THE ROLE OF INSULIN AND GLUCAGON IN THE REGULATION OF HEPATIC DRUG AND STEROID METABOLISM

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ABAS BIN HAJI HUSSIN B. Pharm., M.Sc.

Department of Pharmacology,
The University.
Glasgow.

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ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

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CONTENTS

SUMMARY

Diabetes mellitus is known to affect drug and steroid metabolism in the rat liver. It was first reported in 1961 by Dixon et.al. that chemically-induced diabetes mellitus produces changes in hepatic microsomal drug metabolism. These results were later confirmed by other workers (Kato et.al., 1971; Weiner et.al., 1972; Warren et.al., 1983). Reports from various studies have indicated that streptozotocin-induced diabetes mellitus can also influence hepatic steroid metabolism in the rat (Subbiah and Yunker, 1984; Skett, 1986). The effect of diabetes on hepatic drug and steroid metabolism can be reversed by treating the diabetic animals with insulin. Drug and steroid metabolism by the rat liver is known to be sex-dependent (Kato, 1974) and the effect of diabetes mellitus has been shown also to be sex-dependent with regard to drug and steroid metabolism (Skett and Joels, 1985; Skett, 1986). It is well recognized that glucagon can influence drug metabolism *in-vivo* (Weiner et.al., 1972). Since hyperglucagonaemia is usually present in non-insulin-dependent diabetes mellitus, this hormonal excess has been implicated in the overall pathophysiology of the disease (Unger and Orci, 1981). However, up to date, no study has been conducted to investigate the effect of insulin and glucagon on drug and steroid metabolism in a cell culture system. This is necessary bearing in mind the extensive interactions of hormones within the body making it difficult to assign the effects seen to any particular hormones in an *in-vivo* study. The object of this thesis is to ascertain the role of insulin and glucagon in the regulation of steroid metabolism in the rat liver.

Most of the previous work performed to observe the effect of diabetes on steroid and drug metabolism was done on liver microsomes prepared from treated animals. As described above, it is difficult to ascribe the effect of diabetes on hepatic steroid metabolism to a single action of insulin or glucagon in an *in-vivo* study. The use of isolated hepatocytes is essential to examine the effect of one hormone alone.

In order to achieve our objective, we had to develop a cell culture system which is hormone- and serum-free. We have developed and characterized four different types of culture medium containing animal serum, synthetic multihormone serum substitute (Ultroser G, LKB) or bovine serum albumin only. When the liver cells were cultured in basic Ham's F-10 culture medium supplemented with foetal calf and horse serum or with Ultroser G, the steroid enzyme activities were reduced to less than 50 % of control on day 3 of culture in normal male rat hepatocytes. We discovered that the steroid enzyme activities were best maintained in Ham's F-10 culture medium supplemented with 0.1 % bovine serum albumin only. The basal level of enzyme activities was maintained for at least 3 days in culture. We, therefore, have a hepatocyte culture method to assess the effects of insulin and glucagon on hepatic steroid metabolism in serum- and hormone-free, chemically defined medium. Only male rats were used throughout the project because previous study had shown that the effect of diabetes on hepatic steroid metabolism is only seen in the male rat (Skett, 1986). The substrate chosen for the investigation is androst-4-ene-3,17-dione because its metabolites are well defined and easily separated and the labelled and unlabelled substrate are easily available. The enzymes involved in its metabolism have been shown to be both cytochrome P-450-dependent and -independent and sex-specific. Moreover, insulin has been demonstrated to affect its metabolism *in-vivo* (Skett, 1986).

The effect of insulin on androst-4-ene-3,17-dione metabolism in normal rat hepatocytes over the period studied was characterized by the presence of two peaks of increased activity at 1/2 and 24 hours. Importantly, these effects of insulin are clearly within the physiological range.The dose-response curves at 1/2 and 24 hour insulin preincubation suggest that these two peaks are probably generated by different mechanisms. Biochemical studies performed indicated that there is no correlation between the increase in enzyme activities by insulin at 1/2 hour and changes in cyclic AMP, cytochrome P-450 concentrations or phosphoinositide hydrolysis. It seems that insulin's

effect on androst-4-ene-3,17-dione metabolism at 1/2 and 24 hour involves a phosphorylation reaction since the protein kinase inhibitor, K-252a, completely inhibited the effect of insulin. However, the exact molecular mechanism of action is yet to be determined. Our results did not show any selective changes in the male-specific and female-specific enzyme activities which is in contrast to the result found *in-vivo* (Skett, 1986). This is probably attributed to the continuous exposure of the parenchymal liver cells to many different hormones *in-vivo,* which is absent in our system. The data obtained suggest that insulin has a direct effect on hepatic steroid metabolism and the hormone, *in-vitro*, act as a general stimulator of the enzymes in the liver which metabolize androst-4-ene-3,17-dione.

Since STZ-induced diabetes has been shown to alter phase 1 drug metabolism (Kato, 1974) and steroid and drug metabolism share the same metabolizing enzyme system (Skett et.al., 1984) it is of interest to investigate whether steroid metabolism is similarly affected by STZ-induced diabetes. Hepatocytes from acutely (3 days) STZ-treated diabetic rats were resistant, with respect to androst-4-ene-3,17-dione metabolism, to the effect of insulin addition *in-vitro* and the responsiveness was partly restored by treating the diabetic animals with insulin. Hepatocytes from chronically (21 days) STZ-diabetic rats responded differently; the overall effect of chronic diabetes is much less than that seen in the acutely STZ-treated animals. This result is strikingly similar to that observed for microsomal drug metabolism (Skett and Joels, 1985; Dixon et.al., 1963). Preincubation of hepatocytes from 3 days STZ-diabetic rat with insulin for up to 1 hour still resulted in the absence of insulin responsiveness relative to control. However, in the 21 days diabetic rat, a small but significant increase in enzyme activities was observed after 2 hours of insulin preincubation. Thus, the effect of diabetes mellitus on phase 1 steroid metabolism is time-dependent.

As explained above, glucagon has also been found to affect drug metabolism (Weiner et.al., 1972). In normal rat hepatocytes, the effect of glucagon on androst-4-

ene-3,17-dione metabolism is time-and concentration-dependent. The effects began to be manifested at 1/2 hour of preincubation. Glucagon at 1/2 and 24 hour preincubation decreases all the enzyme activities at physiological concentrations (10^{-10} -10^{-8} M). Higher concentrations are less effective. Thus, glucagon at 1/2 and 24 hours preincubation exhibited V-shaped dose-response curves. Recently, Wakelam et.al. (1986), with the aid of TH-glucagon, a glucagon antagonist, unravelled a new hypothesis on the mechanism of action of glucagon in the liver. They suggested that the effects of glucagon in the liver are mediated by its interaction with two distinct receptors, GR-1 (a receptor coupled to the stimulation of inositol phospholipid breakdown) and GR-2 (a receptor coupled to the stimulation of adenylate cyclase activity). The dose-dependent effect of TH-glucagon on androst-4-ene-3,17-dione, in analogy to the effect of TH-glucagon on inositol phosphate turnover, suggests that the effect of glucagon on androst-4-ene-3,17-dione metabolism is mediated via the GR-1 receptor linked to phosphatidylinositol-4,5 phosphate turnover. The ability of 4β-phorbol-12β-myristate-13α-acetate, a known activator of protein kinase C, but not A 23187, a calcium ionophore, to mimic the effect of glucagon and TH-glucagon on the enzyme activity suggests that glucagon's effect is to activate protein kinase C via the generation of diacylglycerol. Similar to insulin, preincubation with glucagon for 1/2 hour in the presence of K-252a resulted in the abolition of the above effect suggesting that glucagon's effect on the steroid metabolism is mediated by phosphorylation.

In the diabetic rat hepatocytes, glucagon no longer exhibits the V-shaped dose-response curves. The second, inhibitory phase was abolished, probably due to a defect in the adenylate cyclase system coupled to the GR-2 glucagon receptor.

The pattern of plasma hormone levels and the interaction of more than one hormone may be physiologically important for hormone action. We, therefore, tested the effect of insulin and glucagon by adding them together in various combinations. When different concentrations of insulin and glucagon are added together, selective changes in

the activity of the male-specific and female-specific enzymes are observed. This is a first indication of the possible importance of hormonal interaction in expressing sexual differences in steroid metabolism.

Since biguanides and sulphonylureas are the most commonly prescribe hypoglycaemic agents for the treatment of diabetes mellitus, we have decided to investigate whether any of these group of anti-diabetic agents have any influence in the regulation of steroid metabolism. We have chosen phenformin and tolbutamide as representatives of biguanides and sulphonylureas respectively.

In normal rat hepatocytes, phenformin was shown to exert a direct effect on the liver in elevating androst-4-ene-3,17-dione metabolism. It is able to mimic the effect of insulin in normal and diabetic rats although there was a reduction in responsiveness and sensitivity in the latter indicating the existence of receptor and post-receptor defects. In normal rat hepatocytes, phenformin caused a dose-dependent increase in androst-4-ene-3,17-dione metabolism with maximum response observed at 5×10^{-5} M concentration. Physiological insulin concentration (10^{-9} M) potentiated the effect of phenformin on almost all of the enzyme activities.

Tolbutamide has the same effect as phenformin in normal rat hepatocytes. It elicited a dose-dependent increase in androst-4-ene-3,17-dione metabolism with maximum response attained at 10^{-4} M concentration. In the diabetic rat, tolbutamide was still able to increase all the enzyme activities despite a reduction in responsiveness. However, unlike phenformin, tolbutamide reduced the responsiveness of the liver cells towards insulin.

In conclusion, both insulin and glucagon have been shown to have a direct effect on the liver in regulating androst-4-ene-3,17-dione metabolism in the rat. This together with the partial restoration of insulin responsiveness in insulin treated streptozotocin-diabetic rats support our hypothesis that both hormones, insulin and glucagon, are major factors in the regulation of steroid metabolism in the rat liver.

However, the exact molecular mechanism of how insulin mediates its action here is far from being solved. A more thorough investigation of the second messenger system and especially of the inositol phosphate-glycan, is needed to unravel the mystery of how insulin acts on hepatic steroid metabolism.

LIST OF TABLES

LIST OF FIGURES

Page

(contd.)

INTRODUCTION

1.0 HISTORICAL BACKGROUND : CYTOCHROME P-450

The first reported study on cytochrome P-450 was done by Williams in 1955 at the Johnson Foundation for Medical Physics, University of Pennsylvania. Using the double-beam recording spectrophotometer he noticed the appearance of a broad but intense absorption band at 450 nm upon bubbling carbon monoxide into a dithionite-reduced rat liver microsomal suspension. Although this novel observation was not published, it was reported by Klingenberg (1958) who was working in the same laboratory as Williams. However, it was only after four years that Omura and Sato (1962) published a preliminary account of their studies on this pigment which was a new b-type cytochrome, in which they presented conclusive spectral evidence for its hemeprotein nature and proposed the name ' P-450 '. The discovery of its physiological function as a principal enzyme responsible for the hydroxylation or oxidative dealkylation of various drugs by liver microsomes (Cooper et.al., 1965) provided a great impetus into the research of this newly discovered hemeprotein.

The liver is the major site of the biotransformation of foreign compounds into more polar derivatives and the enzymes effecting many of these transformations were suggested to be located in the endoplasmic reticulum (Brodie et.al., 1955) of the parenchymal cells. Although first discovered in the rat liver, it was later demonstrated to exist in the mitochondria of the adrenal gland (Harding et.al., 1964) and in the kidney, lung and intestine (Paine, 1981). Recently, it was demonstrated that the non-parenchymal cells of the liver also have the capacity, though lower than the parenchymal cells, to metabolize a wide range of drugs and chemicals (Steinberg et.al., 1987). In the later half of the 1960's, successive reports on the discovery of this hemeprotein (molecular weight 45,000-60,000) in various forms of life, demonstrated that the distribution of cytochrome P-450 among living creatures was much wider than initially

thought. The presence of cytochrome P-450 in the yeast, *Saccharomyces cerevisiae*, was described by Lindenmayer and Smith (1964). Subsequently, the discoveries of cytochrome P-450 in several species of bacteria (Dus et.al., 1970), plants (Murphy and West, 1970) and insects (Morello et.al., 1971) have been reported.

Interest in the research on this hemeprotein was shared by pharmacologists, endocrinologists, toxicologists and biochemists because its discovered functions included some of the most important metabolic reactions in their respective research fields. Cytochromes P-450 are needed for the synthesis of prostaglandins and steroid hormones (Kupfer, 1980) and are responsible for the oxidative conversion of many foreign compounds such as drugs, pesticides, carcinogens and environmental pollutants. These foreign compounds are generally converted into a form that can be more readily excreted from the body. However, some compounds, which are themselves inert, are metabolized to form potential carcinogenic by-products and sometimes result in the formation of pharmacologically active end-products.

The capacity to metabolize such a broad spectrum of substrates is in part due to the existence of many forms of cytochrome P-450, which exhibit unique but overlapping substrate specificities (Lu and West, 1980). The use of different inducers such as phenobarbital, 3-methylcholanthrene and pregnenolone 16α-carbonitrile, to manipulate the biochemical and biophysical properties of the microsomal hydroxylation system has played a major role in establishing the existence of multiple forms of cytochrome P-450. The finding of the successful stabilization of cytochrome P-450 by glycerol (Ichikawa and Yamano, 1967) permitted solubilization and purification experiments on cytochrome P-450. Recent techniques have helped in the isolation and purification of a number of cytochrome P-450 isoenzymes such as cyt. P-450 15β-hydroxylase (MacGeoch et.al., 1984), cyt. P-450 16α-hydroxylase (Morgan et.al., 1985a) and cholesterol 7α-hydroxylase (Ogishima et.al., 1987).

