulum (Exton 1980) . Mobilisation of calcium , however , is unlikely to mediate the inhibitory effects of IBMX upon steroid metabolism because neither the calcium ionophore , A23187 (Hussin

et al 1988) , nor adrenaline acting via α_1 receptors (see above) had any effect upon steroid metabolism in isolated rat hepatocytes .

Additional actions of forskolin have been little studied . It is conceivable , however , that the effects of forskolin upon hepatic monooxygenase activity may be mediated by previously undocumented effects upon other intracellular second messengers or may be due to a direct action of forskolin itself .

Due to these potential additional actions it would be erroneous to conclude that the effects upon steroid-metabolising enzymes mediated by these agents are attributable only to progressive rises in intracellular cyclic AMP level .

The problems of additional actions of the agents have been partially overcome in this present study by using the cyclic AMP analogue , 8-bromo-cyclic AMP . Cyclic AMP is thought to mediate most , if not all , of its intracellular effects by activation of cyclic AMP-dependent protein kinase (PKa) . 8-bromo-cyclic AMP has been reported to pass freely across the plasma membrane of the cell and directly activate PKa in an analogous manner to cyclic AMP (Beebe et al 1985) . 8-Bromo-cyclic AMP has not been reported to have any additional actions .

Quantitative assessment of the effects of 8-bromo-cyclic AMP , however , is difficult as it has been reported to be much more potent in activating cyclic AMP-dependent processes than cyclic AMP itself e.g. in stimulating adipocyte lypolysis , the EC_{50} of 8-bromo-cyclic AMP has been reported to be 3.9mM whereas the EC_{50}

for cyclic AMP has been reported to be in excess of 15mM (Beebe et al 1986) . In addition , 8-bromo-cyclic AMP is 15 times less susceptible to the degradative effects of cyclic AMP phosphodiesterase (Beebe et al 1986) and the continuous activation of PKa by this agent may , therefore , prolong a response which is limited under physiological conditions . It has recently been reported that PKa is not a single species but is a family of closely related enzymes which have been classified into two groups , type 1 and type 2 (Robinson-Steiner et al 1984) . The liver has been reported to contain both type 1 and type 2 protein kinases . Different cyclic AMP analogues appear to have different affinities for the two types of PKa e.g. 8-thio-substituted analogues preferentially activate type 2 enzymes whereas 8-amino-substituted analogues preferentially activate type 1 enzymes (Beebe et al 1986) . It is possible , therefore , that the ratio of type 1/type 2 protein kinases activated by 8-bromo-cyclic AMP differs from the activation of these two types of protein kinase by cyclic AMP . Although different physiological roles for the two types of protein kinase have not been elucidated , it is possible that differences between activation of type 1 and type 2 protein kinases by 8-bromo-cyclic AMP and by cyclic AMP may result in qualitative and quantitative differences in cellular response .

Another drawback of using these indirect mechanisms to elevate cyclic AMP is that the cyclic AMP concentrations evaluated and used to establish a concentration/effect relationship in this

present study were determined in the whole cell and almost certainly do not reflect the concentrations of cyclic AMP at specific sites within the cell . This is due to the known compartmentalisation of cyclic AMP within the cell which has been illustrated well in a study by Yamatani et al (1987) . This group postulated that glucagon was linked to two functionally separate types of adenylate cyclase , one mediating the effects of glucagon upon glycogenolysis and the other having no effect upon glycogenolysis . It was postulated that the cyclic AMP generated by activation of the two types of adenylate cyclase were separated by compartmentalisation within the cell .

4.3(b) Direct addition of cyclic AMP to electropermeabilised hepatocytes .

(i) Problems of direct addition of cyclic AMP .

The problems associated with indirect elevation of cyclic AMP discussed in the previous section , could be overcome by using the direct addition of cyclic AMP . The major problem of this approach , however , is that cyclic AMP penetration of the plasma membrane is very poor and cyclic AMP that does penetrate the plasma membrane will be rapidly degraded within the cell by the action of cyclic AMP phosphodiesterase (Beebe et al 1986) . The concentration of cyclic AMP within the cell , therefore , will be considerably lower than that in the extracellular medium .

(ii) Theory of electropermeabilisation .

The problems of access of small , non-permeant molecules across the cell membrane has been addressed previously and one method which has been used to permit entry of these molecules into the cell from the extracellular medium is electropermeabilisation of the plasma membrane .

In 1974 , Zimmerman et al reported that the application of potential difference of 1V across a physiological membrane resulted in membrane breakdown . This process is known as electropermeabilisation and was used by Knight and Baker (1982) to allow access of calcium into bovine adrenal medullary cells . A diagrammatic representation of a cell placed in an electric field is shown in figure 7 (see Materials and Methods) . Knight and Baker (1982) used the following formula to calculate the potential difference across the plasma membrane :

$$V_p = C\ b\ E \cos \theta$$

Where V_p is the potential difference at any point (p) around the circumference of the cell , b is the radius of the cell , E is the applied field strength , θ is as shown in figure 7 and C is a constant which depends upon the relative conductivities of the extracellular fluid , the cytosol and the membrane with relation to cell size and membrane thickness . It can be assumed that the

conductivities of the extracellular and intracellular fluids are much greater than that of the membrane and that the dimensions of the cell are much greater than the thickness of the membrane . Under these conditions C has been shown to be approximately 1.5 (Knight and Baker 1982) .

$$\text{Therefore} \quad : \quad V_p = 1.5\ b\ E \cos\theta$$

The potential difference across the cell membrane will be maximum at the centre of the cell in line with the electric field (A-B in figure 7) at which point $\cos\theta = \pm 1$.

$$\text{Therefore} \quad : \quad V_{(A/B)} = 1.5\ b\ E$$

Using this formula , Knight and Baker calculated that an applied field strength of 1000-2000V/cm across a bovine adrenal medullary cell (b = 10μm) would produce a transmembrane potential of 1.5V-3V at opposite poles of the cell in line with the electric field which is sufficient to mediate membrane breakdown .

One possible problem of this method is that potential differences occur across all of the cell membranes and so intracellular damage may occur . The radii of the intracellular

organelles , however , are much less than that of the whole cell e.g. mitochondria have a radius of approximately 0.5 μm (Knight and Baker 1982) . Using the above formula , the potential difference across the membrane of the mitochondria in a field strength of 1000-2000V/cm is 75mV-150mV . This potential difference is theoretically much less than that required for membrane breakdown and so intracellular damage is minimal .

Since the work of Zimmerman et al (1974) and Knight and Baker (1982) , the method of electropermeabilisation has been widely used in a number of different cell types e.g. NIH-3T3 cells (Mir et al 1988) , NHIK 3025 cells (Melvik et al 1986) , medullary thyroid carcinoma cells (Haller-Brem et al 1987) , neutrophils (Grinstein and Furuya 1988) and chinese hamster ovary cells (Teisse and Rols 1986) and in 1982 , Gordon and Seglen first characterised a system for permeabilisation of isolated rat hepatocytes .

(iii) Histological characteristics of permeabilised cell membranes .

In the study of Knight and Baker (1982) it was postulated that the application of a strong electrical field across bovine adrenal medullary cells punctured two pores in the plasma membrane of the cell at opposite poles (see previous section) . Due to the random motion of the cells within the suspension the cells will rotate and the application of a second pulse will puncture another pair of pores at two more sites on the plasma membrane .

The number of pores created , therefore , will increase in ratio to the number of pulses applied . if the face of the cell in line with the electric field contains a previously punctured pore close to the orientation of the electric field , however , the pore is of lower resistance that the surrounding membrane and the lines of the electric field will pass through the pore and no new pore will be created . The number of pores created by successive discharges, therefore , is limited by this effect .

In contradiction to the creation of pores in the membrane as proposed by Knight and Baker above , Gordon et al (1985) reported that electropermeabilisation of isolated rat hepatocytes resulted in the formation of protruberances or blebs on the plasma membrane of the cell . This was thought to indicate the sites of cell damage and similar blebs were noted on the cell membrane immediately following isolation of the hepatocytes by collagenase perfusion . It was proposed by these workers , that stretching of the plasma membrane at the site of the blebs may be the reason for increased permeability .

Two different models have been proposed for the mechanism of electropermeabilisation of cell membranes . These differences may be due to (a) tissue differences , (b) differences in the electropermeabilisation process or (c) differences in data interpretation .

(iv) Size of permeable areas and markers .

Electropermeabilisation of bovine adrenal medullary cells has been reported to produce pores of radius 1 to 2 nm (Knight and Baker 1982) whereas Gordon and Seglen (1982) reported that permeabilised areas of the hepatocytes cell membrane allowed free influx or efflux of molecules with a molecular weight of less than 1000 but occluded larger molecules . In both of these cases electropermeabilisation of the membrane results in the free passage of small molecules such as cyclic AMP and calcium down a concentrations gradient but large molecules such as proteins will be retained within the cell .

A number of markers have been used to assess electropermeabilisation in different cell types . Most workers have measured the rate of influx of these markers in electropermeabilised compared to intact cells . Markers , therefore , are all small molecules (Mr < 1000) which are relatively poor at penetrating the intact cell . Markers tend to be either radiolabelled compounds e.g. (^{14}C)sucrose (Gordon and Seglen 1982 ; Gordon et al 1985) and ^{45}Ca-EGTA (Knight and Baker 1982) which are detected by scintillation counting , or fluorescent dyes e.g. Lucifer Yellow (Mir et al 1988) which are detected optically or fluorometrically .

Trypan blue is unsuitable as a marker as its molecular weight is very close to the maximum for access . It can , however , be used for the assessment of cell viability following electropermeabilisation and its ability to differentiate between

viable and non-viable cells will be minimally affected by the permeabilisation process .

(v) Duration of permeability .

Reports on the length of time that the membrane remains permeable following electropermeabilisation vary . Knight and Baker (1982) reported that following electropermeabilisation of bovine adrenal medullary cells the membrane was permeable to Ca-EGTA for over 60 minutes (the longest time period studied) at both 0°C and 37°C . This was postulated to be due to the membrane proteins surrounding the "pore" (see section 4.3(b)(iii)) being denatured by the electropermeabilisation process . Teisse and Rols (1986) , however , reported that following electropermeabilisation of chinese hamster ovary cells , the cell membranes had reverted to their non-permeable state after 20 minutes at room temperature . Following electropermeabilisation of isolated rat hepatocytes , the cells have been reported to remain permeable to (^{14}C)sucrose for at least 2 hours at room temperature or below but become non-permeable to the marker after a 15 minute incubation period at 37°C (Gordon and Seglen 1982) and this is accompanied by disappearance of the blebs thought to be associated with permeabilised areas of the membrane (Gordon et al 1985) . It is interesting to note that increased blebbing and permeabilisation of the hepatocyte plasma membrane to (^{14}C)sucrose have also been reported to occur immediately following the isolation of hepatocytes by collagenase perfusion (Gordon et al

1985) but the membranes revert to their intact , non-permeable state during the 30 minute incubation period at 37°C which is routinely used to allow the hepatocytes to recover from the isolation process .

These varying reports of the duration of permeability may be due to one or a combination of (a) tissue differences , (b) electropermeabilisation technique or (c) marker used .

(vii) Permeabilisation media .

As discussed in section 4.3(b)(ii) , the ionic strength (and therefore the conductivity) of the extracellular medium is one of the factors which determines the potential difference across the plasma membrane of a cell placed in an electric field . The ionic strength of the medium determines the duration of the electric field to which the cells are exposed , i.e. the lower the ionic strength , the greater the resistivity and the longer the duration of the pulse .

The electropermeabilisation technique used by Gordon and Seglen (1982) for isolated rat hepatocytes was standardised using an electrolyte rich buffer which results in a very brief duration of the pulse . In the same study , Gordon and Seglen also examined the effects upon cellular structure of electropermeabilising hepatocytes in a 10% sucrose solution which has very high resistivity and therefore will result in very long exposure of cells to the electric field . They reported that one discharge of 2000V/cm resulted in marked destruction of cellular

structure and they termed this process electrodisruption .

Most studies upon the electropermeabilisation of cells have used media of intermediate ionic strengths e.g. Teisse and Rols (1986) used a 10mM phosphate buffer containing sucrose (250mM) and magnesium chloride (1mM) to effect permeabilisation of chinese hamster ovary cells and a buffer of similar ionic strength and composition was used by Knight and Baker (1982) to effect electropermeabilisation of bovine adrenal medullary cells .

(vii) Standardisation of electropermeabilisation in this present study .

In this present study , electropermeabilisation was carried out in an extracellular medium of Hams F10 culture medium supplemented with 0.1% bovine serum albumin or phosphate-free incubation medium supplemented with 0.1% bovine serum albumin (section 2.19(f)) . These media are of higher ionic strength than the sucrose buffers used by Knight and Baker (1982) or Teisse and Rols (1986) but of lower ionic strength than the electrolyte-rich saline buffer used to effect permeabilisation hepatocytes by Gordon and Seglen (1982) (section 4.3(b)(vi)) .

Cyclic AMP was used as a marker for the assessment of permeabilisation . This marker differs from those discussed in section 4.3(b)(iv) in that it is not fluorescent and is not radiolabelled . Cyclic AMP levels were detected by radioimmunoassay .

Using a range of electropermeabilisation parameters (2-10

pulses , 1000-3000V/cm) and increase in the intracellular concentrations of cyclic AMP as a measure of the degree of permeabilisation , we were able to show that 5 pulses of 1000V resulted in almost 100% permeabilisation subject to the assumption that (a) the hepatocyte is a sphere of volume $4/3\pi^3$, (b) the volume of the intracellular organelles impermeable to cyclic AMP is very small in comparison to the total volume of the cell and (c) that cyclic AMP degraded by cyclic AMP phosphodiesterase is replaced by cyclic AMP from the surrounding medium .

The strength of the applied field is lower than that routinely used by Gordon and Seglen (1982) to effect electropermeabilisation of isolated rat hepatocytes (2000V/cm) and lower than that used by Knight and Baker (1982) to effect permeabilisation of bovine adrenal medullary cells (2000V/cm) which are of a similar diameter to rat hepatocytes (20μm) . The field strength of 1000V/cm can be calculated by the formula shown in section 4.3(b)(ii) to produced a potential difference across the cell membrane at opposite poles the cell in line with the electric field of 1.5V which is above the minimum voltage of 1V reported to mediate membrane breakdown (Zimmerman et al 1974) and use of this lower voltage will minimise damage to intracellular membranes . Minimising damage to intracellular membranes is of particular importance in this present study because the cytochrome P450-dependent monooxygenases and the 5α-reductase enzyme complex are membrane bound .

Cell viability following electropermeabilisation was assessed by trypan blue exclusion which , as discussed in section

4.3(b)(iv) , is too large to readily pass into the electropermeabilised cell . It ability to differentiate between viable and non-viable cells , therefore , is little affected by the electropermeabilisation process . Cell viability was reduced from >97% to 75%-80% following electropermeabilisation . This may be due to the electropermeabilisation process or due to the penetration of some components of the external medium (standardised for intact cells) into the interior of the cell .

Using this technique we were able to overcome the problems of cyclic AMP access across the cell membrane and examine the direct effects of different concentrations of cyclic AMP upon steroid metabolising enzyme activity . Cyclic AMP allowed access in this manner will still be subject to rapid degradation by cyclic AMP phosphodiesterase . The concentration gradient which results from cyclic AMP degradation , however , will replace that which is lost and so the overall effects of degradation by cyclic AMP phosphodiesterase will be negligible . The intracellular concentration , assumed to be homogenous throughout the hepatocyte , therefore , will be the same as that in the extracellular medium .

4.3(c) Effects of elevating cyclic AMP (direct) in electropermeabilised hepatocytes .

(i) Effects upon steroid-metabolising enzymes .

Using the electropermeabilised hepatocyte as our model we were able to demonstrate that the direct application of cyclic AMP has a concentration- and time-dependent biphasic effect upon steroid metabolism .

5 x 10^{-5}M Cyclic AMP (figure 20 , panel A) had an non-specific inhibitory effect upon the metabolism of 4-androstene-3,17-dione in an analogous manner to adrenaline , IBMX and the combination of adrenaline and IBMX and this concentration may correlate with those produced by hormones in the physiological situation as discussed in section 4.3(a)(i) .

Incubation with 5 x 10^{-4}M cyclic AMP had less of an inhibitory effect than did 5 x 10^{-5}M cyclic AMP and this was followed by slight stimulation of all of the enzyme activities (figure 20 , panel B) . This intermediate concentration of cyclic AMP , therefore , produced identical effects to forskolin in that slight secondary stimulatory effects were apparent .

5 x 10^{-3}M Cyclic AMP had only very slight inhibitory effects but this was followed by a much greater stimulatory effect than that produced by 5 x 10^{-4}M cyclic AMP (figure 20 , panel C) . These effects , therefore , correlate with those produced by the combination of forskolin and IBMX and these high concentrations of cyclic AMP are unlikely to be generated by hormones under

physiological conditions but may correlate with those generated in the liver by in vivo administration of phenobarbitone , 3-methylcholanthrene (Byus et al 1976) and Arochlor-1254 (Costa et al 1976) (section 1.17(b)) . As discussed previously in section 4.3(a)(i) , however , the concentrations of cyclic AMP generated by these agents in vivo were apparently considerably lower than those necessary to mediate stimulation of steroid-metabolising enzyme activity in this present study .

The concentrations of cyclic AMP which were required to produced identical effects upon steroid metabolism in the electropermeabilised hepatocytes and in intact hepatocytes were approximately 100-fold higher in the electropermeabilised hepatocytes and this may reflect the degree of compartmentalisation of agent-generated cyclic AMP within the intact cell .

The mechanism by which cyclic AMP mediates both inhibition and stimulation of steroid-metabolising enzymes will be discussed in sections 4.4 to 4.8 .

(ii) Drawbacks of using electropermeabilised cells as a physiological model .

One drawback is the amount of time that cyclic AMP remains elevated within the cell . Knight and Baker (1982) reported that electropermeabilisation of bovine adrenal medullary cells produced pores which remained stable for in excess of 1 hour following electropermeabilisation . Gordon and Seglen (1982) , however ,

reported that following electropermeabilisation of rat hepatocytes , the cell membrane remained permeable for 15 minutes when incubated at 37°C . Intracellular cyclic AMP levels , therefore , may be expected to remain constant for at least 15 minutes following its addition to the extracellular medium and this contrasts markedly with the transient elevation of cyclic AMP generated by indirect methods in intact hepatocytes . The constant elevation of cyclic AMP over this extended time period , therefore , may prolong a cellular event which is limited under physiological conditions . It is interesting , therefore , that the effects of cyclic AMP upon steroid metabolism in electropermeabilised hepatocytes correlate well with the effects of corresponding elevations of cyclic AMP in intact hepatocytes . This further suggests that it is the initial concentration of cyclic AMP within the cell which determines the later effects upon steroid-metabolising enzymes and that the rate of decline of cyclic AMP levels is functionally unimportant for regulation of steroid-metabolising enzymes .

Another drawback of the system is that the cell membrane becomes freely permeable to all molecules of molecular weight less than 1000 . The intracellular milieu equilibrates with the extracellular medium and this may result in efflux of important components from the cell and influx of unwanted components from the extracellular medium . Correlation between the effects of cyclic AMP upon steroid metabolism in intact cells and in electropermeabilised cells indicates that these possible changes in the cytosolic constituents have no apparent effect upon the

activities of hepatic monooxygenases and their regulation by cyclic AMP .

It has previously been reported that electropermeabilisation of both chinese hamster ovary cells (Teisse and Rols 1986) and rat hepatocytes (Gordon and Seglen 1982) may result in fusion of the cells and the production of multinucleated giant cells . This was thought to be due to changes in the plasma membrane and not the electric field (Teisse and Rols 1986) . Fusion of the hepatocytes was reported to be increased if the cells were in tight contact (Gordon and Seglen 1982) and Teisse and Rols (1986) reported that sedimentation of chinese hamster ovary cells by centrifugation was necessary to produce cell fusion . In this present study the hepatocytes were not centrifuged following electropermeabilisation and microscopic examination of the cells revealed no giant cells which would have indicated that fusion had occurred .

Another effect of electropermeabilisation noted by Gordon and Seglen (1982) was that the procedure resulted in decrease in hepatocyte protein synthesis and degradation . This may be an important consideration in this present study because stimulation of both the synthesis and degradation of cytochrome P450 is a mechanism by which high concentrations of cyclic AMP mediate their effects upon steroid-metabolising enzymes (section 4.7) and these actions may be depressed in electropermeabilised hepatocytes . Gordon and Seglen (1982) , however , noted that protein synthesis and degradation were reduced by 15 to 20% following the application of 10 discharges of 2000V/cm . In this

present study we have used 5 discharges of 1000V/cm to effect permeabilisation and the effect upon protein synthesis and degradation may be expected to be correspondingly less than that noted by Gordon and Seglen (1982) . In addition we noted little difference between the effects of a combination of forskolin and IBMX upon steroid metabolism and cytochrome P450 levels in intact hepatocytes and those of 5 x 10^{-3}M cyclic AMP in electropermeabilised hepatocytes (section 4.7) . This may indicate that the reduction of protein synthesis and degradation is minimal using these electropermeabilisation parameters and over the time periods used in this present study .

Problems of cell fusion and reduction of protein synthesis and degradation , therefore , may have been minimised in this present study by the protocol used whereas possible changes in the cytosolic constituents and extended elevation of cyclic AMP appear to have little effect upon steroid-metabolising enzyme activities and their regulation by cyclic AMP . We , therefore , consider that the electropermeabilised hepatocytes , although not perfect , is a valid model for examining the regulation of steroid-metabolising enzymes by cyclic AMP under the conditions used in this present study .

4.3(d) Conclusions to section 4.3 .

We have shown by both indirectly elevating cyclic AMP in intact rat hepatocytes and by directly elevating cyclic AMP in electropermeabilised hepatocytes that cyclic AMP has a time- and concentration-dependent biphasic effect upon the metabolism of 4-androstene-3,17-dione . The effect upon steroid-metabolising enzyme activity occurring at ½ to 2 hours is related to the concentration of cyclic AMP present in the cell at 2 to 5 minutes. Low (physiological) concentrations of cyclic AMP , generated by adrenaline , IBMX , and a combination of adrenaline and IBMX and simulated by 8-bromo-cyclic AMP in intact cells , correlating with 5×10^{-5}M cyclic AMP in electropermeabilised cells , mediate early inhibition of enzyme activity .

Intermediate concentrations of cyclic AMP , generated by forskolin in intact cells and correlating with 5×10^{-4}M cyclic AMP in electropermeabilised cells mediate weak early inhibitory effects possibly followed by weak stimulatory effects upon enzyme activity .

High (supraphysiological) concentrations of cyclic AMP , generated by a combination of forskolin and IBMX in intact hepatocytes and correlating with 5×10^{-3}M cyclic AMP in electropermeabilised cells mediate little or no early inhibitory effects but this is followed by marked later stimulatory effects upon enzyme activity .

4.4 THE ROLE OF PROTEIN KINASES AND PROTEIN SYNTHESIS IN THE ACTIONS OF CYCLIC AMP .

Cyclic AMP is thought to mediate most , if not all , of its effects in the cell by activation of cyclic AMP-dependent protein kinase (PKa) . In 1983 , Pyerin et al reported that the purified rabbit liver cytochrome P450 , P450 LM_2 , in a reconstituted membrane system was phosphorylated by incubating with the catalytic subunit of PKa and this was accompanied by decrease in the metabolism of 7-ethoxycoumarin . Later work by Pyerin and co-workers (1987) indicated that the catalytic subunit of PKa could also phosphorylate many rat liver cytochrome P450 isozymes. Activation of PKa , therefore , may be a mechanism by which cyclic AMP mediates its inhibitory effects upon steroid metabolising enzymes in this present study . Pyerin and co-workers (1987) also reported that many rat liver cytochrome P450 isozymes were phosphorylated by protein kinase c (PKc) . Many interactions exist between PKa-activating and PKc-activating pathways (section 1.14(b)) and it is possible that some of the effects of cyclic AMP may be mediated by modulation of PKc-activating pathways .