Many factors have been shown to affect the metabolism of xenobiotics and these have been reviewed in detail by others (Paine, 1981; Gibson and Skett, 1986) and amongst these factors are hormones. The many alteration in drug and steroid metabolism have been attributed to the imbalance of the hormones of the endocrine system as a result of endocrinological disorders. One of the most widely researched endocrine organ, whose secretions affect xenobiotic metabolism, is the pancreas. At least 4 peptides with hormonal activity are secreted by the islets of Langerhans in the pancreas, two of these hormones are insulin and glucagon. Absolute or relative deficiency of insulin results in a disease called diabetes mellitus. Numerous studies have demonstrated the influence of diabetes mellitus on xenobiotic metabolism in experimental animals and this aspect of metabolism will be discussed in detail in the next section.

1.1 THE EFFECT OF DIABETES MELLITUS ON XENOBIOTIC AND STEROID METABOLISM

The first reported effect of diabetes mellitus on xenobiotic metabolism was made in 1961 when Dixon and co-workers demonstrated an alteration in drug metabolism in the rat after acute treatment with the β-cell toxin, alloxan. Livers from diabetic animals were able to metabolize hexobarbital, codeine and chlorpromazine at only a fraction of the normal rate. The metabolism of the drugs returned to normal 24 hours after treatment of the animals with insulin. As the effect on drug metabolism was usually accompanied by a decreased in hepatic glycogen content, the authors suggested a relationship between the decrease in the drug metabolism and hepatic glycogen content since the latter is stored in parts of the smooth-surfaced endoplasmic reticulum (SER). Changes in the hepatic glycogen levels is accompanied by structural alterations of the SER (Porter and Bruni, 1959) and this may result in structural alteration and orientation of the cytochrome P-450. This is substantiated by a similar decrease in the metabolism of a variety of drugs in starved male rats (Kato and Gillette, 1965a). The observation of the reduction of drug metabolism in diabetic animals were also confirmed by other workers (Kato and Gillette, 1965b; Weiner et.al., 1972). However the effect of diabetes is not always to decrease drug metabolism as documented by Dixon et.al. (1963). Aniline hydroxylation was reported to be stimulated in the diabetic condition. Furthermore their work revealed that the effect on the metabolism of codeine was time-dependent. The metabolism of codeine was decreased in the acutely diabetic rats but was found unaltered in the chronically diabetic rats.

The paradoxical effect of diabetes on aniline metabolism was later resolved by Kato and Gillette (1965b) where they demonstrated the existence of sex-differences in the effect of diabetes on drug metabolism in the rat. They confirmed the works of Dixon

et.al. (1961) by observing a decrease in hexobarbital metabolism and an increase in aniline metabolism in the diabetic male rats . They also observed a decrease in aminopyrine metabolism. However, all these three parameters were found to increase in the diabetic *female* rats. They have shown previously that there is a marked sex difference in the metabolism of aminopyrine but little, if any, sex difference in the hydroxylation of aniline (Kato and Gillette, 1965a). They postulated that alloxan diabetes impairs mainly the markedly sex-dependent enzymes. It is interesting to note that a similar decrease in the hexobarbital hydroxylation was also observed in castrated male rats and the administration of methyltestosterone to such rats restored the activity to the level of the intact males. Alloxan diabetes did not produce a further decrease in the hexobarbital hydroxylation in the castrated rats but produced a marked decrease in the hexobarbital hydroxylation both in the methyltestosterone treated castrated rats and the intact males suggesting that the effect of alloxan diabetes could probably be mediated through the impairment of an androgen-dependent stimulating mechanism (Kato and Onoda, 1970). A later study by Kato et.al. (1971) demonstrated that the magnitude of the difference spectrum of cytochrome P-450 induced by hexobarbital and aminopyrine was markedly decreased in the male, but not in the female, alloxan-treated rats. This suggests that the binding capacities of cytochrome P-450 for hexobarbital and aminopyrine, which are normally stimulated by androgen (Schenkman et.al., 1967), are decreased in the microsomes of the diabetic male rats.They also reported that treatment with alloxan decreases the V_{max} value for hexobarbital hydroxylation by liver microsomes of male rats, whereas it increased the K_m and K_s (spectral dissociation constant) values. Previous work by Kato and Onoda (1970) indicated that the administration of androgen increases both the K_m and K_s values for hexobarbital and aminopyrine. These results indicate that the actions of androgen to increase the binding capacity and affinity of cytochrome P-450 with the drug substrates are impaired by

diabetes, resulting in the decrease in the oxidation of hexobarbital and aminopyrine.

In 1972, Weiner et.al. demonstrated that the inhibitory effect of alloxan diabetes and starvation on hepatic drug metabolism could be mimicked by glucagon. This inhibitory effect of glucagon could be partially reversed by insulin, an effect similarly seen with diabetic rats (Dixon et.al., 1961; Dixon et.al., 1963). Insulin antagonism of the effects caused by diabetes and glucagon provided an insight into the role of cyclic nucleotides and the possible mechanism of action, at the membrane level, of diabetes mellitus on hepatic drug metabolism since both glucagon and diabetes have been shown to increase liver cyclic AMP concentration (Weiner et.al., 1972; Jefferson et.al., 1968).

All of these studies described above, relating to the effect of diabetes mellitus on drug metabolism in experimental animals, employed alloxan as a diabetogenic agent. Alloxan has been criticized for its overt toxicity since it not only affects the β-cells of the pancreas but has also been reported to have a toxic effect on the kidney, making it uncertain whether the effects seen were related to its diabetogenic action. It thus may not serve as a good model of diabetes. A relatively new diabetogen, streptozotocin (STZ), which has a more β-cell-selective action with less general toxic effects, has become more popular as a diabetogenic agent (Hoftiezer and Carpenter, 1973). Thus, STZ-treated animals have been used as a model for type 1 diabetes in this study.

Although the microsomal metabolism of the androgenic hormones, testosterone and androst-4-ene-3,17-dione, has been studied in several laboratories (Conney et.al., 1973; Einarsson et.al.,1974), Reinke et.al. (1978) were the first to report an alteration in hepatic steroid metabolism in STZ-diabetic male rats. The diabetic state resulted in an overall decrease in the rate of microsomal metabolism of androst-4-ene-3,17-dione, compared to controls. Differential effects on metabolism were observed with individual metabolites. The hepatic microsomes from the diabetic rats exhibited an increased and decreased activities of the female-specific 7α-hydroxylase (Stenberg,1976) and male-

specific 16α-hydroxylase respectively, indicating a sex-differentiation in the effect of the diabetic state. All of these alterations were restored to normal levels when the diabetic rats were treated with insulin. This result is in good agreement with previous reported work relating to drug metabolism where the diabetic state produces the sex-dependent effects by interfering with androgen-dependent microsomal pathways in the rat (Kato and Gillette, 1965b; Kato et.al., 1971). Skett (1986) also reported the sex-dependent effect of diabetes mellitus on steroid metabolism in rat where alterations were only seen in the male and the effect is always to abolish the sex differences in steroid metabolism found in the intact animals.

In the late 1970s and early 1980s work was concentrated on the relationship between the changes in the cytochrome P-450 content and the changes seen in drug metabolism in diabetic animals. Inconsistent reports on the effect of diabetes mellitus on hepatic microsomal cytochrome P-450 content were presented. Ackerman and Liebman (1977), Kato et.al. (1971) and Faas and Carter (1980) reported no alteration in cytochrome P-450 content in diabetic male rats but increased levels in the diabetic female rats whereas Reinke et.al. (1978) reported an increase in cytochrome P-450 content in both the male and female diabetic rats. The reason for this lack of agreement was pointed out by Skett (1987) as being due to the difference in the time the animals were killed after STZ treatment. In 1980, by using sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis, Past and Cook managed to separate different forms of cytochrome P-450. In this study they discovered that diabetes mellitus induced, in both the male and female rat hepatic microsomes, specific increases and decreases in the cytochrome P-450 isoenzymes. The notion of specific alteration of cytochrome P-450 isoenzymes by diabetes mellitus was substantiated by the discovery by the same workers in 1982 of a diabetes-dependent form of cytochrome P-450 which they were able to isolate. This cytochrome P-450, with a molecular weight of 52,000 , was also induced in the diabetic female rat (Past and Cook, 1983) but was not detected in hepatic microsomes from

normal, 3-methylcholanthrene- or phenobarbital-treated rats. This cytochrome P-450 was, however, not detected in the insulin-treated diabetic rats. When compared to normal P-450 in a reconstituted system, this unique 52,000 molecular weight cytochrome P-450 was shown to increase the hydroxylation of aniline and decrease the N-demethylation of morphine. In another laboratory, Warren et.al. (1983) found that the diabetic females, in comparison to the controls, showed an increase in the 52,000 molecular weight band without a decrease in the 46,000 and 61,000 molecular weight regions. On the other hand, in the diabetic males, there was a similar rise in the 52,000 molecular weight band, but there was a marked reduction in the 46,000, 56,000 and 61,000 regions. Insulin treatment reversed these changes in both male and female diabetic animals. Further evidence of alterations in specific constitutive cytochrome P-450 isoenzymes in the diabetic state was provided by Favreau and Schenkman (1987) when they measured cytochrome P-450- dependent hydroxylation of testosterone in hepatic microsomes of control, diabetic and insulin-treated diabetic rats. Cytochrome P-450 RLM5 has been shown to be the major form of cytochrome P-450 responsible for 16α-hydroxylase activity (Waxman, 1984). Favreau and Schenkman (1987) showed that testosterone 16α-hydroxylase activity was decreased in diabetes and this was accompanied by a dramatic decrease in immunodetectable cytochrome P-450 RLM5. Insulin treatment resulted in partial restoration of the 16α-hydroxylase activity and the cytochrome P-450 RLM5 levels. In that same year, Favreau et.al. (1987) also reported the isolation of two specific forms of cytochrome P-450, both of which were found to be elevated in the streptozotocin-induced diabetic male rats. One enzyme, cytochrome P-450 RLM6, metabolizes aniline and acetol, but not testosterone, and insulin treatment of diabetic rats for 1 week lowered the immunologically detectable levels of cytochrome P-450 RLM6 to levels found in the control rat. The second form, cytochrome P-450 RLM5b does not metabolize aniline and only poorly metabolizes acetol and testosterone. However, 1 week

of treatment of the diabetic animals with insulin only partially restored the microsomal content to that of the normal rat. Recently, Favreau and Schenkman (1988) reported three different types of responses by constituents of the cytochrome P-450 population to diabetes. The immunodetectable levels of cytochrome P-450 RLM3 and RLM5 were decreased when the animals were made diabetic by STZ . In contrast, the levels of cytochrome P-450 RLM5b and RLM6 were elevated in diabetes and the level of the fifth enzyme, cytochrome P-450 RLM5a, was not markedly altered by diabetes. Two forms of sex-dependent cytochrome P-450 have been described in the rat, P-450 male and P-450 female. They are developmentally controlled by androgenic and estrogenic hormones (Kamataki et.al., 1983), as well as by growth hormone (Morgan et.al., 1985b). Since the diabetic state is usually associated with alteration of testosterone (Warren et.al.,1983) and growth hormone (Press et.al., 1986) levels, this could explain the changes in the levels of the constitutive cytochrome P-450 isoenzymes.

Alteration of drug metabolism has also been demonstrated in the liver of STZ-diabetic mice (Rouer and Leroux, 1980) and these modifications were completely corrected by insulin treatment of the diabetic mice. Interestingly, they have also demonstrated that normal male mice treated with glucagon by injection or continuous infusion, exhibited alteration of drug metabolism. These hyperglucagonemic animals showed a decrease in cytochrome P-450 content but also an increased synthesis of a specific cytochrome P-450 isoenzyme which differs from those induced by the two classical chemical inducers, phenobarbital and 3-methylcholanthrene (Rouer et.al., 1985). The increases in aniline hydroxylation and benzphetamine N-demethylation seen in the STZ-diabetic mice were also seen in the glucagon-treated animals suggesting that the alteration of the glucagon level in the diabetic state could play a part in the altered xenobiotic metabolism.

Since diabetes affects only the androgen-responsive enzymes in the male rat and

has little effect in the female, it has been postulated that diabetes in some way interferes with the androgenic stimulation of drug metabolism in the male and, thus, exerts its sex-dependent effect (Kato, 1974). This is supported by observations that diabetes alters the serum androgen levels by interfering with the release or production of testosterone from the testes (Murray et.al., 1981). However, the newly discovered system, the hypothalamo-pituitary-liver axis regulating the sex differences in steroid and xenobiotic metabolism in the liver, as suggested by Gustafsson and co-workers (1983), has led to a restyling of this idea. Skett et.al. (1984), in their study to link serum testosterone and the effect of diabetes on drug metabolism, discovered that there was a complete dissociation of change in serum testosterone and effect on hepatic drug metabolism. The level of androgen does not change in the acute diabetic phase when a marked alteration of drug metabolism is seen but serum androgen levels are markedly reduced in the chronic phase with little effect on hepatic drug metabolism. Skett and Gustafsson (1979) found that androgens do not exert a direct effect on the liver but probably have an indirect effect via the hypothalamo-pituitary axis. It has been suggested that androgens and insulin affect drug metabolism by acting through a common mediator - and this mediator has been proposed to be growth hormone (Skett, 1987). Diabetes mellitus is associated with reproductive disorders and lesions of the testes and accessory sex organs in man (Schoffling et.al., 1963) and in male animal models (Foglia et.al., 1963). A later study has shown that STZ-diabetes induces different neuroendocrine and morphological alterations in the hypothalamo-pituitary axis of male and female rats (Bestetti et.al., 1985). From the anatomical data, this study also indicated that the brain and pituitary are less severely affected by diabetes in female than in male rats. According to the hypothalamo-pituitary-liver axis concept , these differential effects, resulting from insulin deficiency, and the effect of diabetes on androgen turnover would somehow influence the drug metabolism in both sexes of the rat.