Cyclic AMP activation of PKa has also been implicated in the induction of hepatic monooxygenases mediated by Arochlor-1254 (Costa et al 1976) , phenobarbitone and 3-methylcholanthrene (Byus et al 1976) and the inductive effects of these agents are thought to be due , at least in part , to increase in protein synthesis (section 1.9(b)) . Activation of PKa and subsequent

increase in protein synthesis , therefore , may be a mechanism by which cyclic AMP mediates stimulation of steroid-metabolising enzymes in this present study .

We , therefore , examined the roles of protein kinase and protein synthesis in the actions of cyclic AMP upon steroid metabolism in isolated rat hepatocytes .

4.4(a) Agents .

(i) Protein kinase inhibitors .

The role of protein kinases in the actions of cyclic AMP were assessed using the recently developed K-252 compounds . The first of these compounds to be characterised was K-252a which is a indole carbazole compound produced by the microbe , Nocardiopsis species K-252 (Kase et al 1986) . This compound was first characterised as a potent inhibitor of Protein kinase c (PKc) with an IC_{50} of 32.9nM (Kase et al 1986) and a Ki of 25nM . It has been subsequently reported that K-252a has comparative inhibitory effects upon both cyclic AMP-dependent protein kinase (PKa) and cyclic GMP-dependent protein kinase (PKg) with a Ki of 18nM and 20nM respectively (Kase et al 1987) . K-252a , therefore , appears to a general protein kinase inhibitor of both PKc and cyclic nucleotide-dependent protein kinases .

K-252a is also an inhibitor of calmodulin and subsequently of calmodulin-dependent cyclic AMP phosphodiesterase (Kase et al 1986) and this may be an unwanted effect of the compound . The

IC_{50} of K-252a for heart calmodulin-dependent cyclic AMP phosphodiesterase , however , is 1.3μM and so inhibitory effects against calmodulin-dependent phosphodiesterase will only occur at concentrations far in excess of those required to inhibit protein kinases .

A related species of Nocardiopsis (species K-290) was also reported to produce compounds with PKc-blocking activity and these were termed K-252b , c and d (Nakanishi et al 1976) , all of these compounds were found to possess PKc-blocking activity but K-252b was found to be the most potent with an IC_{50} of 38.3nM compared with 214nM for K-252c and 337nM for K-252d . Structural analysis of the K-252b has revealed that it is the 9-carboxylic acid derivative of K-252a (Kase et al 1987) . It differs from K-252a , however , in that it is relatively specific for PKc with a Ki of 20nM , whereas its Ki for PKa is 90nM and its Ki for PKg is 100nM (Kase et al 1987) . At a concentration of 20nM , as used in this present study , K-252b will be a selective inhibitor of PKc and will have minimal blocking activity against cyclic nucleotide-dependent protein kinases .

Chemical modification of K-252a yielded two more derivatives with protein kinase-blocking activity . KT5720 is the 9-n-hexyl ester derivative of K-252a and has been found to be a highly selective inhibitor of PKa with a Ki of 60nM whereas its Ki for PKc and PKg are in excess of 2μM (Kase et al 1987) . KT5822 is the 9-methoxy derivative of K-252a and has been found to be a highly selective inhibitor of PKg with a Ki of 2.4nM whereas its Ki for PKc and PKa are 79nM and 37.4nM respectively . (Kase et

al 1987) .

These protein kinase inhibitors are thought to act by competing with ATP for the active site of their respective protein kinases (Kase et al 1987) . In this present study we used the K-252 compounds at concentrations correlating with the Ki for their respective protein kinases thereby eliminating problems of lack of selectivity at higher concentrations . None of the K-252 compounds were found to adversely affect cell viability . In studies utilising these agents , test cell samples were compared to control cells which received the inhibitor alone to eliminate any possible direct effects of the protein kinase inhibitors against hepatic monooxygenase activity . Comparison between untreated control cells and control cells which received the inhibitor alone , however , indicated that the protein kinase inhibitors appear to have little or no direct effect against hepatic monooxygenases .

One of the major problems noted in preliminary experiments was that none of the protein kinase inhibitors (K-252a, K-252b and KT5720) had any effect upon the actions of 8-bromo-cyclic AMP in the intact cell but we have later shown that these agents have marked effect upon the actions of cyclic AMP in the electropermeabilised cell . This may indicate that 8-bromo-cyclic AMP is mediating its inhibitory effects upon steroid metabolism by a protein kinase-independent mechanism . This explanation seems unlikely , however , because no protein kinase-independent effects of 8-bromo-cyclic AMP have been reported and 8-bromo-cyclic AMP is thought to simulate the effects of cyclic AMP . A more likely

explanation , therefore , is that the protein kinase inhibitors are unable to penetrate the intact cell membrane . The assay procedure used by Kase et al (1987) to establish the inhibitory potencies of the K-252 compounds were carried out using in vitro models and no studies were carried out to establish the effects of these compounds in the intact cells . The inability of the compounds to penetrate into intact cells , as implied by this study , may be a serious drawback to the use of these compounds in the investigation of physiological processes .

(ii) Cycloheximide .

Cycloheximide is a glutaramide antibiotic and inhibits protein synthesis at the level of translation . It is thought to inhibit both the initiation of protein synthesis and polypeptide chain elongation by interacting with the 60s ribosomal subunit (Peshka 1971) . In this present study cycloheximide was not found to adversely affect cell viability . One problem of using a protein synthesis inhibitor in studying hepatic monooxygenase activity is that the turnover of the proteinaceous components , e.g. cytochrome P450 , will be disrupted and so a decline in the levels of these components may be expected . This would result in decrease in the metabolising capacity of the hepatic monooxygenases . In studies utilising this agent , therefore , test samples were compared to controls which received cycloheximide alone thereby overcoming direct effects of

cycloheximide perhaps due to disruption of enzyme turnover .

4.4(b) Identification of a multicomponent system .

(i) Effects of protein kinase inhibitors and cycloheximide .

As stated in the introduction , cyclic AMP is thought to produce most of its effects in the cell by activation of PKa . The inhibition of both the initial inhibitory effect and the later stimulatory effect of cyclic AMP upon enzyme activity in the presence of K-252a (figure 23) and KT5720 (figure 24) confirms both that cyclic AMP mediates these two effects by activation of a protein kinase and that the identity of the protein kinase appears to be PKa .

In the presence of KT5822 (figure 27) the inhibitory and stimulatory effects of cyclic AMP upon enzyme activity were unaffected indicating that cyclic AMP does not produce any of its effects via activation of PKg .

In the presence of K-252b (figure 25) the inhibitory effect of 5 x 10^{-5}M cyclic AMP was completely inhibited and instead slight stimulation of enzyme activity occurred . It appears , therefore , that the activation of PKc is necessary for cyclic AMP-mediated inhibition of steroid-metabolising enzymes at these physiological concentrations . This may indicate that the phosphorylation of PKa by PKc is necessary to activate PKa . This is unlikely , however , because cyclic AMP is not thought to directly activate PKc and it is more likely , therefore , that

activation of PKc occurs secondary to activation of PKa by cyclic AMP . Another explanation may be that phosphorylation of proteins by both PKa and PKc is necessary for enzyme inhibition to occur . This is unlikely because activation of PKc by the phorbol ester , PMA , has been reported to inhibit the metabolism of androstenedione to the same magnitude and over a similar time course as cyclic AMP-mediated inhibition (Allan and Skett 1988) and the effects of PMA were unaffected by the presence of KT5720 indicating that the inhibitory effects of PKc can occur independently of PKa activation (C.J. Allan : personal communication) . A more likely explanation is that the inhibitory effects of cyclic AMP are mediated by secondary activation of PKc by PKa and PKa has no direct inhibitory effects .

This interaction represents an unusual mode of intracellular regulation and the physiological implications of such an interactions will be discussed in more detail in section 4.10(b) .

As shown in figure 25 , in the presence of K-252b the weak initial inhibitory effects of 5 x 10^{-3}M cyclic AMP were also inhibited indicating that PKc activation is also necessary for the inhibitory effects of high concentrations of cyclic AMP . The secondary stimulatory effects of 5 x 10^{-3}M upon steroid-metabolising enzymes , however , were of a similar magnitude to those produced in the absence of K-252b . The stimulatory effects of cyclic AMP , therefore , appear to occur independently of PKc activation . It is also apparent that when the PKc-dependent inhibitory effect is blocked by K-252b , concentration-dependent stimulation of enzyme activity occurs i.e. slight stimulation

mediated by 5 x 10^{-5}M cyclic AMP and marked stimulation mediated by 5 x 10^{-3}M cyclic AMP . This occurs at an earlier time point than the later stimulation produced in the presence of activated PKc and would be masked by the predominant inhibitory effect in the presence of activated PKc .

It appears , therefore , that both inhibition and stimulation occur at all concentrations of cyclic AMP but at low concentrations the predominant inhibitory effect masks the weak stimulatory effect whereas at high concentrations the predominant stimulatory effect masks the inhibitory effect .

As shown in figure 22 , cycloheximide blocks the stimulatory effects and enhances the inhibitory effects of all concentrations of cyclic AMP and this indicates that the stimulatory effects of all concentrations of cyclic AMP are mediated by increase in protein synthesis . Cycloheximide also blocked the early , concentration-dependent stimulatory effects of cyclic AMP , unmasked by inhibiting PKc activation with K-252b (figure 26) , which indicates that these early stimulatory effects are also due to increase in protein synthesis . These results further imply that both inhibition and stimulation of steroid metabolism occur at all concentrations of cyclic AMP .

In the presence of both cycloheximide and K-252b , 5 x 10^{-5}M cyclic AMP had no effect upon steroid metabolism which confirms the previous finding that activation of PKa by physiological concentrations of cyclic AMP has no direct inhibitory effects upon

steroid metabolism . 5 x 10^{-3}M Cyclic AMP , however , did mediate slight inhibition of steroid metabolism in the presence of cycloheximide and K-252b . This may be due to the high concentrations of cyclic AMP overcoming the inhibitory effects of K-252b . It has been reported , however , that PKa mediates inhibition of cytochrome P450-dependent monooxygenases in vitro (Pyerin et al 1983 ; Pyerin et al 1984) . It is possible , therefore , that another explanation for the inhibitory effects of 5 x 10^{-3}M cyclic AMP in the presence of cycloheximide and K-252b is that these supraphysiological concentrations of cyclic AMP mediate some inhibitory effects by a PKc-independent mechanism .

The finding that cycloheximide also blocks the stimulatory effects and prolongs and enhances the inhibitory effects of forskolin and a combination of forskolin and IBMX in intact hepatocytes , indicates that the stimulatory effects of these agents appear to be mediated by an increase in protein synthesis and the stimulatory effects of forskolin and a combination of forskolin and IBMX in intact hepatocytes appear to be mediated in an identical manner to those produced by high concentrations of cyclic AMP in electropermeabilised hepatocytes .

(iii) Components mediating the effects of physiological concentrations of cyclic AMP .

Low (physiological) concentrations of cyclic AMP (5 x 10^{-5}M) , therefore , have a weak PKc-independent stimulatory

effect upon protein synthesis which does not result in overall stimulation of enzyme activity due to the predominance of the inhibitory effect but does accelerate the recovery of the enzymes from inhibition . The weak stimulatory effects , however , can be unmasked by inhibiting the PKc-dependent inhibitory effects . When the weak protein synthesis-dependent stimulation of enzyme activity , mediated by PKa in the absence of activated PKc , is inhibited by cycloheximide , PKa has no effect upon enzyme activity and this further illustrates that the inhibitory effects of physiological concentrations of cyclic AMP are mediated by indirect activation of PKc and PKa has no direct inhibitory effects .

The effects of physiological concentrations of cyclic AMP , therefore , appear to be mediated by two components (a) PKc-independent/protein synthesis-dependent stimulation and (b) PKc-dependent/protein synthesis-independent inhibition .

(iv) Components mediating the effects of supraphysiological concentrations of cyclic AMP .