In 1985, Skett and Joels reported that diabetes mellitus has a transient effect on drug metabolism. Acute diabetes had a marked effect on drug metabolism while the chronic effect was much less than that seen in the acute phase. In another study, MacFarlane and Skett (1986) reported that there is no correlation between STZ-induced changes in drug metabolism and the serum parameters i.e. serum glucose and triglyceride levels.

The diabetic effect on drug and steroid metabolism can be partially or completely restored to normal by treating the animals with insulin. Glucagon infused animals also showed alteration in drug metabolism that resembles the effect of diabetes on the metabolism of some substrates. All this is indicative of the role of both hormones in the regulation of xenobiotic and steroid metabolism in the rat liver. In the next two sections we will focus our discussion on the receptor structure of both of the hormones and their possible mechanism of action at the molecular level.

1.2 INSULIN

1.2.1 THE STRUCTURE OF THE INSULIN RECEPTOR

There is general consensus that the mature insulin receptor is an integral membrane glycoprotein that spans the plasma membrane bilayer of most animal cells. A large body of evidence generated by a variety of approaches is consistent with the concept that the major form of the native insulin receptor complex is a symmetrical heterotetrameric glycoprotein composed of two α and two β subunits covalently linked to one another by $\alpha-\beta$ and $\alpha-\alpha$ disulphide bonds [Kahn, 1985; Czech, 1985]. The isolated receptor consists of two types of subunits : a 135,000 M.W. α subunit and a 95,000 M.W. β subunit [Jacobs et.al., 1979]. A smaller subunit of 45-50 kilodaltons (kDa), identified on electrophoretic gels after affinity-purification and labelling, was found to be derived from the insulin receptor complex [Massague et.al., 1980] and appears to be a proteolytic (elastase) fragment of the β–subunit and has been denoted β_1 [Massague et.al., 1981]. This general model for the insulin receptor subunit configuration was originally proposed independently by Jacobs and co-workers [Jacobs et.al., 1979] using photolabelling and affinity purification techniques and by Massague and colleagues [Massague et.al., 1980; Czech et.al., 1981] using affinity cross-linking techniques. Several observations strongly suggest the $\alpha_2\beta_2$ stoichiometry [Kasuga et.al., 1982a] and the disulphide-linked configuration [Pilch and Czech, 1980]. An alternative structure of the insulin-receptor complex has been put forward by Yip and Moule [1983] whereby evidence supporting a different type of insulin-receptor structure in rat adipocytes was obtained using two photoreactive derivatives of insulin. The authors suggested that the receptor exists in three interconvertible redox forms of 380, 300 and 230 kilodaltons and the interconversion of these three receptor species is the result of oxidation and reduction of the intersubunit

sulphydryl groups. The substantial differences in receptor structure from that obtained in fat cells [Massague et.al., 1981] and liver membranes [Jacob et.al., 1979] signify that further studies are required for its full elucidation.

Recent evidence suggests that the α and β subunits are synthesised in cells as a single polypeptide precursor that is ultimately processed to the mature cell-surface receptor subunits by proteolytic cleavages [Ronnett et.al., 1984]. Data from many studies indicate that the insulin receptor subunits are synthesised as a high M_r polypeptide precursor that is rapidly glycosylated and identified as an endoglycosidase H-sensitive 190 kDa species. This is followed by further glycosylation yielding a 210 kDa precursor. It is known that glycosylation is a prerequisite for the formation of a functional insulin receptor [Reed et.al., 1981; Ronnett et.al., 1984]. The endoglycosidase H-sensitive 210 kDa species is a substrate for one or more proteolytic processing cleavages, which result in the formation of 125 kDa and 83 kDa subunit precursors.These two precursors contain oligosaccharide chains that are ultimately capped with sialic acid residues near the time of their appearance at the cell surface. The insulin receptor has been shown to be a glycoprotein [Reed et.al., 1981; Ronnett et.al., 1984] in which the oligosaccharide side chains are N-linked complex structures. These complex carbohydrate side chains are linked to asparagine in the polypeptide backbone of the receptor and contain N-acetylglucosamine, mannose, galactose, fucose and sialic acid.

The identification of the α subunit as one of the component of the insulin-receptor complex was first suggested by visualisation of protein-stained electrophoretic gels containing a preparation of insulin receptor that had been affinity purified by adsorption and elution from insulin-agarose [Jacobs et.al., 1977]. A band of similar apparent mass was subsequently identified by photoaffinity labelling [Yip and Moule, 1983] and affinity cross-linking techniques [Pilch and Czech 1980]. The α subunit can now be readily identified by photoaffinity labelling [Yip and Moule 1983], affinity purification [Jacobs et.al., 1979], specific immunoprecipitation of insulin

receptors [Kasuga et.al., 1981b], affinity cross-linking [Czech et.al., 1981], and immunoaffinity purification [Harrison and Itin, 1980 ; Heinrich et.al., 1980]. The amino acid sequencing studies indicate that the α subunit contains no transmembrane sequence [Ullrich et.al., 1985; Ebina et.al., 1985] suggesting that it is located entirely external to the plasma membrane, presumably being firmly attached to the external membrane surface through covalent disulphide cross-links to the β subunit. First evidence to indicate that the α subunit is a glycoprotein was demonstrated by an altered electrophoretic mobility after treatment with neuraminidase [Jacobs et.al., 1980]. It was further confirmed by receptor immunoprecipitation techniques using a variety of anti-insulin receptor antibody preparations whereby labelled saccharides were shown to incorporate into the α subunit [Roth et.al., 1982]. Affinity labelling of the receptor using either photosensitive insulin analogues [Jacobs et.al., 1979], or cross-linking insulin with bifunctional reagents [Pilch and Czech,1979] have shown that the α subunit is the predominant labelled species when compared to the β-subunit, the labelling of which is much weaker [Massague et.al., 1981], or even absent [Yip et.al., 1978]. This suggests that the α subunit of the receptor oligomer houses the insulin-binding site which is located on the outer face of the plasma membrane where it is accessible to extracellular insulin. An insulin receptor α subunit of similar features to that found in human and rodent has been identified in tissues of the hagfish, denoting a substantial conservation of its properties over the last 500 million years of evolution [Czech and Massague, 1982].

The identification of the β subunit has been more elusive than the α on the grounds that it is poorly labelled by most [Czech et.al., 1981], although not all, affinity labelling procedures [Yip and Moule, 1983] , is poorly stained by Coomassie blue or silver compared to the α subunit [Fujita-Yamaguchi, 1984], and also is relatively more sensitive to fragmentation by proteases [Massague et.al., 1981]. It was first detected in affinity purification studies as a receptor band [Jacobs et.al., 1979] and was

deduced to be part of the native insulin receptor structure by affinity cross-linking [Czech et.al., 1981] and affinity purification [Jacobs and Cuatrecasas, 1981] techniques and two dimensional gel electrophoresis in the presence and absence of reductant. In intact cells, insulin was found to stimulate the phosphorylation of the insulin receptor purified on wheat-germ-agglutinin-agarose. This was first demonstrated in rat hepatoma cells and human IM-9 lymphoblasts [Kasuga et.al., 1982b], and later in freshly isolated rat hepatocytes [Van Obberghen and Kowalski, 1982]. The cells were preincubated with ^{32}P-orthophosphate to label cellular ATP, then solubilised in detergent and affinity purified on wheat-germ-agglutinin-agarose. Immunoprecipitation of phosphorylated proteins by antibodies to insulin receptor followed by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis under reducing conditions and autoradiography revealed a labelled band (M_r ~ 95,000). Its identity with the insulin receptor β subunit was established for the following reasons. First, non-immune serum did not precipitate a band with a similar electrophoretic mobility. Second, the molecular size was identical with that determined previously, using biosynthetic and affinity labelling methods [Pilch and Czech, 1979; Massague et.al., 1981]. Subsequently, the phosphorylation of the β subunit of the insulin receptor was demonstrated in cell-free systems using [γ-^{32}P]-ATP in solubilised and partially purified receptor preparations from rat liver and human placenta [Kasuga et.al., 1982b; Petruzzelli et.al., 1982]. Phosphoaminoacid analysis of the phosphorylated β-subunit of partially purified receptors showed phosphoserine, phosphothreonine and phosphotyrosine under basal conditions. Insulin induced a several-fold increase in ^{32}P-incorporation into tyrosine, and had in addition a smaller, but consistent stimulating effect on the labelling of serine [Kasuga et.al., 1982b]. An interesting feature is that insulin stimulates the autophosphorylation of the β subunit of its own receptor in both intact cells [Kasuga et.al., 1982b]

and in a cell-free system [Petruzzelli et.al., 1982]. The 90,000-dalton subunit contains oligosaccharide as evidenced by its biosynthetic labelling with labelled monosaccharides [Hedo et.al., 1981]. It is possible that receptor phosphorylation could participate in altering the affinity for insulin, play a role in receptor internalisation, or initiate some of insulin's biological responses. The latter possibility is supported by the demonstration that vanadate ions, which mimic the biological effects of insulin [Shechter and Karlish, 1980; Dubyak and Kleinzeller, 1980], are also potent inhibitors of tyrosine-specific phosphoprotein phosphatase [Swarp et.al., 1982]. Amino acid sequence studies indicate that the β-subunit possesses a single hydrophobic transmembrane domain consisting of 23 amino acid residues which separate the external one-third of its polypeptide chain from the intracellular two-thirds. Hence, the kinase catalytic site is disposed on the inner face of the plasma membrane where it has access to intracellular ATP and target protein substrates.

Peptide-mapping experiments indicated distinct overall peptide maps of the α and β subunits and are consistent with the apparent different functional roles of the subunits, whereby the α subunit binds hormone while the β subunit carries tyrosine kinase activity and is the predominant subunit autophosphorylated. The postulated insulin receptor structure suggests a scenario for transmembrane signalling induced by insulin. The binding of insulin at the 'extracellular' ligand-binding site of the receptor presumably induces a conformational change in the α subunit that is transmitted through the membrane bilayer to the tyrosine protein kinase domain of the β subunit where catalysis is activated. This in turn would allow the receptor to phosphorylate an intracellular target protein(s), thereby initiating the cascade of events eliciting a remarkable array of biological responses.

1.2.2 THE INSULIN RECEPTOR KINASE

The earliest known event that follows the binding of insulin to its receptor is activation of the receptor protein tyrosine kinase. Kasuga and colleagues [Kasuga et.al., 1982b] first demonstrated that incubation of ^{32}P-labelled cultured human lymphocytes and rat hepatoma cells with insulin (at physiological concentrations) leads to the increased incorporation of ^{32}P into the insulin receptor β subunit and it was shown to reflect incorporation of [^{32}P] phosphate into both serine and tyrosine residues of the β subunit. These results are strikingly similar to the ability of epidermal growth factor to stimulate the phosphorylation of its own receptor observed by Cohen et.al., [1980] who pioneered the discovery of growth factor receptors as enzymes that catalyse tyrosine phosphorylation. However, early studies were not conclusive as to whether the insulin receptor is a protein kinase or only a substrate for an intrinsic protein kinase. Two series of observations have led to the idea that the insulin receptor itself is likely to be a protein kinase. Firstly, insulin receptors isolated by specific immunoprecipitation can be phosphorylated and insulin increased several-fold their phosphorylation [Van Obberghen et.al., 1983]. This indicates that anti-receptor antibodies immunoprecipitate an insulin-responsive kinase, which is closely associated with the receptor, or contained in the receptor. Secondly, using covalent affinity labelling with oxidised (α-^{32}P) ATP, a specific ATP binding site on a polypeptide with M_r 95,000 was identified indicating that it contains a catalytic site for the insulin receptor-associated tyrosine kinase [Van Obberghen et.al., 1983]. At the same time it was demonstrated that highly purified insulin receptor from human placenta [Fujita-Yamaguchi et.al., 1983] retained protein kinase activity [Kasuga et.al., 1983]. Further support to show the localisation of the tyrosine kinase on distinct subunits of the insulin receptor comes from the observation

that selective degradation of the β subunit of the receptor by collagenase was accompanied by concomitant loss of kinase activity, whilst exerting no effect on the α subunit and insulin binding [Roth et.al., 1983b].