High (supraphysiological) concentrations of cyclic AMP (5 x 10^{-3}M) have a much greater PKc-independent/protein synthesis-dependent stimulatory effect upon steroid-metabolising enzyme activity which masks the inhibitory effects . When protein synthesis is blocked by cycloheximide , inhibitory effects are unmasked . When PKc-dependent inhibition is blocked by K-252b and

the protein synthesis-dependent effects of PKa are blocked by cycloheximide , PKa does appear to have some PKc-independent inhibitory effects upon steroid-metabolising enzyme activity . The inhibitory effects unmasked by the presence of cycloheximide , therefore , may be mediated by a combination of PKc-dependent and PKc-independent mechanisms and this may indicate that PKa has a direct inhibitory effect upon steroid-metabolising enzymes at supraphysiological concentrations of cyclic AMP .

We have identified three components which contribute to the overall effects of supraphysiological concentrations of cyclic AMP , (a) PKc-independent/protein synthesis-dependent stimulation (b) PKc-dependent/protein synthesis-independent inhibition and (c) PKc- and protein synthesis-independent inhibition .

4.4(c) Conclusion to section 4.4 .

Using the selective protein kinase inhibitors and cycloheximide , we have shown that both physiological and supraphysiological concentrations of cyclic AMP mediate inhibitory and stimulatory effects upon steroid metabolism and that both of these effects appear to be mediated by cyclic AMP activation of PKa . The inhibitory effects of physiological concentrations of cyclic AMP , however , do not appear to be mediated directly by PKa but may be due to secondary activation of PKc by PKa . Supraphysiological concentrations of cyclic AMP do appear to mediate some inhibition of steroid metabolism by a PKc-independent

mechanism which may indicate a direct effect of PKa at these concentrations . Inhibition of steroid metabolism by these supraphysiological concentrations of cyclic AMP , therefore , may be mediated by a combination of direct actions of PKa and indirect actions via secondary activation of PKc .

Stimulation of steroid metabolism occurs independently of PKc activation at all concentrations of cyclic AMP and is due to increase in protein synthesis .

4.5 MEDIATORS OF PROTEIN KINASE C-INDEPENDENT EFFECTS OF CYCLIC AMP .

Although the initial mediator of all of the separate effects of cyclic AMP is PKa , the exact nature of the mediator of PKc-independent effects is unclear . PKa is known to affect other intracellular pathways as documented in the Introduction (section 1.14(b)) and the PKc-independent actions of cyclic AMP , therefore , could be mediated either by PKa directly or indirectly via alteration of the levels or activities of other intracellular mediators .

It is well documented that one of the effects of PKa activation is stimulation of calcium influx (Mauger et al 1985 ; Staddon and Hansford ; Poggioli et al 1986) . The elevation of intracellular calcium , however , is unlikely to mediate the PKc-independent effects of PKa as the inhibitory effects of adrenaline were completely blocked by the presence of propranolol

which blocks β_2 receptor-mediated generation of cyclic AMP . This indicates that adrenaline-stimulated elevation of intracellular calcium levels does not mediate the inhibitory effects of adrenaline on steroid metabolism . It has also been reported that incubation of isolated rat hepatocytes with the calcium ionophore, A23187 , similarly has no effects upon the metabolism of androstenedione (Hussin et al 1988) .

PKa may alter the levels of other second messengers such as arachidonic acid and prostaglandins . The mechanism by which PKa may produce such effects , however , is unclear . The cyclooxygenase inhibitor , indomethacin , has been reported to inhibit monooxygenase activity by mediating a selective loss of haem and one mechanism by which these effects may occur is by elevation of arachidonic acid levels which has also been reported to inhibit monooxygenase activity (Burke et al 1983) . The inhibitory effects of another cyclooxygenase inhibitor , ibuprofen, upon hepatic monooxygenase activity , however , are thought to be due to direct toxic effects upon cytochrome P450 mediated by the drug itself and unrelated to its cyclooxygenase-inhibiting activity (Reinicke and Klinger 1975) . Furthermore phenylbutazone is a known inducer of cytochrome P450 and this effect is not related to its actions as a cyclooxygenase inhibitor (Conney and Schneidman 1964) . In addition PKa has no known cyclooxygenase-inhibiting activity and so would be unlikely to be producing its effects by mediating a subsequent accumulation of intracellular arachidonic acid . It appears unlikely ,

therefore , that alteration in the levels of arachidonic acid and prostaglandins would constitute a major PKa-mediated regulatory pathway .

It appears likely , therefore , that the PKc-independent effects of PKa are due to direct effects of PKa itself and subsequent results will be discussed in this context .

4.6 MECHANISMS OF ACTION (PHYSIOLOGICAL CONCENTRATIONS OF CYCLIC AMP) .

4.6(a) Effects upon cytochrome P450 levels .

One of the mechanisms by which physiological concentrations of cyclic AMP may mediate inhibitory effects is by decreasing levels of cytochrome P450 . The inhibitory effects of 5×10^{-5}M cyclic AMP , however , were not accompanied by alteration in cytochrome P450 levels as determined by assessing whole cell cytochrome P450 levels (table 8) . Furthermore we could detect no alteration in the levels of cytochrome P450 in the presence of cycloheximide or in the presence of K-252b . In addition , the inhibitory effects of 8-bromo-cyclic AMP , adrenaline , IBMX , adrenaline in combination with IBMX , and forskolin in intact hepatocytes similarly did not appear to be mediated by alteration of cytochrome P450 assessed by the same assay procedure (table 7). One explanation may be that inhibitory effects are due to selective decrease in the levels of specific cytochrome P450

isozymes but that the overall depletion of whole cell cytochrome P450 levels is negligible and undetectable by this assay procedure. This explanation seems unlikely due to the pronounced non-specific inhibitory effects of cyclic AMP upon steroid-metabolising enzymes . It is probable , therefore , that the inhibitory effects of cyclic AMP may be due , at least in part , to some alternative mechanism of action which does not involve degradation of cytochrome P450 .

4.6(b) Effects upon phosphorylation of cytochrome P450 .

In 1983 , Pyerin et al reported that incubation of the purified rabbit liver cytochrome P450 , P450 LM_2 , in a reconstituted membrane system , with the catalytic subunit of PKa resulted in phosphorylation of the cytochrome P450 . The identity of the phosphorylated residue has subsequently been shown to be serine 128 (Pyerin et al 1986a) which is on the cytoplasmic side of the cytochrome P450 apoprotein (Nelson and Strobel 1988) and it has been proposed by Jansson et al (1987) that this residue may be at the site of interaction between cytochrome P450 and cytochrome b_5 (section 1.3(b)). Phosphorylation was show to result in the conversion of the cytochrome P450 to the corresponding cytochrome P420 (Taniguchi et al 1985) with a concomitant decrease in the metabolism of benzphetamine and 7-ethoxycoumarin (Pyerin et al 1984) .

In a later study (1987) Pyerin and co-workers reported that

the phosphorylation of cytochrome P450 by PKa was not restricted to rabbit liver isozymes , but that PKa could also phosphorylate a number of rat liver cytochrome P450 isozymes . In the same study , these workers also examined the effects of incubating rabbit or rat liver cytochrome P450 isozymes with PKc and reported that many of these isozymes could also be phosphorylated by PKc . The susceptibility of each of the isozymes to phosphorylation by either PKa or PKc , however , varied . Rat cytochrome P450 PB-3a (the major phenobarbitone-inducible isozyme : table 1) was reported to be phosphorylated by both PKa and PKc . Rat cytochrome P450 PB-3b (phenobarbitone-inducible : table 1) was reported to be phosphorylated by PKa but not by PKc. Rat cytochromes P450 PB-2a (steroid-16α-hydroxylase : table 1) and MC-1a (the major isosafrole-inducible isozyme : table 1) were reported to be phosphorylated by PKc but not by PKa . Rat cytochromes P450 MC-1b (the major 3-methylcholanthrene-inducible isozyme : table 1) and UT-1 (steroid-7α-hydroxylase : table 1) were reported to be refractory to phosphorylation by both PKa and PKc .

These reports imply that phosphorylation of cytochrome P450 by secondary activation of PKc by PKa may be a mechanism by which physiological concentrations of cyclic AMP may inhibit steroid metabolism in isolated rat hepatocytes .

To investigate if secondary activation of PKc by PKa mediated phosphorylation of cytochrome P450 , we examined the incorporation of ^{32}P into cytochrome P450 PB-2a (table 1) which is thought to

mediate up to 90% of the hydroxylation of androstenedione at the 16α position (Waxman 1984) (section 1.5) . We were unable to detect any incorporation of ^{32}P into immunoprecipitated cytochrome P450 PB-2a either in control hepatocytes or in hepatocytes incubated for up to 1 hour with 5 x 10^{-5}M cyclic AMP (figure 33). We also examined the incorporation of ^{32}P into two other isozymes which represent the major cytochrome P450 isozymes induced by the two major classes of inducing agent (section 1.9(a)) . Cytochrome P450 PB-3a is the major phenobarbitone-inducible isozyme (table 1) and cytochrome P450 MC-1b is the major polycyclic aromatic hydrocarbon-inducible isozyme (table 1) . We were unable to detect the incorporation of ^{32}P into either of these isozymes following incubation of hepatocytes for up to 1 hour with 5 x 10^{-5}M cyclic AMP and subsequent immunoprecipitation with specific antibodies (figure 33) .

The above findings may indicate that direct phosphorylation of cytochrome P450 isozymes is not a physiological effect . This proposal remains to be clarified , however , because although we were able to demonstrate that the phosphorylation of intracellular proteins occurred in the whole cell extract (figure 32) , we did not assess the cytochrome P450 content of the immunoprecipitates . We cannot be certain , therefore , that the isolation of the specific cytochrome P450 isozymes by this method was successful . The presence of a large protein band of Mr 45,000-50,000 , however , indicates that the immunoprecipitation process has resulted in the isolation of a protein with similar

molecular weight to cytochrome P450 . In addition , it has recently been reported that the immunoprecipitate contains haem (C.J. Allan : personal communication) . Both of these findings imply that the immunoprecipitation of cytochrome P450 isozymes has been successful by the method used . Additional evidence also exists that implies that phosphorylation of cytochrome P450 may not be the underlying mechanism of cyclic AMP-mediated , PKc-dependent inhibition of steroid-metabolising enzymes :

(1) Cytochrome P450 UT-1 (table 1) is thought to mediate , almost exclusively , the hydroxylation of androstenedione at the 7α position (Waxman et al 1987 ; Nagata et al 1986) (section 1.5) and Pyerin et al (1987) reported that this isozyme was refractory to phosphoryation by both PKa and PKc . We have previously reported , however , that both 5 x 10^{-5}M cyclic AMP and agents acting physiologically to elevate cyclic AMP in intact cells have a marked inhibitory effect upon the activity of this enzyme .

(2) We have reported that the activities of 5α-reductase and 17α/β hydroxysteroid dehydrogenase are also inhibited following incubation with physiological concentrations of cyclic AMP . These enzymes do not contain cytochrome P450 (Golf and Graef 1978) (section 1.5) and so inhibition cannot be attributed to phosphorylation of cytochrome P450 .

(3) Taniguchi et al (1985) reported that phosphorylation of cytochrome P450 resulted in its conversion to cytochrome P420 . As discussed previously (section 4.6(a)) , however , we were unable to detect any increase in the levels of cytochrome P420 when we assessed whole cell cytochrome P450 levels nor were we able to detect any decrease in cytochrome P450 levels which would be the ultimate result of the conversion of cytochrome P450 to cytochrome P420 and its rapid degradation by haem oxygenase (Maines and Anders 1973b ; Kutty et al 1988) (section 1.8(b)) .

One of the mechanism by which cyclic AMP may be producing its effects is by depletion of the cofactors for the electron transport chain of hepatic monooxygenases . Homogenisation of hepatocytes following incubation with 8-bromo-cyclic AMP and the readdition of excess cofactors using a NADPH-generating system , however , had no effect upon the 8-bromo-cyclic AMP-mediated inhibition of steroid metabolism (figure 11) . This indicates that the inhibitory effects of cyclic AMP at physiological concentrations are not due to depletion of cofactors .

The possibility exists that the phosphorylation of other enzymes in the electron transport chain of hepatic monooxygenases may mediate cyclic AMP-mediated , PKc-dependent inhibition of enzyme activity . In this context both NADPH-cytochrome P450 reductase and NADH-cytochrome b_5 reductase have been reported to be weak substrates for phosphorylation by PKc although cytochrome b_5 appears to be a very poor substrate for PKc (Pyerin et al

1987) .

Phosphorylation of these additional components may explain the cyclic AMP-mediated inhibition of 5α reductase which although a non-cytochrome P450-dependent enzyme , is the terminal enzyme of an electron transport chain which exhibits many similarities to the cytochrome P450 monooxygenases (Golf and Graef 1978) (section 1.5) .

Another mechanism by which cyclic AMP may mediate enzyme inhibition is by activation of phosphatases which have been shown to mediate inhibition of cytochrome P450 monooxygenases in a reconstituted system (Pyerin et al 1986b) . This effect appears to be mediated at the level of NADPH-cytochrome P450 reductase and may be due to the conversion of FMN to riboflavin (Taniguchi and Pyerin 1987) . This would result in disruption of the transfer of electrons from the reductase to the cytochrome P450 (Backes and Reker-Backes 1988 ; Iyanagi et al 1981 ; Vermilion et al 1981) (section 1.2(d)) and subsequent inhibition of the activity of the enzyme .

One of the mechanism by which phosphatase activation may occur is by PKa-mediated elevation of intracellular calcium levels (Aitkin et al 1984 ; Tonks and Cohen 1983) . This does not explain the dependence of inhibition of steroid metabolism upon PKc activation , however , and PKc has not been reported to have any effect upon intracellular calcium levels . In addition , as stated previously (section 4.8) , agents which directly elevate intracellular calcium levels (A23187 and adrenaline acting at α_1

receptors) have been reported to be without effect upon steroid-metabolising enzymes . This suggests that the activation of phosphatases by secondary elevation of calcium is unlikely to mediate the inhibitory effects of cyclic AMP .

More recently Yang et al (1987) have reported the existence of a directly-acting activator of the membrane-bound protein phosphatase-1 in the liver which appears to be a membrane-bound protein kinase and has been termed Fa . The endogenous ligand for Fa was not elucidated by Yang and co-workers but it is possible that activation of Fa may be a mechanism by which PKa-mediated activation of PKc may directly activate protein phosphatase-1 independently of any alteration in the levels of cytosolic free calcium . Alternatively , PKc may directly phosphorylate protein phosphatase-1 in an analogous manner to Fa .

4.6(c) Conclusion to section 4.6 .

In this present study we have been able to show that the inhibition of steroid-metabolising enzymes mediated by physiological concentrations of cyclic AMP do not appear to be mediated by changes in the levels of whole cell cytochrome P450 , direct phosphorylation of cytochrome P450 or by depletion of cofactors for the electron transport chain . The precise cellular mechanisms of cyclic AMP-mediated effects , therefore , still remains to be clarified but they may involve some reversible inactivation of the enzyme complex .

4.7 MECHANISMS OF ACTION (SUPRAPHYSIOLOGICAL CONCENTRATIONS OF CYCLIC AMP) .

4.7 (a) Effects upon cytochrome P450 levels .

As stated in the previous section , one of the mechanisms by which supraphysiological concentrations of cyclic AMP (5 x 10^{-3}M) may mediate their effects upon steroid-metabolising enzyme activity is by altering levels of cytochrome P450 . The effects of 5 x 10^{-3}M cyclic AMP and the effects of the components which have been postulated to mediate the overall effects of 5 x 10^{-3}M cyclic AMP upon both steroid-metabolising enzyme activity (7α hydroxylase) and whole cell cytochrome P450 are illustrated in figure 34 . This figure is composed of the results previously illustrated in figures 20 (panel c) ; 22 (panel c) ; 25 (panel b) ; 26 (panel b) ; 29 ; 30 and 31 and is included to illustrate more clearly the effects of the different components in producing the overall response to high concentrations of cyclic AMP .

(i) Effects of cyclic AMP .

Measurement of the whole cell cytochrome P450 levels following incubation with both 5 x 10^{-3}M cyclic AMP in electropermeabilised hepatocytes and a combination of forskolin and IBMX in intact hepatocytes , indicated that the protein synthesis-dependent

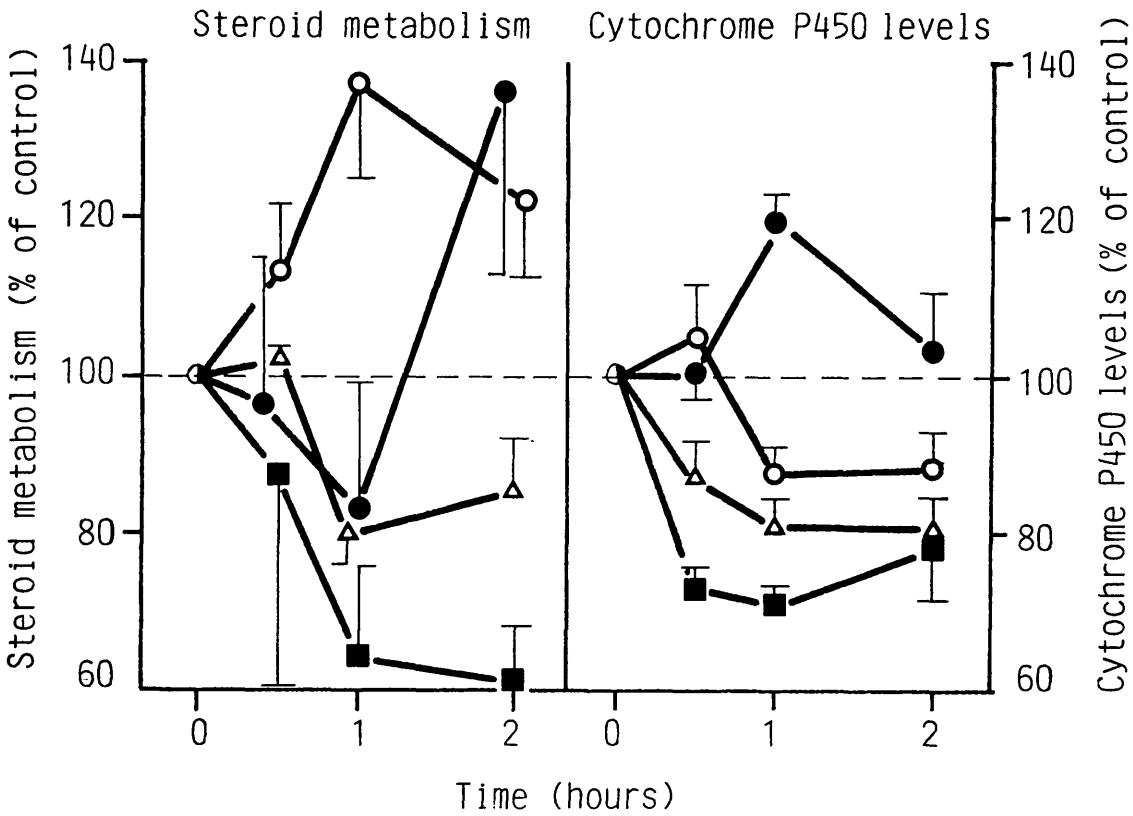

Figure 34 : Changes in steroid metabolism (7α-hydroxylase activity) and cytochrome P450 levels with time in electropermeabilised hepatocytes following addition of 5 X 10^{-3}M cyclic AMP (-●-) , 5 X 10^{-3}M cyclic AMP and cycloheximide (10^{-5}M) (-■-) , 5 X 10^{-3}M cyclic AMP and K-252b (2 X 10^{-8}M) (-○-) and 5 X 10^{-3}M cyclic AMP with a combination of cycloheximide and K-252b(-△-) . Results are expressed as percentage of relevant control ± S.D. for at least 4 different cell samples . Control activities and cytochrome P-450 levels at all time points are as listed previously (figures : 20 , 22, 25, 26 , 29 , 30 , and 31) .

stimulatory effects upon steroid metabolism are due , at least in part , to induction of cytochrome P450 .

The hypothesis that the induction of cytochrome P450 is mediated by cyclic AMP activation of PKa was confirmed by the fact that cyclic AMP-mediated increase in cytochrome P450 levels was inhibited by the presence of the PKa blocker , KT5720 (table 10). The percentage increase in the levels of whole cell cytochrome P450 , however , was not as great as the stimulatory effects of high concentrations of cyclic AMP upon steroid metabolism . This may be due to the induction of other protein factors which act in a stimulatory manner upon steroid-metabolising enzymes . It is more probable , however , that cyclic AMP is acting in a selective manner to induce steroid-metabolising cytochrome P450 isozymes but having little effect upon other constitutive cytochrome P450 isozymes and the overall increase in whole cell cytochrome P450 does not reflect this selective induction .

The development of specific antibodies raised against individual cytochrome P450 isozymes should help to clarify this matter using the technique of Western blotting and will give a clearer indication of the induction profile of high concentrations of cyclic AMP .

(ii) Effects of cyclic AMP in the presence of cycloheximide .

In the presence of cycloheximide , supraphysiological concentrations of cyclic AMP (5×10^{-3}M) mediated a marked decrease in the levels of whole cell cytochrome P450 . The

inhibitory effects of these concentrations of cyclic AMP upon steroid metabolism , therefore , appear to be due , at least in part , to degradation of cytochrome P450 . This differs markedly from the inhibitory effects of physiological concentrations of cyclic AMP which did not appear to be mediated by degradation of cytochrome P450 and were postulated to be due to some reversible inhibitory effect upon steroid-metabolising enzymes (section 4.9). The percentage decrease in whole cell cytochrome P450 levels is not as great as its effects upon steroid-metabolising enzymes . This may indicate that cyclic AMP has a selective degradative effect upon some cytochrome P450 isozymes which is not detected by determining whole cell cytochrome P450 content . It is more likely , however , that , in addition to degradation of cytochrome P450 , the non-degraded cytochrome P450 is further inactivated by the same mechanisms postulated to mediate physiological inhibition of enzyme activity (section 4.9) .

It seems probable , therefore , that at least two mechanisms mediate the inhibitory effects of cyclic AMP upon cytochrome P450 monooxygenases (a) inactivation of cytochrome P450 by a direct or indirect mechanism which occurs at all concentrations of cyclic AMP and (b) degradation of cytochrome P450 which occurs at high concentrations of cyclic AMP .

(iii) Effects of cyclic AMP in the presence of a combination of cycloheximide and K-252b .

In the presence of both cycloheximide and K-252b , the degradative effects of supraphysiological concentrations of cyclic AMP were weaker than in the presence of cycloheximide alone . This indicates that secondary activation of PKc by PKa mediates at least some of the degradative effects of cyclic AMP at these concentrations . The levels of cytochrome P450 were still decreased over the whole time course of ½ to 2 hours , however , although to a lesser degree than in the presence of PKc , and it appears , therefore , that the degradative effects of pharmacological concentrations of cyclic AMP upon cytochrome P450 are mediated by a combination of direct degradation by PKa and indirect degradation due to secondary activation of PKc .

These findings correlate with the inhibitory effects of supraphysiological concentrations of cyclic AMP upon steroid-metabolising enzyme activity which were also proposed to be mediated by a combination of PKa and PKc (section 4.8) .

(iv) Effects of cyclic AMP in the presence of K-252b .

In section 4.4(b) it was shown that in the absence of activated PKc , activation of PKa by supraphysiological concentrations of cyclic AMP mediated marked stimulation of steroid metabolising enzyme activity . When the effects of supraphysiological concentrations of cyclic AMP upon cytochrome

P450 levels were examined in the presence of K-252b to inhibit the secondary activation of PKc , however , we were unable to detect any corresponding increase in the levels of whole cell cytochrome P450 . This may imply that the early protein synthesis-dependent stimulatory effects of PKa upon steroid-metabolising enzymes are not due to synthesis of cytochrome P450 . This differs from the mechanism proposed to mediate the later stimulatory effects of high concentrations of cyclic AMP upon steroid metabolism in the control cyclic AMP time course (as discussed in section 4.7(a)(i)).