1.2.2.1 REGULATION OF THE INSULIN RECEPTOR KINASE

1.2.2.1.1 *In-vivo* studies

In intact cells, insulin stimulates not only the phosphorylation of its own receptor on tyrosine but also the subsequent phosphorylation of the receptor on serine and threonine residues. A rapid rise in the level of phosphotyrosine is followed by a slower increase in the content of phosphoserine [Denton, 1986]. The insulin receptor from insulin-treated cells can be isolated in its phosphorylated, active form [Stadmauer and Rosen, 1986] indicating that the phosphorylation-induced activation of the receptor kinase occurs *in-vivo*. Czech [1984] found at least seven phosphorylation sites on the insulin receptor upon addition of high concentrations of [γ-^{32}P]ATP. Also in their study, serine and tyrosine, as well as a much smaller number of threonine residues, were found to be phosphorylated by ATP during tyrosine kinase activation. When the phosphorylated insulin receptor, that exhibited elevated tyrosine kinase activity, was dephosphorylated by incubation with alkaline phosphatase, deactivation of the receptor-associated tyrosine kinase activity could be achieved. Under these conditions of tyrosine kinase deactivation, about 90 % of the phosphate removed from the receptor could be accounted for by tyrosine phosphate in the insulin receptor, indicating that tyrosine phosphorylation of the insulin receptor is directly regulating the receptor - associated tyrosine kinase activity *in-vitro*. Although the location of all of the sites phosphorylated in intact cells are not known, the tyrosine-containing peptide whose phosphorylation correlates with *in-vitro* activation of the kinase is also phosphorylated in response to insulin in intact cells

[Herrera et.al., 1985]. When the insulin receptor is phosphorylated in intact cells in the absence of insulin, phosphoserine and phosphothreonine are found in the receptor β subunit. While direct data are now implicating tyrosine phosphorylation in the regulation of the insulin receptor kinase, indirect data obtained suggest that serine phosphorylation leads to decreased receptor kinase activity in the presence of insulin [Takayama et.al., 1988]. Van Obberghen and Gammeltoft [1986] hypothesised that the insulin receptor-kinase complex contains two separate protein kinases, a tyrosine- and a serine-specific kinase, the latter of which is non-covalently associated with the receptor in the cell membrane but would not be an integral part of the receptor. Serine phosphorylation of the receptor has been demonstrated to inhibit its kinase activity [Stadtmauer and Rosen, 1986].

1.2.2.1.2 *In-vitro* studies

In cell-free systems, the differences with which phosphopeptide residues of the β subunit are phosphorylated, is dependent on the nature of the receptor preparation used as a protein kinase source. The β subunit of the insulin receptor has been demonstrated to be phosphorylated by insulin in a cell-free system using [γ-^{32}P]ATP and solubilised and partially purified receptor preparations from human placenta and rat liver. Predominant labelling of the tyrosine residues, and to a smaller extent the serine residues, was observed as shown by phosphoaminoacid analysis [Petruzzelli et.al., 1982; Van Obberghen et.al., 1983; Roth and Cassell, 1983]. In highly purified insulin receptors, phosphorylation occurred exclusively on tyrosine residues under basal conditions and the insulin stimulatory action was accounted for by a several-fold increase in phosphotyrosine [Kasuga et.al., 1983], indicating tyrosine kinase as a constituent of the insulin receptor. The tyrosine kinase activity is an intrinsic property of the insulin receptor

as demonstrated by the ability of the insulin receptor of high purification to incorporate ^{32}P in response to added insulin. Further studies supporting this hypothesis include affinity labelling with ATP analogues [Roth and Cassell, 1983], sequence homology with other known tyrosine kinases [Ullrich et.al., 1985; Ebina et.al., 1985] and by its copurification to homogeneity with insulin binding activity [Petruzzelli et.al., 1984]. Insulin receptor kinase exhibits a preference for tyrosine residues that are close to acidic amino acid residues in exogenous substrates which is similar, but not identical, to EGF receptor kinase or $pp60^{src}$ [Bishop, 1985; Hunter and Cooper, 1985]. Immunoprecipitated insulin receptors obtained from patients with severe insulin resistance and Acanthosis nigricans [Van Obberghen et.al., 1983], or monoclonal immunoglobulin G directed against insulin receptors [Roth et.al., 1982; Roth et.al., 1983a] have been shown to exhibit insulin stimulated protein kinase activity which catalysed phosphorylation of both the β subunit and exogenous substrates like histones, synthetic tyrosine-containing peptides and casein [Kasuga et.al., 1982b; Kasuga et.al., 1983; Petruzzelli et.al., 1984]. Rosen et.al. [1983] showed directly that incubation of insulin receptor with unlabelled ATP, prior to a second incubation with [γ-^{32}P]ATP plus substrate, resulted in markedly enhanced substrate phosphorylation by the insulin receptor. They also demonstrated that the phosphorylated, activated insulin receptor kinase no longer exhibits sensitivity to modulation by insulin itself. Thus, the insulin receptor appears to be converted, upon tyrosine phosphorylation of its β subunit, to a form that is hormone-independent with respect to its kinase activity. The phosphorylation of the receptor does not affect insulin binding. Dephosphorylation of the receptor restores its insulin dependency with concomitant loss of the activated state indicating that the hormone-induced activation is reversible [Rosen et.al., 1983; Yu and Czech, 1984]. Site-specific antibodies generated to different domains of the β subunit of the receptor have been used to elucidate the structural basis for the kinase activity of the

receptor. Herrera and Rosen [1986] were able to inhibit the kinase activity of the insulin receptor by using an antipeptide antibody directed to a domain surrounding tyrosine 960 which lies between the transmembrane region of the molecule and the nucleotide binding site [Ullrich et.al., 1985; Ebina et.al., 1985]. This antibody is able to bind to the phosphorylated receptor but is unable to inhibit the kinase activity of this form of the receptor suggesting conformational changes may result from receptor phosphorylation. An antipeptide antibody directed to a domain surrounding Tyr 1150 recognises only the phosphorylated form of the receptor under nondenaturing conditions. The major *in-vitro* autophosphorylation sites on the β subunit of the insulin receptor are located at the carboxyl terminal and in the domain that includes Tyr 1150 and phosphorylation of the latter is correlated with the activation of the kinase [Pang et.al., 1985] implying that the Tyr 1150 domain plays a critical role in the mechanism of signal transduction.

At present, it seems plausible that the receptor kinase activity is involved in a cascade of events that leads to phosphorylation and / or dephosphorylation of other cellular proteins that control the metabolic pathways regulated by insulin. Collected evidence from recent years has reinforced the role of the kinase activity of the insulin receptor in insulin action :

i) The insulin receptor-kinase activity appears to be widespread and has been characterized in insulin receptor preparations solubilised from a variety of tissues such as liver plasma membranes [Kasuga et.al., 1982a; Van Obberghen and Kowalski, 1982] and microsomes [Blackshear et.al., 1983], adipocytes [Haring et.al., 1982], 3T3-L1 adipocytes [Petruzzelli et.al., 1982; Rosen et.al., 1983], muscle [Haring et.al., 1984a], placenta [Roth and Cassell, 1983 ; Petruzzelli et.al., 1984], erythrocytes [Grigorescu et.al., 1986], lymphocytes [Grunberger et.al., 1984a], brain cortex [Rees-Jones et.al., 1984], monocytes [Grunberger et.al., 1984b] and several cultured cell lines [Kasuga et.al., 1982a; White et.al., 1985]. Besides that, the kinase activity of

the insulin receptor has also been detected in non-mammalian species such as birds [Aiyer, 1983] and insects [Petruzzelli et.al., 1986].

ii) The insulin-induced activation of the receptor kinase is a rapidly reversible process [Rosen et.al., 1983; Yu and Czech, 1984].There is a correlation between the concentrations of insulin required for kinase activation and the specificity of the ligand stimulation with the binding of insulin to the receptor and generation of biological activity [Haring et.al., 1982; Petruzzelli et.al., 1984; Kahn, 1985].

iii) The impairment of the receptor kinase activity in cells from various insulin resistant states including cultured melanoma cells [Haring et.al., 1984b], gold-thioglucose-treated mice [LeMarchand-Brustel et.al., 1985], human patients with insulin resistance associated with acanthosis nigricans type A [Grunberger et.al., 1984a] and streptozotocin diabetic rats [Kadowaki et.al., 1984].

iv) Microinjection of an inhibitory monoclonal antibody into Xenopus oocytes was found to inhibit insulin's ability to stimulate oocyte maturation [Morgan et.al., 1986] possibly by binding to an antigenic region containing an important phosphorylation site of the insulin receptor (tyrosines 1162 and 1163) [Ellis et.al., 1986].

v) Monoclonal insulin receptor antibody did not stimulate the phosphorylation of the insulin receptor either in intact IM-9 cells or in purified receptor preparations. In contrast, three agents which mimic insulin action in intact cells (concanavalin A, wheat germ agglutinin, and polyclonal insulin receptor antibody), mimicked insulin's ability to stimulate the kinase activity of purified insulin receptors [Roth et.al., 1983a].

vi) When antibodies to the insulin receptor tyrosine kinase are incorporated into various cells by osmotic lysis of pinocytotic vesicles, there is a decreased ability of insulin to stimulate the uptake of 2-deoxyglucose in Chinese hamster ovary (CHO) cells and freshly isolated rat adipocytes; of ribosomal protein S6 phosphorylation in CHO cell; and glycogen synthesis in the human hepatoma cell line HepG2 [Morgan and Roth,

1987].

vii) Mutant human insulin receptors deficient in kinase activity fail to stimulate glucose transport [Ellis et.al., 1986] ; expression of normal human receptors enhances the insulin responsiveness in these cells.

However, efforts to establish a role for the receptor kinase in insulin action have not been entirely successful. It has been suggested that the receptor kinase may not be important in mediating certain rapid effects of insulin. Certain polyclonal antisera have been shown to stimulate glucose uptake in rat adipocytes without stimulating receptor phosphorylation or receptor kinase activity [Simpson and Hedo, 1984; Zick et.al., 1984].

1.2.3 MECHANISMS OF ACTION OF INSULIN

Insulin was discovered more than 60 years ago. Much is known about the physiological and biochemical consequences of insulin action ; however, the molecular mechanism by which the interaction of insulin with its receptor is transduced into an insulin response has yet to be elucidated. Insulin has both long and short term effects on the metabolism of its target cells. The long term effects involve changes in both general and specific protein synthesis and breakdown, while short term effects are those brought about solely through changes in the activity of pre-existing enzymes and membrane transporters. There are many theories to explain how insulin works and below are descriptions of the prevailing hypotheses of the mechanism of insulin action.

1.2.3.1 Changes in cyclic nucleotides

Many of the metabolic effects of insulin on liver, skeletal muscle, and adipose

tissue oppose the actions of cyclic AMP. Early reports indicated that insulin could reduce hormonally- elevated levels of cyclic AMP in adipose tissue (Butcher et.al., 1966; Soderling et.al., 1973) which is consistent with the situation *in-vivo*. However, insulin alone has little or no effect on cyclic AMP levels in fat or liver cells although many effects of insulin are manifested under these conditions (Denton et.al., 1981). In rat diaphragm, insulin had no effect on total cyclic AMP content but the physiological effect of insulin was demonstrable (Larner, 1975). Thus, it must be concluded that although insulin may exert some of its effects by decreasing cyclic AMP levels in adipose and hepatic tissue, the simple lowering of previously elevated concentrations of cyclic AMP cannot explain many well-established physiological actions of insulin.

The ability of insulin to depress cellular cyclic AMP may be a result of inhibition of adenylate cyclase (Illiano and Cuatrecasas, 1972) and/or the stimulation of a specific membrane-bound cyclic nucleotide phosphodiesterase (Zinman and Hollenberg, 1974) - see Figure 1. In a recent report, Gawler et.al. (1987) demonstrated the ability of insulin to inhibit adenylate cyclase in streptozotocin- or alloxan-induced diabetic rats despite the loss of G_i, the guanine nucleotide-binding regulatory (G) protein that mediates the inhibitory effects on adenylate cyclase activity. It appears, therefore, that insulin inhibits adenylate cyclase via a G protein different from G_i. Indeed, there is evidence that the insulin receptor does interact with, and activate, a specific G protein called G_{ins} which has tentatively been identified as having a 25 kDa subunit (Houslay, 1986). However, there are conflicting results as to the activity of adenylate cyclase in response to insulin. Pilkis and Park (1974) failed to see any inhibitory effect of insulin on the adenylate cyclase.