The ability of PKa to stimulate protein synthesis , however , did delay the PKa-mediated degradation of cytochrome P450 which occurred when protein synthesis was blocked by the presence of cycloheximide . Thus , as shown in figure 34 , following an incubation period of ½ hour with 5 x 10^{-3}M cyclic AMP in the presence of K-252b , the levels of cytochrome P450 did not differ significantly from corresponding controls . When cycloheximide was included , however , (as discussed in section 4.7(a)(ii)) cytochrome P450 levels were decreased to 84% of corresponding controls following an identical time period . The difference between these two levels is due to PKa-mediated protein synthesis. The time course of the early stimulatory effects upon steroid metabolism correlates well with the delay of PKa-mediated degradation of cytochrome P450 . It is possible , therefore , that the direct degradative effects of PKa are relatively non-specific for cytochrome P450 isozymes whereas its inductive effects are relatively specific for steroid-metabolising cytochrome P450

isozymes and so no overall increase in the whole cell cytochrome P450 levels occurs over this early time period .

This was also thought to be a likely explanation for the discrepancy between steroid-metabolising enzyme activity and cytochrome P450 levels in the control cyclic AMP time course (section 4.7(a)(i)) .

(v) Components mediating the effects of supraphysiological concentrations of cyclic AMP upon cytochrome P450 levels.

In section 4.4 it was postulated that the effects of high concentrations of cyclic AMP upon steroid-metabolising enzyme activity were mediated by a number of separate components and the sum of the effects of these components at any one time determined the overall effect upon enzyme activity .

We have subsequently shown that the overall effects of cyclic AMP upon the cytochrome P450-dependent steroid-metabolising enzymes and the separate components which determine the overall effect are due , at least in part , to alterations in the levels of cytochrome P450 .

Using the PKc inhibitor , K-252b , and cycloheximide we have identified three of these components , namely , (a) PKa-mediated, protein synthesis-dependent induction of cytochrome P450 and concomitant stimulation of steroid metabolism , (b) PKa-mediated, protein synthesis-independent degradation of cytochrome P450 and concomitant inhibition of steroid metabolism and (c) PKc-mediated , protein synthesis-independent degradation of cytochrome

P450 and concomitant inhibition of steroid metabolism.

4.7(b) Proposed mechanisms mediating the effects of cyclic AMP upon cytochrome P450 levels .

(i) Induction .

The PKa-mediated , protein synthesis-dependent stimulatory effects upon steroid-metabolising enzyme activities have been proposed to be due to selective increase in the levels of steroid metabolising cytochrome P450 isozymes . One of the mechanisms by which this may occur is by PKa-mediated phosphorylation of acidic nuclear proteins (Johnson and Allfrey 1972) . This is thought to stimulate gene transcription as correlation exists between the decrease in the binding of these proteins to specific initiation sites when phosphorylated by PKa and the subsequent enhancement of messenger RNA synthesis (Teng et al 1971) . In addition , increase in messenger RNA has been reported to occur in the liver 1 hour following the "in vivo" administration of cyclic AMP (Dokas and Kleinsmith 1971) . It is interesting to note that phenobarbitone , which has been reported to elevate cyclic AMP levels and/or activate PKa (Byus et al 1976) , has also been reported to increase the phosphorylation states of acidic nuclear proteins (Blankenship and Bresnick 1974) .

It has been proposed that an alternative mechanism by which phenobarbitone and 3-methylcholanthrene may mediate induction is by stabilisation of some extranuclear event which occurs beyond

the level of gene transcription (Iversen et al 1987) perhaps by stabilising messenger RNA or by stimulating messenger RNA translation at the level of the ribosome . Although Dokas and Kleinsmith (1971) noted an increase in the levels of messenger RNAs in the liver following injection of cyclic AMP , as we have previously discussed (section 4.5) , cyclic AMP penetration of the intact plasma membrane is very poor and cyclic AMP which does penetrate the plasma membrane will be rapidly degraded by cyclic AMP phosphodiesterases (Beebe et al 1986) . The mechanism by which cyclic AMP may mediate this elevation of messenger RNA levels in the liver , therefore , is unknown . It is possible , therefore , that the inductive effects of PKa upon cytochrome P450 levels may be mediated , at least in part , by stabilisation of post-transcriptional events in an analogous manner to the effects reported for phenobarbitone and 3-methylcholanthrene .

Using cycloheximide , which blocks the translation of messenger RNA at the level of the ribosomes (Peshka 1971) , we were unable to determine if the increase in the levels of steroid-metabolising cytochrome P450 isozymes was due to an increase in gene transcription or due to extranuclear effects .

The development of complementary DNA probes for cytochrome P450 genes and Northern blotting will allow the effects of cyclic AMP upon the levels of messenger RNA corresponding to the isozymes which are induced by cyclic AMP , to be determined , and so will give an indication of whether the effects of cyclic AMP are mediated by an increase in gene transcription or by stabilisation of post-transcriptional events .

Other workers , in attempting to elucidate the mechanisms by which the classic inducing agents mediate their effects , have proposed that induction of apoprotein occurs secondary to induction of ALA-synthase . The subsequent elevation of haem levels in the cytosolic haem pool has been postulated to induce cytochrome P450 apoproteins (Dwarki et al 1987 ; Satyabhama et al 1986 ; Jayarama-Bhat and Padmanaban 1988 ;) (section 1.9(c)) . The possibility exists , therefore , that the inductive effects of PKa upon cytochrome P450 apoprotein synthesis are not mediated directly by PKa but that induction of cytochrome P450 occurs secondary to the induction of ALA-synthase as has been proposed for the classic inducing agents above .

The possibility that the effects of cyclic AMP are mediated secondary to induction of ALA-synthase may be evaluated by using cobalt chloride which inhibits ALA-synthase as one of its actions (Drummond and Kappas 1982) (section 1.10(c)) .

(ii) Degradation .

The mechanisms of the degradative effects of PKa and PKc upon cytochrome P450 isozymes are unknown .

We did not determine the effects of supraphysiological concentrations of cyclic AMP (5 x 10^{-3}M) upon the phosphorylation states of cytochrome P450 isozymes because cyclic AMP-mediated alteration of the levels of the cytochrome P450

isozymes would have made comparison between control and treated groups difficult due to the limitations of the assay procedure used . This problem may be overcome in future studies by qualitative assessment of the levels of individual cytochrome P450 isozymes by Western blotting . Any increase in the phosphorylation of the cytochrome P450 isozyme mediated by supraphysiological concentrations of cyclic AMP may , therefore , be correlated to the level of that particular isozyme .

It is conceivable that although we did not detect any increase in the phosphorylation of cytochrome P450-2a (table 1) mediated by physiological concentrations of cyclic AMP (section 4.9) , phosphorylation may occur at these high concentrations of cyclic AMP . The subsequent conversion of cytochrome P450 to the corresponding cytochrome P420 (Taniguchi et al 1985) and degradation by haem oxygenase (Maines and Anders 1973b ; Kutty et al 1988) may , therefore , be the underlying mechanism mediating degradation of cytochrome P450 . It has been reported , however , that haem oxygenase is located predominantly in the reticuloendothelial cells of the liver (Guengerich 1977) and although carrier proteins have been postulated to convey cytochrome P450 (or the corresponding cytochrome P420) to the site of haem oxygenase in the intact liver (Maines and Anders 1973b) this cannot occur in the isolated rat hepatocyte model which we have utilised in our studies .

Another mechanism which has been postulated to mediate degradation of cytochrome P450 and which does not rely on the

postulated transportation of cytochrome P450/P420 to the reticuloendothelial cells , is by an increase in lipid peroxidation (Schacter et al 1973) . Lipid peroxidation is mediated by NADPH-cytochrome P450 reductase in the presence of NADPH and molecular oxygen . Subsequent increase in the generation of hydrogen peroxide in the vicinity of the haem mediates lipid peroxidation and degradation of cytochrome P450 (Guengerich 1977) (section 1.8(c)) . This is the mechanism by which carbon tetrachloride has been postulated to mediate its degradative effects upon cytochrome P450 (Davies et al 1985) . In addition , 3-methylcholanthrene , which has been postulated to mediate its inductive effects upon cytochrome P450 by elevation of cyclic AMP (Byus et al 1976) , also mediates degradation of cytochrome P450 by uncoupling the components of the electron transport chain which results in the accumulation of hydrogen peroxide (De Mattheis 1988) . PKa- and PKc-mediated degradation of cytochrome P450 , therefore , may also be due to increase in lipid peroxidation perhaps by uncoupling the electron transport chain and subsequent increase in hydrogen peroxide generation .

It is possible that phosphorylation of additional components of the cytochrome P450 electron transport chain e.g. NADPH-cytochrome P450 reductase , which has been reported to be weakly phosphorylated by both PKa (Pyerin et al 1983) and PKc (Pyerin et al 1987) , may lead to uncoupling of the electron transport chain , an increase in the production of hydrogen peroxide and degradation in this manner .

Cyclic AMP has also been postulated to mediate the effects of glucagon upon cellular autophagy i.e. lysosomal breakdown of intracellular proteins by proteolytic enzymes (Ballard 1980) . Phenobarbitone has been postulated to mediate its inductive effects upon cytochrome P450 isozymes by elevation of cyclic AMP (Byus et al 1976) and has also been reported to increase cellular autophagy . This effect has been proposed to mediate the rapid removal of the induced proteins following cessation of phenobarbitone treatment (Bolender and Weibel 1973) . Increase in the proteolytic breakdown of cytochrome P450 apoprotein , therefore , may be an alternative mechanism by which PKa and PKc may mediate the degradative effects of cyclic AMP . The effects of glucagon upon cellular autophagy , however , occur at physiological concentrations (Ballard 1980) and as discussed in section 4.6(a) , physiological concentrations of cyclic AMP do not appear to mediate any detectable degradation of cytochrome P450 . It seems unlikely , therefore , that an increase in the proteolytic breakdown of cytochrome P450 apoprotein would be a "first line" mechanism of PKa- and PKc-mediated degradation . It is conceivable , however , that some other mechanism of action of these supraphysiological concentrations of cyclic AMP may mediate a conformational change in the cytochrome P450 molecule which does not occur at physiological concentrations and which results in an increase in the susceptibility of the apoprotein to proteolytic breakdown . This effect has been reported for allylisopropylacetamide- and 3,5-dicarbethoxy-2,6-dimethyl -4-ethyl-1,4-dihydropyridine-mediated degradation of cytochrome

P450p (Bornheim et al 1987 ; Correia et al 1987) (section 1.10(a)) and is thought to occur subsequent to breakdown of haem by either haem oxygenase or lipid peroxidation in the physiological situation (Abraham et al 1988) (section 1.8(c)).

The assay procedure used to determine the whole cell content of cytochrome P450 did not enable us to determine if the degradation of cytochrome P450 was due to the proteolytic breakdown of the cytochrome P450 apoprotein or due to loss of haem from the apoprotein active site . The possible role of proteolytic breakdown of the apoprotein in the PKa- and PKc-mediated degradation of cytochrome P450 , therefore , remains to be elucidated .

4.7(c) Conclusion to section 4.7

Measurement of whole cell cytochrome P450 levels has shown that the effects of supraphysiological concentrations of cyclic AMP and the components which determine the overall effect are mediated , at least in part , by alteration in the levels of cytochrome P450 . It is proposed that the inductive effects of cyclic AMP are mediated directly by PKa and are relatively specific for steroid-metabolising cytochrome P450 isozymes . The degradative effects of supraphysiological concentrations of cyclic AMP appear to be less specific than the inductive effects and are mediated by a combination of PKa acting directly and secondary activation of PKc .

In addition to degradation of cytochrome P450 , cytochrome

P450 may also be inactivated by the same mechanism that mediates the inhibitory effects of physiological concentrations of cyclic AMP. The overall inhibitory effects of supraphysiological concentrations of cyclic AMP upon steroid metabolism , therefore , may be due to both degradation and reversible inactivation of cytochrome P450 .