Treatment of hepatocytes with insulin has been reported to activate two distinct cyclic AMP phosphodiesterases, one termed the peripheral plasma membrane phosphodiesterase and the second, ' dense-vesicle 'phosphodiesterase, since it is associated with an intracellular vesicle fraction (Heyworth and Houslay, 1983). Insulin

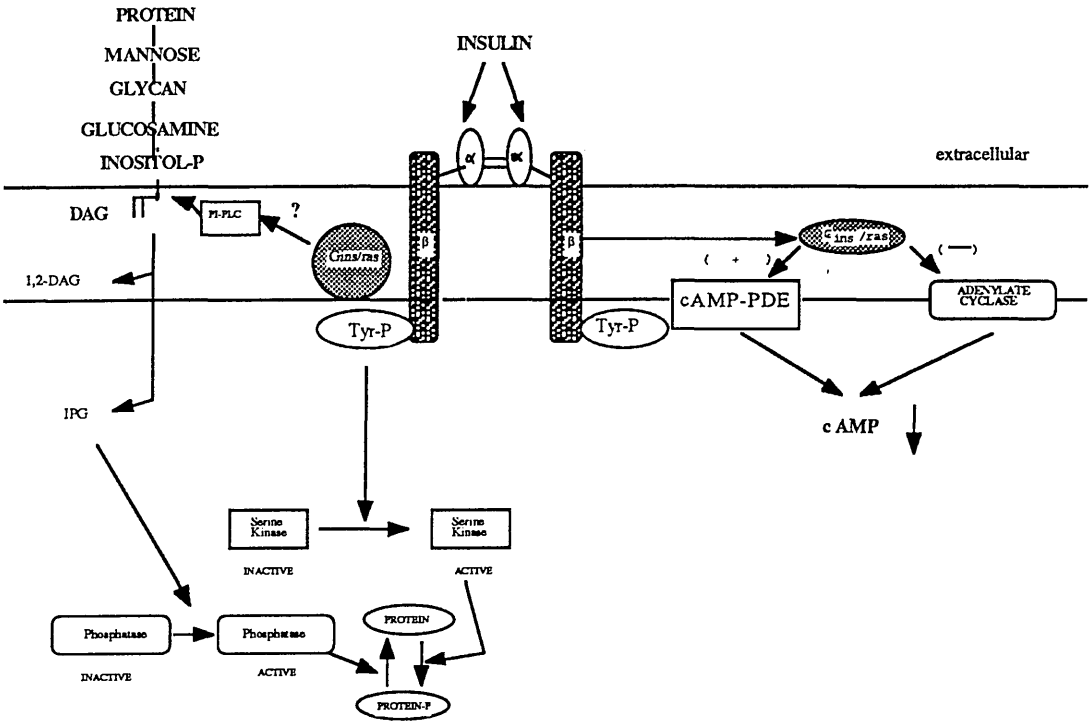


FIGURE 1. A HYPOTHESIS FOR THE MECHANISM OF ACTION OF INSULIN

Abbreviations : cAMP = cyclic AMP, cAMP PDE = cyclic AMP-dependent phosphodiesterase, DAG = diacylglycerol, Gins = guanine nucleotide regulatory protein specific for insulin, IPG = inositol phosphate-glycan, P = phosphate, PI-PLC = phosphatidylinositol-specific phospholipase C, Tyr-P = insulin receptor tyrosine kinase

(Adapted from Espinal, 1987)

activation of the peripheral plasma membrane phosphodiesterase is dose-dependent, and requires the presence of GTP, ATP and intriguingly, also requires cyclic AMP which is consistent with the observation that insulin can lower elevated but not basal levels of intracellular cyclic AMP. It has been suggested that insulin might exert its action on adenylate cyclase and on the peripheral cyclic AMP phosphodiesterase through a distinct guanine nucleotide regulatory protein called G_{ins} (Heyworth and Houslay, 1983).

Insulin might also antagonise the effects of cyclic AMP without changing the concentration of cyclic AMP in target cells. One mechanism by which this could occur is the inhibition of cyclic AMP dependent protein kinase by insulin. However, the exact mechanism of this response still remains obscure. It has been suggested that insulin decreases the affinity of the protein kinase for cyclic AMP in isolated hepatocytes (Mor et.al., 1981). Another study has also reported a similar effect, but also added that insulin raises the K_a without influencing the V_{max} of the enzymes, resulting in a decrease in the capacity of protein kinase to be dissociated (Gabbay and Lardy, 1987). In addition, they also suggested that insulin may act to decrease the affinity of the protein kinase for cyclic AMP through a possible regulation of intrachain site 2 binding on the enzyme. The site 2 intrachain cyclic AMP binding domain has been shown to be involved in protein kinase activation (Ogreid et.al., 1983). Another mechanism which has been proposed by Walkenbach et.al. (1978) involves the generation of a second messenger which may be directly associated with the holoenzyme protein kinase. This association results in the formation of a second messenger-protein kinase complex which has a reduced ability to bind cyclic AMP.

1.2.3.2 Generation of inositol-glycans

The concept of the ' insulin mediators ', a term coined by Larner (1972), was

modelled on the cyclic AMP -second messenger system. In 1974, Larner and co-workers reported that an acid- and heat-stable inhibitor of cyclic AMP-dependent protein kinase was generated in rat muscles within a few minutes of insulin administration. Since then numerous papers have reported the isolation and purification of these chemical mediators (Jarett and Seals, 1979; Seals and Czech, 1980). However, the exact nature of the mediators was not elucidated until recently. They were initially thought to be peptides, but recent studies by Saltiel and Cuatrecasas (1986) indicate that insulin stimulates the generation of a glycan containing inositol phosphate which has an apparent molecular weight between 1500 and 2500. The generation of this glycan, termed IPG by Saltiel, is associated with a concomitant release of diacylglycerol, containing myristate. The molecular events that bring about the release of IPG and diacylglycerol , are unclear. It is thought that insulin receptor activation leads to stimulation of the membrane phospholipase C probably via a G protein, resulting in the formation of IPG and diacylglycerol - see Figure 1. IPG can mimic the effects of insulin on several insulin-sensitive enzymes in liver and hepatoma broken cell preparations. They have been shown to inhibit adenylate cyclase and the catalytic subunit of cyclic AMP-dependent protein kinase (Malchoff et.al., 1987) while stimulating cyclic AMP-dependent phosphodiesterase and pyruvate dehydrogenase (Saltiel, 1987). The IPG has also been shown to mimic insulin-induced changes in phosphorylation of phospholipid methyltransferase (Kelly et.al., 1987b), ATP-citrate lyase, hormone-sensitive lipase and glycogen phosphorylase (Alemany et.al., 1987) thus supporting the role for IPG in some of insulin actions. The ability of this long list of insulin-sensitive enzymes to be modulated by IPG substantiates the claim that the latter functions as a second messenger of insulin action.

1.2.3.3 Effect of insulin on inositol phospholipid metabolism

As described above insulin-mediated activation of a specific phospholipase C results in the formation of diacylglycerol which is known to act as a second messenger. Diacylglycerol which remains in the membrane, together with the cofactor phosphatidylserine, activates protein kinase C. It has been shown that phorbol esters, which are known activators of protein kinase C, are able to imitate several of insulin's effects on metabolism (Farese et.al., 1985a; Sato et.al., 1988). In fact, insulin has also been shown to stimulate protein kinase C activity in myocytes (Cooper et.al., 1987).

In-vivo (Takayama et.al., 1988) and *in-vitro* (Bollag et.al., 1986) studies have revealed that phorbol ester activation of protein kinase C stimulates the phosphorylation of serine in the insulin receptor resulting in inhibition by 50-65 % of the insulin receptor's intrinsic tyrosine-specific protein kinase activity without having any effect on the insulin-binding activity (Bollag et.al., 1986). The data suggest that protein kinase C may regulate the function of the insulin receptor. In contrast, protein kinase C-deficient cells have been shown to maintain some of insulin's effect (Blackshear et.al., 1987) indicating that some of insulin actions such as activation of ribosomal S6 protein kinase and increases in ornithine decarboxylase activity, are not mediated by protein kinase C. However, studies from many laboratories have indicated that the effect of insulin on the turnover of diacylglycerol is tissue specific; e.g. it is reported to occur in myocytes and adipose tissue (Cooper et.al., 1987) but not in liver cells (Sakai and Wells, 1986).

1.2.3.4 Insulin-stimulated serine kinases

Many of the effects of insulin ultimately involve altered phosphorylation of target

proteins, usually on serine residues. In intact cells phosphorylation of the insulin receptor on serine residues is increased by insulin (Takayama et.al., 1984) and Denton et.al. (1981) have proposed that insulin actually causes the dissociation of a serine kinase from the plasma membrane. Yu et.al. (1987) have reported the identification of a novel Mn^{2+}-dependent cytosolic serine kinase in rat adipocytes which is stimulated by insulin in a dose-dependent manner. Recently, Sakanoue et.al. (1988) also reported the presence of an insulin-stimulated serine kinase in *Xenopus* oocyte plasma membrane. The insulin-stimulated serine kinase significantly phosphorylated H1 histone and phosphoaminoacid analysis revealed that phosphate was incorporated into serine residues. Despite this evidence, serine kinases activated in cells by insulin are poorly characterized and need further investigation before they can be accepted as playing a role in mediating insulin's effects.

1.3 GLUCAGON

1.3.1 THE STRUCTURE OF THE GLUCAGON RECEPTOR

Glucagon regulates carbohydrate metabolism in the liver by binding to cell surface receptors on the external surface of hepatocytes (Rodbell et.al., 1971). The glucagon-bound receptor activates adenylate cyclase via a guanine nucleotide-regulated mechanism (Rodbell, 1980) resulting in enhanced cyclic AMP synthesis rates. While the glucagon-sensitive adenylate cyclase has been purified and extensively characterized (Sternweis et.al., 1981), relatively little is known about the glucagon receptor. Understanding the basic features of glucagon action is important, particularly since in certain diseases like diabetes, glucagon has been implicated in contributing to the overall pathophysiology (Unger and Orci, 1981).

The glucagon receptor was first identified by Rodbell and his co-workers in 1971 who simultaneously discovered that guanine nucleotides were essential for glucagon activation of adenylate cyclase. Specific binding sites and/or a glucagon-sensitive adenylate cyclase have been described in a variety of mammalian and avian tissues (Desbuquois, 1985). Studies with adipocytes (Desbuquois and Laudat, 1974) and hepatocytes (Rouer et.al., 1980) indicated that both the glucagon receptor and adenylate cyclase are localized in the plasma membrane of these cells although glucagon-sensitive adenylate cyclase has been detected in endoplasmic reticulum and Golgi membranes (Yunghans and Morre, 1978).

The delay in the identification of the glucagon receptor is due to the lack of a purification technique that is able to monitor the receptor after it is extracted from the membrane with detergents. An added complication is that the amphipathic nature of the glucagon receptor makes it difficult to distinguish true receptor binding from interactions with lipids and detergents. Covalent labelling of receptors using cross-linking agents,

^{125}I-labelled hormones, and ligands has become a very useful tool for the study of the structure of receptors. By cross-linking [^{125}I-Tyr^{10}]monoiodoglucagon ([^{125}I] MIG) to liver membrane receptors with hydroxysuccimidyl-*p*-azidobenzoate, Iyengar and Herberg (1984) were able to identify a M_r = 63,000 peptide on the SDS-PAGE gels as the glucagon receptor since unlabelled glucagon added prior to cross-linking of labelled hormone abolished appearance of the labelled protein. In another study (Herberg et.al., 1984) demonstrated that if the liver membranes were preincubated at 32 ^{0}C for 20 minutes before the addition of [^{125}I] MIG, then a labelled band with an apparent M_r = 113,000-115,000 was observed. Labelling of the M_r = 113,000 band was decreased by the inclusion of GTP, abolished by the inclusion of glucagon, and unaffected by insulin, indicating that the M_r = 113,000 protein also recognized glucagon specifically in a GTP-dependent manner. They concluded that the hepatic glucagon receptor is a dimer of the M_r = 63,000 hormone-binding subunit. Furthermore, the size of the glucagon receptor determined by hydrodynamic measurements is similar to the molecular weight obtained for the functional receptor by target size analysis (Schlegel et.al., 1979). However, definitive proof to support the authors contention must await purification of the glucagon receptor. Treatment of the covalently labelled M_r = 63,000 peptide with endo-β-*N*-acetylglucosaminidase F (Endo F) results in the appearance of four discrete bands suggesting that the peptide is a glycoprotein and contains four *N*-linked glycans (Iyengar and Herberg, 1984) having *N*-acetylglucosamine and/or sialic acid residues (Herberg et.al., 1984).

Structural analysis of the hepatic glucagon receptor revealed that elastase treatment of the unlabelled M_r = 63,000 peptide yields a M_r = 24,000 peptide that binds [^{125}I] MIG specifically and is sensitive to GTP but not ATP, indicating that the fragment retains the capacity to interact with the stimulatory guanine nucleotide regulatory protein, G_s. It is not sensitive to Endo F treatment suggesting that this peptide does not contain *N*-linked

glycans. The presence of detergent is needed for its solubilization implying that at least this part of the glucagon receptor monomer is embedded in the bilayer. The M_r = 24,000 peptide is said to be contained within a M_r = 33,000 peptide, the latter is formed upon treatment of the hormone-occupied M_r = 63,000 peptide with proteases (Iyengar and Herberg, 1984).

In order to understand the structure of the glucagon receptor, many chemical derivatives of glucagon have been prepared by treating native hormone with conventional protein-modifying reagents. Simultaneous modification of both free amino groups of the hormone causes a great decrease of binding affinity and ability to activate adenylate cyclase. An example is N^{α}-trinitrophenyl, 12-homoarginine glucagon (TH-glucagon) which is a competitive antagonist of glucagon at the glucagon receptor (Bregman et.al., 1980). It is generally accepted that the actions of glucagon on the liver cell metabolism are primarily mediated through changes in intracellular cyclic AMP levels. In man and experimental animals, the enhanced hepatic production of cyclic AMP observed after glucagon injection accounts for almost all of the increase in plasma concentration and urinary and biliary excretion of the nucleotide (Desbuquois, 1985). TH-glucagon has essentially no ability to activate adenylate cyclase from rat liver nor to increase the levels of cyclic AMP in isolated hepatocytes nor to stimulate cyclic AMP-dependent protein kinase activity (Cote and Epand, 1979). The ability of TH-glucagon to mimic the effect of glucagon on glycogenolysis, gluconeogenesis and urea production have cast some doubts on the involvement of cyclic AMP as the sole factor mediating the actions of glucagon (Corvera et.al., 1984) while others have suggested the existence of two glucagon receptor populations (Musso et.al., 1984). A recent discovery by Wakelam et.al. (1986) has altered fundamentally the idea on the postreceptor effects of glucagon. They demonstrated that both TH-glucagon and glucagon caused a rapid dose-dependent hydrolysis of phosphatidylinositol and that glucagon-stimulated production of inositol

1,4,5-trisphosphate is biphasic. Inhibition occurs at higher glucagon concentrations when adenylate cyclase activity is maximally stimulated where excess cyclic AMP production inhibits the polyphosphoinositide phosphodiesterase. They have suggested that hepatocytes possess two distinct receptors for glucagon, a GR-1 receptor coupled to the stimulation of inositol phospholipid breakdown and a GR-2 receptor coupled to the stimulation of adenylate cyclase activity. At physiological glucagon concentrations it is probable that a significant proportion of glucagon activity on cellular metabolic processes will be mediated via the GR-1 receptors. At higher glucagon concentrations, the functioning of the GR-2 receptors coupled to adenylate cyclase activation predominates. Thus, glucagon is able to exert its action on liver via two signal-transduction systems.