The precise cellular mechanisms by which cyclic AMP mediates induction and degradation of cytochrome P450 have not been elucidated by this study .

4.8 OTHER POSSIBLE COMPONENTS MEDIATING THE EFFECTS OF SUPRAPHYSIOLOGICAL CONCENTRATIONS OF CYCLIC AMP .

It has been shown in previous sections that the effects of physiological concentrations of cyclic AMP upon steroid metabolising enzymes occur as a result of two components , namely, (a) PKa-mediated , protein synthesis-dependent stimulation and (b) PKc-mediated , protein synthesis-independent inhibition . The overall effect upon steroid metabolism appears to reflect the changes in the relative magnitudes of these two components with time .

We have also identified three components of the effects of supraphysiological concentrations of cyclic AMP upon steroid-metabolising enzymes . The overall effect of cyclic AMP and the separate components all reflect , at least in part , changes in the levels of whole cell cytochrome P450 and the three components are (a) PKa-mediated , protein synthesis-dependent induction and

concomitant stimulation of steroid metabolism , (b) PKa-mediated , protein synthesis-independent degradation of cytochrome P450 and concomitant inhibition of steroid metabolism and (c) PKc-mediated , protein synthesis-independent degradation of cytochrome P450 and concomitant inhibition of steroid metabolism . It is apparent from figure 34 , however , that the overall effects of supraphysiological concentrations of cyclic AMP upon both steroid metabolism and cytochrome P450 levels do not appear to be fully explained numerically by these three components and this may imply that one or more , as yet unidentified , components are necessary to explain the overall effect .

One possible discrepancy which arises when assessing the effects of supraphysiological concentrations of cyclic AMP upon steroid-metabolising enzyme activity is that the early PKa-mediated , protein synthesis-dependent stimulation of enzyme activity , unmasked by inhibiting PKc activation with K-252b , is of too transient a duration to account for the pronounced secondary stimulatory effects of 5 x 10^{-3}M cyclic AMP in the control cyclic AMP time course . This discrepancy is more apparent when assessing effects upon cytochrome P450 levels and the PKa-mediated , protein-synthesis-dependent induction of cytochrome P450 is too weak to mediate any overall increase in whole cell cytochrome P450 levels whereas 5 x 10^{-3}M cyclic AMP mediates a marked increase in the levels of cytochrome P450 in the control cyclic AMP time course .

It is possible that this discrepancy may be due either to

another unidentified component , which we propose to be PKc- and protein synthesis-dependent , mediating the secondary stimulatory effects , or that the PKa-mediated , protein synthesis-dependent stimulatory effects upon steroid metabolism are prematurely terminated in the presence of K-252b .

As discussed previously , phenobarbitone has been reported to stimulate cellular autophagy and one of the mechanism by which phenobarbitone has been postulated to produce this effect is by mediating general proliferation of liver membranes and then this increase in membrane proteins triggers cellular autophagy (Bolender and Weibel 1973) . The effects of phenobarbitone upon cellular autophagy , therefore , appear not to be due to a direct effect of phenobarbitone itself but to be due to some secondary cellular event . Cyclic AMP has been reported to mediate the autophagic effects of glucagon (Ballard 1980) and to mediate the effects of phenobarbitone upon cytochrome P450 isozymes (Byus et al 1976) and it may be that when cyclic AMP-mediated inhibition of enzyme activity is partially blocked by the presence of K-252b the early increase in steroid-metabolising enzyme activity , which we have postulated to be due to early induction of cytochrome P450 , triggers cellular autophagy in an analogous manner to phenobarbitone . This increase in cellular autophagy may result in premature termination of the increase in steroid-metabolising enzyme activity due to proteolytic breakdown of cytochrome P450 . We proposed in section 4.7(a)(iv) , however , that these early stimulatory effects of cyclic AMP , unmasked by

inhibition of the PKc-mediated inhibitory effects are due to selective induction of steroid-metabolising cytochrome P450 isozymes and we could not detect any increase in whole cell cytochrome P450 levels which correlated with the early stimulation of enzyme activity . It seems unlikely , therefore , that the premature termination of enzyme activity occurs as a result of selective increase in the levels of steroid-metabolising cytochrome P450 isozymes triggering cellular autophagy .

The possibility exists that the secondary stimulatory effects of supraphysiological concentrations of cyclic AMP upon cytochrome P450 levels and subsequent stimulation of steroid-metabolising enzyme activity are due to another component which is PKc- and protein synthesis-dependent and may be due to PKc-mediated stimulation of gene transcription or extranuclear effect in an analogous manner to that proposed for PKa (section 4.7(b)(i)) . However , although Allan and Skett (1988) reported that direct activation of PKc by PMA and 1,2-dioleoylglycerol mediated inhibition of steroid-metabolising enzyme activity , they did not report any secondary stimulatory effects upon enzyme activity . In addition , Hussin et al (1988) examined the effects of a range of concentrations of glucagon and TH-glucagon upon steroid-metabolising enzyme activity in isolated rat hepatocytes and showed that physiological concentrations of glucagon inhibited enzyme activity but that this effect of glucagon was lost at supraphysiological concentrations . TH-glucagon had an analogous inhibitory effect upon enzyme activity at concentrations

corresponding to physiological concentrations of glucagon but this effect was not diminished as the concentration increased . As TH-glucagon stimulates hydrolysis of inositol phospholipids but has no effect upon cyclic AMP generation , it was postulated that glucagon mediates its inhibitory effects upon steroid metabolism by stimulation of PIP_2 hydrolysis and subsequent PKc activation . The results of this present study suggest that the loss of enzyme inhibition at supraphysiological concentrations of glucagon are due to the high concentrations of cyclic AMP generated by the glucagon mediating stimulatory effects upon steroid metabolism which opposes the inhibitory effects . The absence of this effect in the TH-glucagon treated cells implies that PKc activation does not mediate any stimulatory effects upon steroid-metabolising enzyme activity even at these very high concentrations .

These results imply that PKc alone is unable to mediate the secondary stimulatory effects of cyclic AMP upon steroid-metabolising enzymes . They may indicate , however , that both PKa and PKc are necessary for the sustained stimulatory effects upon steroid-metabolising enzymes and correspondingly upon the induction of cytochrome P450 and that neither of these protein kinases is capable of mediating sustained induction by itself .

It may be possible to differentiate between these two postulated mechanisms by comparing the time course of the effect of cyclic AMP in the presence of K-252b upon protein synthesis with the time course of the effect of the same combination on cytochrome P450 levels . If the time course of the effect of

cyclic AMP upon messenger RNAs are also prematurely terminated in the presence of K-252b this would imply that PKc activation is necessary for sustained induction of cytochrome P450 . However , if the effect of cyclic AMP upon messenger RNA remains stable throughout the time course whilst the effects of cyclic AMP upon cytochrome P450 levels are suppressed then this would imply that some additional effect , not involving termination of protein synthesis , was mediating isozyme degradation perhaps mediated by an increase in proteolysis .

4.9 CYTOCHROME P450-INDEPENDENT ENZYMES .

The mechanisms of action of the separate components proposed to mediate the actions of supraphysiological concentrations of cyclic AMP upon steroid-metabolising enzyme activity , have been examined and discussed only with regards to cytochrome P450-dependent enzymes . The mechanisms which mediate the analogous effects of supraphysiological concentrations of cyclic AMP upon the activities of the non-cytochrome P450-dependent enzymes , 5α reductase and 17α/β hydroxysteroid dehydrogenase , have not been examined . The mechanism of action of cyclic AMP control over the activities of these enzymes and the possibility that the activities of these enzymes also reflect changes in the levels of constitutive enzymes , therefore , have not been elucidated .

4.10 PHYSIOLOGICAL IMPLICATIONS .

4.10(a) Hormonal control of hepatic monooxygenase activity .

We have shown in this study that physiological concentrations of cyclic AMP have a concentration-dependent inhibitory effect upon hepatic monooxygenases in isolated rat hepatocytes . The *in vivo* inhibitory effects of adrenaline (Fouts 1962 ; Kato and Gillette 1965a) (section 1.11(l)) , glucagon (Weiner et al 1972a) (section 1.11(k)) and thyroxine (Kato and Gillette 1965a ; Kato et al 1970) (section 1.11(h)) , therefore , are all likely to be mediated , at least in part , by elevation of hepatic cyclic AMP levels either by direct receptor-mediated mechanisms in the case of adrenaline (Exton et al 1971) (section 1.16(b)) and glucagon (Exton et al 1971) (section 1.16(c)) or by indirectly altering the sensitivity of the hepatocytes to the cyclic AMP-elevating effects of glucagon and catecholamines in the case of thyroxine (Müller and Seitz 1987) (section 1.16(g)) . Similarly the hormone deficiency states , diabetes (Kato and Gillette 1965a ; Kato et al 1971 ; Weiner et al 1972 ; Reinke et al 1978) (section 1.11(i)) and adrenalectomy (Kato and Gillette 1965a ; Kato et al 1971) (section 1.11(j)) , may also mediate inhibitory effects upon hepatic monooxygenases by elevating hepatic cyclic AMP levels . This occurs by derepression of the inhibitory effects of their respective hormones upon either intracellular cyclic AMP levels and effects in the case of diabetes (Heyworth et al 1985 ;

O'Brien et al 1987 ; Malchoff et al 1987 ; Thomas et al 1985) (section 1.16(d)) or β_2 adrenergic receptor responsiveness in the case of adrenalectomy (Studer and Borle 1984 ; Kunos and Ishac 1987 ; Kunos et al 1984) (section 1.16(f)) .

In this present study we have not examined the possibility that a decrease in the intracellular levels of cyclic AMP has the reverse effect upon the activities of steroid-metabolising enzymes i.e. stimulation . It is conceivable , however , that the stimulation of hepatic steroid metabolism that occurs $\frac{1}{2}$ hour after the addition of physiological concentrations of insulin to isolated rat hepatocytes (Hussin and Skett 1987) is also due , at least in part , to a decrease in the intracellular concentration of cyclic AMP (Heyworth et al 1985 ; O'Brien et al 1987 ; (section 1.16(d)) .

4.10(b) Protein kinase c as a mediator of the effects of cyclic AMP .

One of the most interesting findings of this present study has been that , although the cyclic AMP-mediated inhibition of hepatic monooxygenase activity is attributable to cyclic AMP-mediated activation of PKa , PKa appears to have no direct inhibitory effects upon hepatic monooxygenases but inhibitory effects appear to be due to secondary activation of PKc . This interaction between the two protein kinases may represent a novel mode of intracellular regulation because , although many interactions exists between the cyclic AMP-generating and inositol

phospholipid-hydrolysing pathways (section 1.14(b)) , activation of PKc by PKa has not previously been reported to be a mechanism by which cyclic AMP mediates its physiological effects .

One of the mechanisms by which this interaction may occur is by PKa-mediated stimulation of inositol phospholipid hydrolysis . However , reports on the ability of PKa to stimulate inositol phospholipid hydrolysis in isolated rat hepatocytes vary . PKa has been reported to stimulate PIP_2 hydrolysis by phosphorylating and subsequently stimulating the activity of the putative GTP-binding protein linking the hormone receptor to phospholipase c (section 1.12(c)) (Blackmore and Exton 1986) . These workers , however , were attempting to elucidate the mechanisms by which glucagon elevates intracellular calcium levels and the effects of PKa-mediated PIP_2 hydrolysis upon activation of PKc were not examined . In contradiction to the above report , Kaibuchi et al (1982) reported that cyclic AMP had no effect upon hydrolysis of inositol phospholipids in hepatocytes and O'Shea et al (1987) reported that cyclic AMP inhibits hormonal stimulation of PIP_2 hydrolysis by inhibition of PI kinase (section 1.12(c)) and depletion of PIP_2 in the plasma membrane .

In this present study we have shown that the inhibitory effects of adrenaline upon steroid-metabolising enzyme activity are mediated only by activity at the β_2 adrenergic receptor and that adrenaline activity at α_1 adrenergic receptors , which are directly linked to hydrolysis of inositol phospholipids , has no role to play in the regulation of steroid-metabolising enzymes .