1.3.2 MECHANISMS OF GLUCAGON ACTION

Most, if not all, of the effects of glucagon are mediated by cyclic AMP, the synthesis of which is catalysed by the membrane-associated enzyme adenylate cyclase. This nucleotide activates cyclic AMP-dependent protein kinase , which in turn affects, by phosphorylation reactions, the activity of specific enzymes within the cell. Treatment of isolated liver cells with glucagon has been demonstrated to increase the phosphorylation of at least 12 cytoplasmic proteins, many of which are important regulatory enzymes in intermediary metabolism (Garrison and Wagner, 1982). Another mechanism through which glucagon could influence the intracellular cyclic AMP concentration is by modulating the activity of the enzyme, cyclic AMP phosphodiesterase. Recent developments have also shown an effect of glucagon on plasma membrane phospholipid metabolism as a second means of signal transduction of the hormone. The mechanism through which glucagon acts in both signal transduction systems will be discuss in detail below.

1.3.2.1 Changes in cyclic nucleotides

The glucagon-sensitive adenylate cyclase system in rat liver plasma membranes is composed of three functionally and structurally distinct entities : the receptor, which specifically binds glucagon, the guanine nucleotide-regulatory protein which mediates the activation of the catalytic unit of the adenylate cyclase by the glucagon-receptor complex and GTP and the catalytic unit of the cyclase, which converts ATP, the substrate, to cyclic AMP. Treatment of intact hepatocytes with glucagon causes a rapid and transient rise in intracellular cyclic AMP concentrations, which reach a peak within 5 minutes before returning to basal values within 30 minutes (Heyworth et.al., 1983a). This accumulation of cyclic AMP is largely due to the activation of adenylate cyclase by glucagon since the addition of 3-isobutyl-1-methylxanthine (IBMX), a phosphodiesterase inhibitor, greatly enhanced intracellular cyclic AMP accumulation but did not change the time for it to reach the maximal. In membranes, stimulation of adenylate cyclase activity by the hormone exhibits a lag time of several minutes (Rodbell et.al., 1974). The ability of glucagon to increase cyclic AMP concentration in intact cells and to stimulate adenylate cyclase activity in broken cell preparations is saturable with respect to hormone concentration. In the isolated perfused rat liver (Exton et.al., 1971) and in isolated rat hepatocytes (Christofferson and Berg, 1974), glucagon increases cyclic AMP concentration at least 50-fold with a half-maximal effect at about 10 and 1 nM respectively. In human liver membranes, glucagon stimulated the production of cyclic AMP with half-maximal activation elicited by 6 nM glucagon (Livingston et.al., 1985). Both glucagon-stimulated cyclic AMP accumulation in intact hepatocytes (Sonne et.al., 1978) and hormone-stimulated adenylate cyclase activity in liver membranes (Rodbell et.al., 1974) are nonlinearly related to receptor occupancy. Half-maximal stimulation occurs with less than 10 % receptor occupancy.

Glucagon stimulation of the liver adenylate cyclase activity was dependent on GTP as demonstrated in various animal species (Padrell et.al., 1987) and in human liver (Livingston et.al., 1985). In the absence of glucagon, GTP stimulates adenylate cyclase activity about threefold. Rodbell et.al., (1974) found that in the absence of added GTP, 100 % occupancy of glucagon receptors was required to achieve maximal activation of adenylate cyclase. At optimal GTP concentrations, the maximal adenylate cyclase activity found in the presence of glucagon was much greater, but only 10 % receptor occupancy was required. On the other hand, glucagon , at maximal concentrations, causes a marked increase in the ability of guanine nucleotides to stimulate adenylate cyclase activity.

Treatment of intact hepatocytes with glucagon led to a rapid time- and dose-dependent loss of the glucagon-stimulated response of the adenylate cyclase activity seen in membrane fractions isolated from these cells. This particular type of desensitization, by glucagon, does not occur in isolated membranes which exhibit linear rates of cyclic AMP production for at least 20 minutes (Houslay et.al, 1987). Any further addition of glucagon after 5 or 10 minutes failed to increase cyclic AMP production, showing that degradation of glucagon was not responsible for this event. Also, the rate of onset and the extent of desensitization were unaffected by the addition of IBMX. Since it has been found that challenge of hepatocytes with glucagon does not lead to any rapid internalization of glucagon receptors, it has been suggested that the desensitization process may involve a lesion occurring at the coupling interface of either the stimulatory guanine nucleotide regulatory protein, G_s, or the glucagon receptor. TH-glucagon, which has no effect on the intracellular cyclic AMP concentration, was also able to elicit desensitization of adenylate cyclase and this was mimicked by phorbol ester, 4β-phorbol-12β-myristate-13α-acetate (PMA) (Murphy et.al., 1987). Thus the authors suggested that the glucagon GR2 receptor-stimulated adenylate cyclase undergoes desensitization

through a cyclic AMP-independent process, which involves the stimulation of inositol phospholipid metabolism by glucagon acting through GR1 receptors. They proposed that the molecular mechanism for the desensitization of glucagon-stimulated adenylate cyclase may involved the phosphorylation of the glucagon receptor or G_s or both by protein kinase C. This is hardly surprising since it is known that activation of protein kinase C can either inhibit or enhance the accumulation of cyclic AMP promoted by activators of adenylate cyclase in different cell types (Kelleher et.al., 1984; Bell and Brunton, 1987).

Another means by which glucagon could affect cell metabolism is by acting on the cyclic AMP-dependent protein kinase. In hepatocytes, glucagon has been demonstrated to dissociate a holoenzyme of cyclic AMP-dependent protein kinase in a dose-dependent manner (Ciudad et.al., 1987) and by using two cyclic AMP analogues, Connelly et.al. (1987) provided direct pharmacological evidence for the activation of the cyclic AMP-dependent protein kinase in response to glucagon in the intact cells. The increase in free catalytic subunits explains the activation of this enzyme by glucagon in the liver.

Intriguingly, conditions which result in high levels of cyclic AMP in liver and fat tissue also lead to activation of phosphodiesterase (Loten et.al., 1978 ; Solomon, 1975). Allan and Sneyd (1975) reported that activation of the low K_m phosphodiesterase by glucagon was concentration dependent and occurred at the same concentration required to observe an increase in cyclic AMP levels in the cell. The maximal increase in activity occurred within 5 minutes of incubation with glucagon, similar to activation of adenylate cyclase, but the activity of the former was sustained for at least 35 minutes. It seems a reasonable hypothesis that the action of glucagon on hepatic phosphodiesterase is mediated by cyclic AMP via cyclic AMP-dependent protein kinase (Cherrington et.al., 1976). It has been shown that glucagon can stimulate the activity of ' dense-vesicle ' phosphodiesterase which is associated with a unique intracellular vesicle fraction resolved

on Percoll gradients (Wilson et.al., 1983). The mechanism whereby glucagon activates the 'dense-vesicle' enzyme is mediated by cyclic AMP.

1.3.2.2 Glucagon and phosphoinositide metabolism

Wakelam et.al. (1986) have reported that concentrations of glucagon below 10 nM can increase myo-inositol 1,4,5-trisphosphate (IP_3) by a cyclic AMP-independent mechanism and this phosphoinositide breakdown results in the mobilization of calcium from an intracellular pool. Inositol trisphosphate may act as a messenger to cause a release of Ca^{2+} from non-mitochondrial intracellular stores, thereby contributing to the increase in intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ (Berridge and Irvine, 1984). The glucagon action on cellular calcium has been well documented both in isolated hepatocytes and in perfused rat liver. Charest et.al. (1983) reported that glucagon could increase $[Ca^{2+}]_i$ in isolated hepatocytes without causing an increase in the concentration of inositol trisphosphate (Charest et.al., 1985). The results so far accumulated indicated that glucagon action on calcium mobilization is mediated by both cyclic AMP-dependent and -independent mechanisms. The glucagon-stimulated increases in the level of Ca^{2+} can be mimicked by the stimulatory diastereomer of adenosine cyclic 3',5'-phosphorothioate ((S_p)-cAMPS), and inhibited by pretreatment with the inhibitory diastereomer of adenosine cyclic 3',5'-phosphorothioate ((R_p)-cAMPS), a cyclic AMP antagonist (Connelly et.al., 1987). Both of these two compounds ((S_p)-cAMPS and (R_p)-cAMPS) bind to the regulatory subunit of the purified cyclic AMP-dependent protein kinase (O'Brian et.al., 1982), but only the (S_p) diastereomer releases the active catalytic subunit (DeWit et.al., 1982). Their results indicate that glucagon can elevate $[Ca^{2+}]_i$ via cyclic AMP and the cyclic AMP-dependent protein kinase. Blackmore and Exton (1986) have shown that glucagon, forskolin and 8-*p*-chlorophenylthio cyclic AMP produce

small increases in IP_3 in hepatocytes which are sufficient to mobilize intracellular Ca^{2+} (Lynch et.al., 1985).On the other hand, Mine et.al. (1988) reported that glucagon-induced elevation of $[Ca^{2+}]_i$ was mimicked by forskolin and exogenous cyclic AMP but these two agents induced a more prolonged elevation of $[Ca^{2+}]_i$. Glucagon-induced elevation of $[Ca^{2+}]_i$ was accompanied by a small increase in cyclic AMP. 3-isobutyl-1-methylxanthine addition potentiated the cyclic AMP production by glucagon but did not affect the glucagon-induced increase in $[Ca^{2+}]_i$ suggesting that part of glucagon's action on calcium mobilization is independent of cyclic AMP. Staddon and Hansford (1986) found that 4β-phorbol 12-myristate 13-acetate attenuates the increase in $[Ca^{2+}]_i$ caused by glucagon and suggested that it is probably mediated by a mechanism independent of changes in cyclic AMP concentration. However, the effects of glucagon on glycogenolysis and gluconeogenesis are not impaired in Ca^{2+}-depleted hepatocytes (Assimacopoulos-Jeannet et.al., 1977) indicating that Ca^{2+} plays no role in these actions of glucagon.

Another suggested mechanism for the way in which glucagon elevates $[Ca^{2+}]_i$ is by inhibition of the (Ca^{2+}-Mg^{2+})-ATPase activity. However, high concentrations of glucagon ($> 10^{-7}$ M) are required to produce this effect (Lotersztajn et.al., 1984). This effect was neither mimicked by cyclic AMP nor by dibutyryl cyclic AMP nor by TH-glucagon suggesting the absence of correlation between (Ca^{2+}-Mg^{2+})-ATPase inhibition and adenylate cyclase activation and inositol phosphate production. However, in the presence of bacitracin, an inhibitor of glucagon degradation, the Ca^{2+} pump is no longer sensitive to glucagon (Mallat et.al., 1987). They discovered that a proteolytically generated fragment of glucagon is responsible for the inhibition of the (Ca^{2+}-Mg^{2+})-ATPase. The physiological significance of the glucagon fragment in the regulation of liver

cell Ca^{2+} and other processes awaits further developments.

1.4 ORAL HYPOGLYCAEMIC AGENTS

The most commonly used drugs in treating diabetes mellitus come from two groups of compounds, the sulphonylureas and the biguanides. The first sulphonylurea approved by the Food and Drug Administration for use in the management of non-insulin dependent diabetes mellitus (NIDDM) or Type II diabetes mellitus was tolbutamide (in 1957) while a biguanide, phenformin, was approved by the FDA two years later (Kennedy et.al., 1988). Although tolbutamide was the most frequently used oral hypoglycaemic agent in the 1960's, the reported findings of the University Group Diabetes Project (UGDP) in 1970 suggested that the use of tolbutamide was related to an increase in cardiovascular mortality (UGDP, 1970) and later, the same group reported a similar finding for phenformin (UGDP, 1971). About nine years later, a reevaluation study of the first group found that the report unknowingly included cardiovascular high-risk patients in the tolbutamide group (Williamson and Kilo, 1980) thus opening to question the validity of analysis and conclusions of the first group report. The association of phenformin with fatal lactic acidosis ultimately resulted in the product's removal from the market in the United States (Anon, 1977). A recent survey has indicated that chlorpropamide is currently the most frequently used oral hypoglycaemic agent in the United States (Kennedy et.al., 1988) and that the second-generation sulphonylureas such a glyburide and glipizide have been readily accepted.

1.4.1 Molecular mechanisms of action of the oral hypoglycaemic agents

Although the ability of these agents to lower blood glucose is unquestioned, the underlying molecular mechanisms of action are still not fully resolved. Reports from many laboratories have indicated that sulphonylureas increase the number of insulin

receptors (Feinglos and Lebovitz, 1978; Zuber et.al., 1985). However, the effect of sulphonylureas on insulin binding has been variable. Salhanick et.al. (1983) found that tolazamide significantly increased insulin binding in hepatocytes from normal rats cultured in the absence of insulin. Others have reported that sulphonylureas had no significant influence on insulin binding (Vigneri et.al., 1982; Dolais-Kitabgi et.al., 1983). Maloff and Lockwood (1981) reported that tolazamide-potentiated, insulin-stimulated hexose transport is not associated with alteration in insulin binding, suggesting a direct effect of sulphonylureas on the postbinding pathways of insulin action. There is increasing evidence that sulphonylurea drugs exert long term hypoglycaemic actions that are partly extrapancreatic and related to potentiation of the effects of insulin (Lockwood et.al., 1983). Specific binding of sulphonylureas to receptors on the β-cell membrane has been shown (Lebovitz, 1984) and recent developments have indicated that the sulphonylurea receptor may be part of an ATP-sensitive K^+ channel (Sturgess et.al., 1985).