This finding implies that PKa-mediated activation of PKc is not mediated by stimulation of hydrolysis of inositol phospholipids .

In contradiction to the mechanisms proposed for adrenaline in this present study , Hussin et al (1988) reported that the inhibitory effects of physiological concentrations of glucagon upon steroid metabolism could be mimicked by TH-glucagon which stimulates PIP_2 hydrolysis but has no effect upon cyclic AMP levels . It was postulated , therefore , that the inhibitory effects of glucagon upon hepatic monooxygenase activity were mediated by stimulation of inositol phospholipid hydrolysis and not by cyclic AMP generation . This finding implies that glucagon-mediated stimulation of PIP_2 hydrolysis can result in sufficient activation of PKc to mediate the inhibitory effects of the hormone upon steroid metabolism . PKa may be acting in a similar way , therefore , to stimulate inositol phospholipid hydrolysis and activate PKc which then mediates subsequent inhibition of steroid-metabolising enzyme activity .

In view of these contradictory reports , the possibility that PKa-mediated activation of PKc is mediated via stimulation of inositol phospholipid hydrolysis still remains to be clarified. Similarly the possibility that <u>in vivo</u> hormonally-mediated inhibition of enzyme activity proceeds , at least in part , by direct stimulation of inositol phospholipid hydrolysis , as has been postulated for glucagon , cannot be overlooked .

An alternative mechanism by which PKa may activate PKc is via direct interaction between the two protein kinases , presumably involving phosphorylation by PKa . Such a positive interaction has not previously been reported but interaction between the two protein kinases has been reported to occur in skeletal muscle myoblasts which results in PKa-mediated inhibition of PKc (Narindrasorasak et al 1987) . In the GH_4 pituitary cell line cyclic AMP has been postulated to mediate some of its nuclear effects by stimulating the activity of a calcium-dependent protein kinase (Waterman et al 1985) . Although the identity of this protein kinase and the mechanisms by which cyclic AMP may stimulate its activity are unclear , the possibility exists that this represents another example of PKa-mediated activation of a second protein kinase , which may or may not be PKc , and which mediates a physiological effect of cyclic AMP .

The possibility that PKa mediates direct activation of PKc in hepatocytes remains to be clarified .

4.10(c) Sex differences in hepatic monooxygenase activity and the response of these enzymes to hormones .

Another interesting finding from this present study is that cyclic AMP has no effect upon the sexually differentiated enzyme profiles from either male or female rats and had a non-specific inhibitory effect upon all steroid-metabolising enzymes in rats regardless of sex . The inability of cyclic AMP to feminise male

steroid-metabolising enzymes may indicate that alteration of cyclic AMP levels or alteration of the levels of other intracellular mediators that directly activate PKc , are not mechanisms by which the postulated in vivo feminising factor , now thought to be growth hormone (Mode et al 1983 ; Kramer and Colby 1976) (sections 1.11(f) and 1.11(g)) , mediates its effects . Growth hormone , however , has been reported to have no effect upon steroid-metabolising enzymes when added directly to isolated rat hepatocytes cultured under the same conditions as those used in this study (P. Gulati : personal communication) . It appears, therefore , that feminisation of hepatic monooxygenases is a much more complicated process than first thought and may involve many components of the endocrine system . It is conceivable , therefore , that although we have detected no feminisation of enzyme activity in this present study , one of the additional unidentified hormonal components which has been shown to be necessary for the feminising effect of growth hormone , may be mediating effects by elevation of cyclic AMP and inhibition of the activities of 6β hydroxylase and 16α hydroxylase . Protection against the inhibitory effects of cyclic AMP upon 7α hydroxylase and 17α/β hydroxysteroid dehydrogenase and stimulation of the activity of 5α reductase , however , may be due to yet another hormonal factor .

The identical non-specific inhibitory effect of cyclic AMP upon steroid-metabolising hepatic monooxygenases in rats regardless of sex also differs markedly from the findings of in

vivo studies which compared the effects of hormones postulated to mediate their effects , at least in part , by alteration of cyclic AMP in male and female rats . In these studies female hepatic monooxygenases were reported to be refractory to the in vivo inhibitory effects of thyroxine (Kato and Gillette 1965a ; Kato et al 1970) (section 1.11(h)) , diabetes (Kato and Gillette 1965a ; Kato et al 1971) (section 1.11(i)) , adrenalectomy (Kato and Gillette 1965a ; Kato et al 1971) (section 1.11(j)) and adrenaline (Kato and Gillette 1965a) . The inhibitory effects of "in vivo" administration of dibutyryl cyclic AMP also do not occur in the female rat (Ross et al 1973) . This implies that the refractoriness is due to some extracellular effect which does not involve a decrease in hormonally-mediated generation of intracellular cyclic AMP in the female rat and may be due to some hormonal influence which is absent in this present study .

It is conceivable that the hormonal factor which mediates the proposed protective effect against the inhibitory effects of cyclic AMP and/or PKc activation in the female rat is identical to that which we have postulated to mediated selective protection against the inhibitory effects of cyclic AMP upon 7α hydroxylase, 17α/β hydroxysteroid dehydrogenase and 5α reductase in feminising male hepatic monooxygenases .

The nature of this postulated hormonal factor is unknown. Kato et al (1971) , however , reported that the inhibitory effects of adrenalectomy and diabetes upon hepatic monooxygenase activity in the male rat did not occur in castrated male rats .

This implies that one of the unidentified factors may be testosterone and its absence in the female may confer the protective effect . If testosterone is postulated to be the missing factor , however , its absence in the isolated rat hepatocyte model used in this present study would result in refractoriness of hepatic monooxygenases to the inhibitory effects of cyclic AMP in hepatocytes from both male and female rats and we have shown that this does not occur . The loss of the protective effect in hepatocytes isolated from female rats indicates that the protective effect is conferred by a hormonal factor which is present in the intact female but absent in the intact male and this differs from the role postulated for testosterone above .

One obvious possibility is that the identity of the missing factor is oestrogen . Oestrogen , however , is thought to have little effect upon hepatic monooxygenase activity at the level of the liver (Gustafsson and Stenberg 1976) and hepatic oestrogen receptors are thought to be non-functional at physiological concentrations of the hormone (Lax 1987) . It appears unlikely , therefore , that the postulated missing hormonal factor is of ovarian origin although Ross and Oppelt (1973) reported that pretreatment of castrated male rats with oestradiol reduced the inhibitory effects of dibutyryl cyclic AMP upon hepatic monooxygenase activity in comparison to the inhibitory effects of the cyclic AMP analogue in non-pretreated castrates .

It has recently been reported that the combination of growth hormone , thyroxine and a glucocorticosteroid (dexamethasone) when added directly to cultures of hepatocytes isolated from male

rats mediated feminisation of steroid-metabolising enzymes (P. Gulati : personal communication) . It is conceivable , therefore , that the identity of the missing hormonal factor is one or a combination of these three hormones .

The hormonal influences mediating "in vivo" refractoriness of female hepatic monooxygenases to the inhibitory effects of cyclic AMP and those mediating "in vivo" feminisation of male hepatic enzyme profiles in addition to a possible role of cyclic AMP , PKa activation and PKc activation in the production of the latter, remain to be elucidated .

4.11 PHARMACOLOGICAL IMPLICATIONS .

In 1976 it was postulated that the inductive effects of Arochlor-1254 (Costa et al 1976) , 3-methylcholanthrene and phenobarbitone (Byus et al 1976) were mediated by elevation of cyclic AMP and/or activation of PKa . The results of this present study have indicated that supraphysiological concentrations of cyclic AMP are able to mediate stimulation of steroid-metabolising enzyme activity by induction of cytochrome P450 and so correlate with the above report that the effects of the classic inducing agents may be mediated by elevation of cyclic AMP and subsequent activation of PKa . The concentrations of hepatic cyclic AMP generated by in vivo administration of phenobarbitone and 3-methylcholanthrene , however , were reported to be only 150% to 200% of corresponding control levels (Byus et al 1976) and

these levels differ markedly from the concentrations of cyclic AMP which we have found necessary to mediate induction of cytochrome P450 and associated stimulation of steroid metabolism in this present study .

Despite these discrepancies in concentrations , however , the effects of supraphysiological concentrations of cyclic AMP also exhibit other similarities with those of the classic inducing agents . Both phenobarbitone and 3-methylcholanthrene have been reported to increase the degradation of cytochrome P450 either by induction of haem oxygenase (Lincoln et al 1988 ; Shedlovsky et al 1987) or by uncoupling the cytochrome P450 electron transport chain which mediates degradation of cytochrome P450 by increasing peroxide generation (De Mattheis 1988) . The ultimate result of increase in degradation of cytochrome P450 by either of these two mechanisms in conjunction with induction of specific cytochrome P450 isozymes is an increase in the rate of turnover of cytochrome P450 (Sadano and Omura 1983) (section 1.9(d)) . We have noted a similar combination of inhibitory and stimulatory effects upon steroid-metabolising enzymes and cytochrome P450 levels mediated by supraphysiological concentrations of cyclic AMP in this present study .

One discrepancy which does arise between the effects of high concentrations of cyclic AMP and the effects of phenobarbitone and 3-methylcholanthrene , however , is that no inhibitory effects of low concentrations of either phenobarbitone or 3-methylcholanthrene have been reported which would be expected if the effects of these inducing agents were mediated only by

elevation of cyclic AMP . In an early study by Kato et al (1964) , however , a time-dependent biphasic effect upon the metabolism of xenobiotics was reported for a wide range of different inducing agents which would be expected if cyclic AMP was mediating the effects of these agents . In addition , low doses of phenobarbitone have been reported to have different effects in male and female rats in that females appeared to be refractory to the inductive effects of low doses of phenobarbitone whereas higher doses of phenobarbitone had the same inductive effect in rats of both sexes (Shapiro 1986) . This may indicate that low doses of phenobarbitone mediate different effects to high doses . More recently it has been reported that phenobarbitone inhibits PKc activation in rat brain (Chauhan and Brockerhoff 1986) and so a possible explanation for the lack of an inhibitory effect of phenobarbitone upon hepatic monooxygenases at low concentrations is that the PKc-dependent inhibitory effects are blocked by the presence of phenobarbitone and so only PKa-mediated induction occurs .

It is possible , therefore , that the effects of phenobarbitone , 3-methylcholanthrene and Arochlor-1254 upon hepatic monooxygenases are mediated , at least in part , by elevation of cyclic AMP and activation of PKa and perhaps also by concomitant inhibition of PKc . It will be necessary to examine the effects of phenobarbitone , 3-methylcholanthrene and Arochlor-1254 upon the levels of cyclic AMP in isolated rat hepatocytes and to determine if the inductive effects of these agents are inhibited by KT5720 (PKa inhibitor) to verify this

proposal . In addition the fact that these agents induce different cytochrome P450 isozymes indicates that the inducing agents must have some additional cyclic AMP-independent effects which confer isozyme specificity .

4.12 GENERAL CONCLUSIONS .

We have shown by both indirect methods in intact hepatocytes and by direct methods in electropermeabilised hepatocytes that cyclic AMP has a concentration- and time-dependent biphasic effect upon steroid-metabolising enzymes . The overall effect produced is the sum of the effects of a number of stimulatory and inhibitory components mediated either by PKa directly or by secondary activation of PKc by PKa . The changes in the relative magnitudes of these components with time results in the biphasic nature of the response .

Low (physiological) concentrations of cyclic AMP mediate predominant inhibition of steroid-metabolising enzymes and these concentrations correlate with those produced by hormones which have an inhibitory effect upon hepatic monooxygenases *in vivo* .

High (supraphysiological) concentrations of cyclic AMP mediate predominant stimulation of steroid-metabolising enzyme activity which we have shown may be due to induction of cytochrome P450 . Elevation of cyclic AMP , therefore , may be one mechanism by which phenobarbitone , 3-methylcholanthrene and Arochlor-1254

mediate their inductive effects upon hepatic monooxygenases *in vivo*.

It is proposed , therefore , that elevation of cyclic AMP has a role in both the hormonally-mediated inhibition of hepatic monooxygenase activity and in the actions of xenobiotics in hepatic monooxygenase induction .

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