The effect of biguanides on insulin receptor binding has also been inconsistent. It has been reported that metformin increased insulin receptor binding in erythrocytes of normal subjects (Holle et.al., 1981) and in various cell lines (Vigneri et.al., 1982). However, metformin can increase the hypoglycaemic response to insulin without a measurable effect on insulin receptor binding (Lord et.al., 1983; Lord et.al., 1985). The latter results suggest that metformin can influence postreceptor sites of insulin action independent of insulin receptor binding.

The effect of sulphonylureas on adenylate cyclase activity may be variable, depending on the tissue studied and the experimental system used. Lasseter et.al. (1972) reported a stimulation of myocardial adenylate cyclase activity by sulphonylureas whereas an inhibition of renal adenylate cyclase activity was noted by Leichter and Chase (1978). Luly et.al. (1977) have reported that chlorpropamide decreased the intracellular cyclic

AMP concentration of isolated hepatocytes without being effective on plasma membrane-bound adenylate cyclase. This effect is also seen with phenformin.

The effect of sulphonylureas on cyclic AMP-dependent phosphodiesterase has also been inconsistent. Goldfine et.al. (1971) observed that tolbutamide inhibits cyclic AMP-dependent phosphodiesterase activity in preparations of rat brain, liver, lung, and kidney. Brooker and Fichman (1971) demonstrated that chlorpropamide and tolbutamide inhibited cyclic AMP-dependent phosphodiesterase in rat kidney. However, Luly et.al. (1977) demonstrated that chlorpropamide, as well as phenformin, stimulated low K_m cyclic AMP-dependent phosphodiesterase activity in the rat liver plasma membrane and this is associated with a marked decrease in intracellular cyclic AMP level. A second generation sulphonylurea, glyburide, was found to restore low K_m cyclic AMP-dependent phosphodiesterase activity in the liver and fatty tissue of streptozotocin-treated diabetic rat (Solomon et.al., 1986a).

Tolbutamide has been shown to increase soluble and particulate guanylate cyclase activity in rat liver, lung, colon, pancreas, kidney cortex, heart and spleen (Vesely, 1986). Further development is needed to elucidate the role of cyclic GMP in sulphonylurea action.

1.5 AIMS OF THE PROJECT

These may be summarized as follow :-

i) to develop a hormone- and serum-free cell culture system for cultivation of hepatocytes from adult rats under maximally controlled conditions to make it possible to study , *in-vitro*, the effect of the individual hormones, insulin and glucagon, on steroid metabolism. Under controlled conditions, this will enable us to ascribe any change in androst-4-ene-3,17-dione metabolism to the added hormone.

ii) to investigate the acute and chronic effect of insulin on steroid metabolism in hepatocytes from normal, 3- and 21-days STZ-diabetic rats and insulin-treated STZ-diabetic rats.

iii) to investigate the possible molecular mechanisms of action of insulin that brought about the changes in androst-4-ene-3,17-dione metabolism on acute exposure to the hormone in normal rat hepatocytes.

iv) to investigate the acute and chronic effect of glucagon on androst-4-ene-3,17-dione metabolism in hepatocytes from normal and 3 days STZ-treated diabetic rats.

v) to investigate the acute and chronic effect of TH-glucagon, a glucagon antagonist, on androst-4-ene-3,17-dione metabolism in hepatocytes from normal rats.

vi) to investigate the effect of insulin in the presence of glucagon on androst-4-ene-3,17-dione metabolism in normal rat hepatocytes.

vii) to investigate the effect of phenformin or tolbutamide on androst-4-ene-3,17-dione metabolism in hepatocytes from normal and 3 days STZ-treated diabetic rats.

viii) to investigate the effect of phenformin or tolbutamide in the presence of physiological insulin concentration (10^{-9} M) on androst-4-ene-3,17-dione metabolism in hepatocytes from normal and 3 days STZ-treated diabetic rats.

ix) to investigate the effect of insulin in the presence of phenformin or

tolbutamide on androst-4-ene-3,17-dione metabolism in hepatocytes from normal and 3 days STZ-treated diabetic rats.

METHODS AND MATERIALS

2.0 EXPERIMENTAL ANIMALS

Mature male or female rats of the Wistar strain, bred in the department were used throughout the study. The animals were allowed free access to water and food (CRM Nuts, Labsure, Croydon) and housed in light- and temperature-controlled conditions (light 0800-2000; 19 ± 1^0 C) and were used when they were 10-12 weeks old at the beginning of the experiment. When comparing groups age-matched animals were used, and each group consisted of at least four animals. The weights of individual rats ranged from 250 to 300 g and variation within each group was ± 25 g.

In experiments where blood was required for detection of serum glucose levels, the animals were asphyxiated by carbon dioxide in a sealed box. Immediately after cervical dislocation, the throat was cut and blood was collected by using a 10 ml syringe. The blood was allowed to clot at + 4^0 C and serum prepared by centrifugation at 2000*g* for 10 minutes at 4^0 C (in a DAMON/IEC Model DPR-6000 refrigerated centrifuge). The upper serum layer was removed using a Pasteur pipette and then stored at -20^0 C. The assay was usually done within 3 days of serum collection.

In experiments where diabetes mellitus was induced by streptozotocin (STZ), the rats were weighed before treatment and then 3 days or 21 days post-treatment prior to sacrifice.

2.1 INDUCTION OF DIABETES BY STREPTOZOTOCIN

Diabetes mellitus could be induced in experimental animals by administering to the animals the β-cell toxins, alloxan or streptozotocin. The diabetogenic action is mediated by selective destruction of pancreatic β-cells. Alloxan, however, does not

produce a good model of diabetes because of its overt toxicity (Hoftiezer and Carpenter, 1973). Streptozotocin may provide a better model for type 1 (insulin-dependent) diabetes than alloxan because of its more selective β-cell toxicity (Arison and Feudale, 1967).

Since STZ is unstable in solution, it was dissolved in distilled water immediately before use and then injected intravenously (100 mg/kg) into the tail vein of the rat under halothane/nitrous oxide anaesthesia. The tail was rubbed with cotton wool dampened with xylene to dilate and expose the vein before injecting the drug. Control animals were treated identically except that they were injected with vehicle only. The animals were left for 3 and 21 days after injection before sacrifice.

2.2 INSULIN TREATMENT OF STZ-DIABETIC RATS

Insulin-treated STZ diabetic rats received 2, 12 or 16 units of insulin subcutaneously (Neulente Insulin Zinc Suspension), given one hour after STZ and at 24 hour intervals for two days, after which they were sacrificed. The international unit of insulin is the activity contained in 0.04167 mg of the Fourth International Standard Preparation (1958).

2.3 ISOLATION OF RAT HEPATOCYTES

Isolated hepatocytes from control, 3 - days and 21 - days STZ - treated and insulin - treated STZ - diabetic male and female (where appropriate) rats were prepared using collagenase digestion, a modification of the method described by Seglen (1973). While the rat is under anaesthesia (3.5 % halothane : 400 ml/min oxygen : 800 ml/min nitrous oxide - Mini Boyle anaesthetic machine), the abdomen was cut open and the portal vein was freed of any fat. A cannula was inserted into the vein at a position just before it branches into the liver. Perfusion (using a Watson Marlow 501 U perfusion pump) was commenced with calcium-free Hank's balanced salt solution

(calcium-free HBSS) at a rate of 90 ml/min until the liver reached twice its normal size after which the vena cava was cut and the perfusion rate reduced to 50 ml/min. The perfusion with calcium-free HBSS was allowed to continue for another 7 minutes. The perfusion was then switched over to warm (37^0 C) collagenase buffer (calcium-free HBSS supplemented with 4 mM calcium chloride and 0.5 mg/ml collagenase). The collagenase buffer was recycled for about 12.5 minutes before the liver was removed from the animal into a petri dish containing calcium-free Hank's balanced salt solution. The liver capsule was gently removed and the cells dispersed using a steel comb. The suspension of cells was filtered through gauze and centrifuged at 200*g* for 2 minutes to clear the parenchymal cells of debris. The isolated cells were resuspended in incubation medium (calcium-free HBSS supplemented with 1 g/L glucose, 100 mg/L $MgSO_4$, 100 mg/L $MgCl_2$ and 185 mg/L $CaCl_2$) and their number counted in a haemocytometer with average yield of 1 x 10^8 cells/g liver. Viability was estimated by their exclusion of a vital dye, trypan blue, and cell preparations which showed an initial viability greater than 90 % were used in the experiments.The buffers were bubbled for 20 minutes with oxygen : carbon dioxide (95 % : 5 %) before use and continuously during perfusion and care was taken not to introduce any air bubble into the portal vein. Viability of the cells was also checked after preincubation and the number of cells at this stage was used in the final calculation.

2.4 DEVELOPMENT AND CHARACTERIZATION OF HORMONE-FREE CULTURE MEDIUM

Suitable medium for maintaining androst-4-ene-3,17-dione metabolism in primary cultures of adult rat hepatocytes was investigated. Ham's F-10 nutrient media supplemented with amino acid or serum were chosen and tested. Hepatocytes from

normal *male* and *female* rats were cultured in Ham's F-10 medium supplemented with either 2.5 % foetal calf serum and 15 % horse serum **or** a multihormone serum substitute, 2% Ultroser G (LKB Products, Sweden) for 3 and 6 days. Medium was changed after 3 days. In a second set of experiments, *male* rat hepatocytes were cultured with Ham's F-10 medium supplemented with 2 % Ultroser G for 24 hours. The medium was removed and then replaced with Ham's F-10 supplemented with 0.1 % bovine serum albumin and left for a further 24, 48 and 72 hours. In another similar time-scale experiment, *male* rat hepatocytes were cultured in Ham's F-10 medium supplemented with 0.1 % bovine serum albumin only.

Androst-4-ene-3,17-dione metabolism by normal male rat hepatocytes using five different amounts of hepatocytes (1, 2, 3, 5 and 10 million cells/incubation) and five time periods of incubation (5, 10, 20, 30 and 60 minutes) was also studied.

2.5 ADDITION OF HORMONES AND DRUGS

2.5.1 Preincubation with insulin *or* glucagon *or* TH-glucagon for 1/2, 1 and 2 hours

The serum-free nutrient medium used was Ham's F-10 medium containing the following additions/liter : 100 U of penicillin and 100 µg of streptomycin. The cells were plated out on sterile petri dishes (NUNCLON, Denmark, 9 cm diameter) at a density of 3 x 10^5 cells/cm^2 in Ham's F-10 nutrient medium supplemented with 0.1 % bovine serum albumin (10 ml final volume). The plates were swirled carefully to ensure even distribution of cells over the surface of the plates. The additions of hormones and drugs were made directly to the cell culture and in all cases the compounds were added in the smallest volume possible and controls were treated with a similar amount of vehicle. Insulin, glucagon and TH-glucagon were dissolved in 0.1 M hydrochloric acid and their additions did not alter the pH of the medium. The hormones and drugs were added at concentrations ranging from 10^{-10} to 10^{-6} M. The plated cells were subsequently

incubated at 37^0 C under 95 % air and 5 % CO_2 in a humidified incubator (Forma Scientific Model 3028) for the various time period, i.e. 1/2, 1 and 2 hours.

2.5.2 Preincubation with insulin *or* glucagon *or* TH-glucagon for 24, 48 and 72 hours

The method was essentially similar to the short period incubation as described above (**2.5.1**) except that cells were initially plated in Ham's F-10 nutrient medium supplemented with 2 % Ultroser G (LKB), which is a multihormone serum substitute, to promote monolayer cell formation. After a period of 24 hours, the medium was removed and replaced with Ham's F-10 supplemented with 0.1 % bovine serum albumin. The hormones or drug (concentrations ranging 10^{-10} to 10^{-6} M) were then added and the cells preincubated for a further 24,48 or 72 hours. In one set of experiments, insulin (10^{-9} M) was added at 24-hour intervals from 0 to 72 hour.

2.5.3 Preincubation with glucagon for 1/2, 1, 2, 5 and 10 minutes

The method was essentially similar to that described in (**2.5.1**) except that due to the very short preincubation time intervals involved, experiments were conducted in glass tubes. Isolated hepatocytes (~ 2 x 10^7 cells/ml) were suspended in Ham's F-10 nutrient medium containing 0.1 % bovine serum albumin (final volume of 10 ml). Glucagon was added (at various concentrations between 10^{-10} and 10^{-6} M) and the tubes inverted a few times to ensure uniform distribution of the hormone. The tubes were then incubated at 37^0 C in a rotatory shaking water bath (Citenco, U.K.) for the indicated time period.

2.5.4 Preincubation with insulin *and* glucagon for 1/2 hour

Hepatocytes from normal or 3-days STZ-treated rats were plated out in petri

dishes in Ham's F-10 nutrient medium supplemented with 0.1 % bovine serum albumin. Various combinations of insulin and glucagon concentrations were added directly to the petri dish and then left in the incubator for 1/2 hour.

2.5.5 Preincubation with insulin *or* glucagon in the presence of K-252a for 1/2 and 24 hours

Primary cultures of hepatocytes were established by plating cells in the petri dishes in Ham's F-10 nutrient medium supplemented with 0.1 % bovine serum albumin. K-252a (20 nM) was added to the plated cells and then left in the incubator for 15 minutes. The hormones were then added (10^{-10} to 10^{-6} M) and the cells reincubated at 37^0 C for a further 1/2 or 24 hours. K-252a was dissolved in dimethylsulphoxide and controls were treated with a similar amount of vehicle.

2.5.6 Preincubation with oral hypoglycaemic agents in the absence or presence of insulin

Phenformin and tolbutamide were dissolved in 0.1 M NaOH. Phenformin or tolbutamide (concentration ranging from 10^{-6} to 10^{-3} M) was added to primary cultures of hepatocytes obtained from normal or STZ-treated rats, and the culture was left in the incubator for 24 hours. In another set of experiments, the cells were further challenged with insulin for 1/2 hour after preincubating with the drugs for 24 hours.

2.6 ASSAY OF STEROID METABOLISM

After treatment with the hormones or drugs for the various time periods, the cells were harvested by scraping the plates with a rubber policeman. The suspensions were centrifuged (200*g*; 2 min; 4^0 C) and the supernatant layer discarded. The cells were washed once with incubation medium and finally resuspended in the same medium.

The cells were counted, assessed for viability and diluted with incubation medium to a concentration of 3 x 10^6 cells/ml and incubated with the substrate, androst-4-ene-3,17-dione, by a modification of the method of Berg and Gustafsson (1973) as described below. Triplicate samples at each hormone or drug concentration were taken in all cases. The calculated final substrate concentration in the incubation was 0.58 mM and this represents a saturating concentration of substrate for some, but not all, of the enzymes studied (Skett et.al., 1978). This concentration of substrate was employed as this is the limit of solubility of androst-4-ene-3,17-dione under the conditions used in the assay.

2.6.1 Incubation and assay procedure

Each incubation tube contained the following reagents in a total volume of 3 ml :

1.0 ml of the diluted cell suspension

50 µl of androst-4-ene-3,17-dione (500 µg, dissolved in acetone)

10 µl of [4-^{14}C]-androst-4-ene-3,17-dione (10^5 c.p.m, dissolved in acetone)

1.94 ml of incubation medium

The tubes were incubated in a rotatory shaking waterbath at 37^0 C for 30 minutes. The incubations were terminated by the addition of 10 ml of Folch's solution (chloroform : methanol; 2:1; v/v) and 1 ml of 0.9 % sodium chloride. The tubes were shaken gently and left to stand overnight for phase separation. The tubes were centrifuged (500*g*; 5 minutes) for complete separation between the aqueous and organic phases. The lower organic layer was transferred to another tube and dried at 40^0 C under oxygen-free nitrogen using a Techne Dri-Block. The extract was redissolved in 5 drops of chloroform (in ultrasonic waterbath for at least 30 seconds) and then spotted on silica gel thin layer

chromatography plates (20 cm x 10 cm; Merck, Darmstadt, F.R.G.). A mobile phase consisting of chloroform : ethyl acetate (4 : 1; v/v) was used for chromatographic separation of substrate and metabolites. The tanks were equilibrated with the solvent mixtures for one hour before commencing the separation. The samples were spotted (3 samples on a plate) at the lower side of the t.l.c. plate (~1.5 cm in), using a 10 µl disposable micropipette. After the solvent front had run to about 1 cm from the edge of the plates they were dried in warm air. Individual substrate and metabolites were visualised by autoradiography (Kodak, France) and have previously been identified (see Figure 2) by gas chromatography - mass spectrometry using authentic standards (Berg and Gustafsson,1973). The radio-labelled bands (see Figure 3) were scraped into polythene scintillation vials and suspended in Ecoscint liquid scintillant. Radioactivity in each sample was measured in a Packard Tri-Carb (Model 2000 CA) scintillation counter and enzyme activity calculated using a custom-made computer program.

2.7 DETERMINATION OF CYTOCHROME P-450 CONTENT

Cytochrome P-450 concentrations were determined from the carbon monoxide difference spectra of the reduced protein by the method described by Omura and Sato (1964), with an extinction coefficient of 91 mM^{-1} cm^{-1} for the wavelength couple 450-490 nm. All solutions and utensils were pre-cooled on ice before the start of the experiment. Cells suspended in incubation medium (5 x 10^6 cells/ml) were homogenised using a Potter-Elvehjem homogeniser with a tight-fitting Teflon pestle for 10 complete strokes. Trypan blue inclusion was used as a method to indicate the cells were broken.

A base line was then determined between 400 nm and 500 nm. The sample

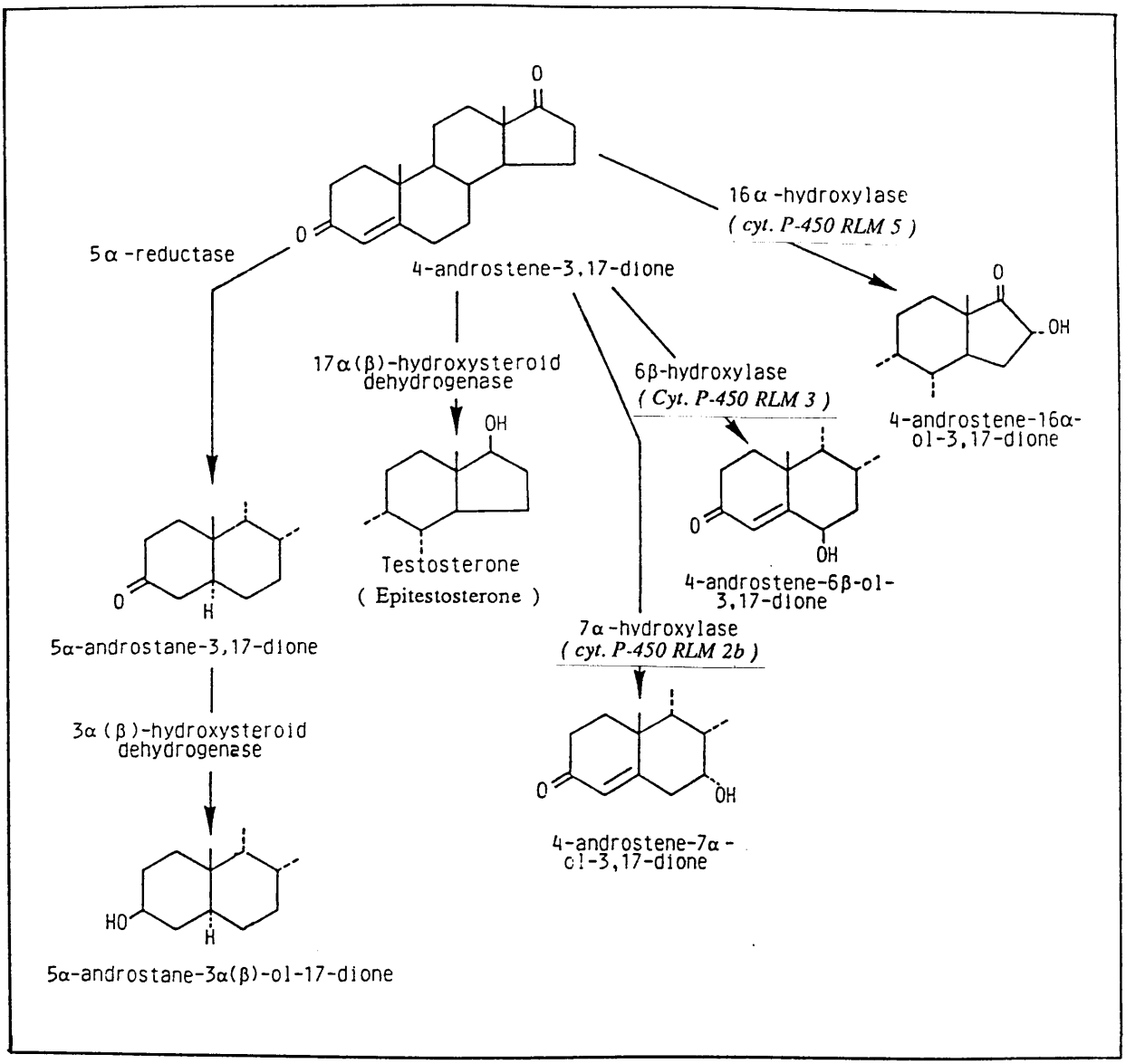


Figure 2. The phase 1 metabolism of androst-4-ene-3,17-dione in the liver Specific isoenzymes thought to produce the various metabolites are given in the *italic*.

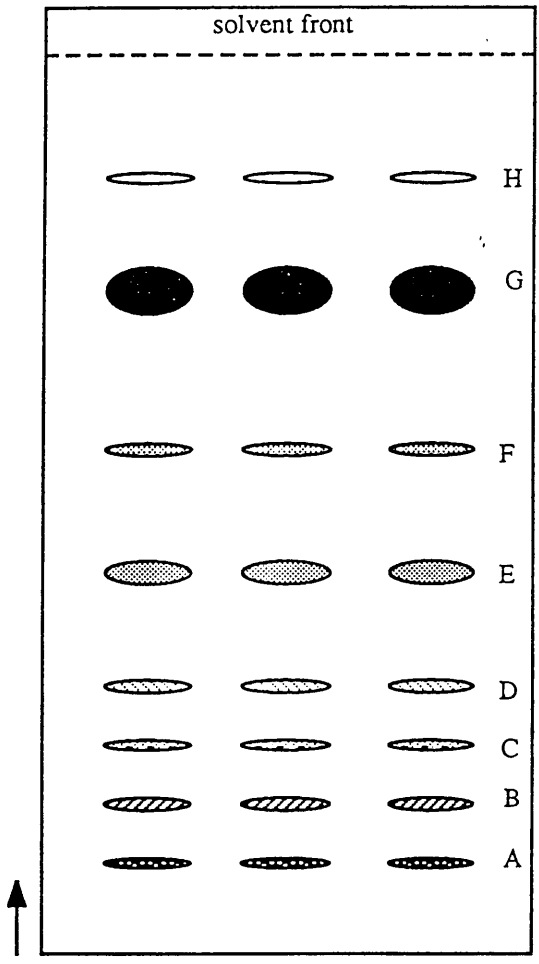


Figure 3. Schematic representation of androst-4-ene-3,17-dione metabolites separation by one-dimensional thin layer chromatography

specific bands identified by autoradiography are indicated :

A. Spotting band
B. Androst-4-ene-7 α -ol-3,17-dione
C. Androst-4-ene-6 β -ol-3,17-dione
D. Androst-4-ene-16 α -ol-3,17-dione
E. Testosterone / Epitestosterone
F. 5 α -Androstane-3 α (β)-ol-17-dione
G. Unchanged substrate Androst-4-ene-3,17-dione
H. 5 α -Androstane-3,17-dione

suspension was put into two cuvettes; 1 ml aliquots in each. Similar amount of the reducing agent, sodium dithionite, was added to both sample and reference cuvettes with gentle stirring and the sample cuvette only was gently bubbled with carbon monoxide for approximately 60 seconds. Cytochrome P-450 content of the homogenised cell preparation was then determined using a dual beam Shimadzu UV-240 recording spectrophotometer. The cytochrome P-450 content being expressed as nanomoles P-450/ million cells.

2.8 HEPATOCYTE CYCLIC AMP CONTENT

Following incubation with insulin as described in Section **2.5**, cell samples were pooled and counted and then centrifuged (200*g* ; 2 min ; room temperature). The supernatant was removed and the cells were resuspended in sodium acetate buffer to give an approximate concentration of 1×10^7 cells per ml (an expected concentration of 20 nM cyclic AMP). 0.5 ml of 10 % perchloric acid was added to each sample and vortex-mixed. 1 M potassium hydroxide in 0.9 % sodium chloride (to precipitate excess perchloric acid as potassium perchlorate) was added to adjust the pH to 7 and the final volume was adjusted to 2 ml by adding 0.9 % sodium chloride. The mixture was centrifuged (500*g* ; 5 min ; room temperature) and a third of the supernatant was collected and resuspended in sodium acetate buffer (final volume of 1 ml ; pH 5) The samples were then stored at -20^0 C prior to assay for cyclic AMP.

The cyclic AMP concentration was estimated by radioimmunoassay essentially as described by Brooker et.al. (1979). Authentic cyclic AMP standard concentrations (0.01-100 *p*moles/100 µl ; triplicate samples) were prepared in sodium acetate buffer. To maximise assay sensitivity, samples (100 µl) were acetylated by the addition of 5µl of a freshly made mixture of acetic anhydride and triethylamine, 1: 2 by

volume (Frandsen & Krishna, 1976) and the samples were rapidly vortex-mixed. To each tube was added 150 µl of a 1/10,000 dilution (0.1 % bovine serum albumin) of anti-cyclic AMP serum (raised in goats against human serum albumin conjugated to succinyl cyclic AMP) and 25 µl of a 1/175 dilution of [^{125}I] - iodotyrosine methyl ester succinyl cyclic AMP (approximately 3000 cpm/sample). Samples were mixed and incubated for 16 hours at 4^0 C.

After this incubation period, 0.5 ml of a suspension of washed charcoal (1 % , w/v) in potassium phosphate buffer containing 0.25 % bovine serum albumin (at 4^0 C) was added. The samples were mixed and the charcoal sedimented by centrifugation (4000*g* ; 4 min ; 4^0 C). Aliquots (0.5 ml) of the supernatant were taken to assess antibody -bound [^{125}I] - cyclic AMP which was counted (2 min) in a gamma counter (LKB 1275 Gamma Counter). Standard calibration curves in each experiment were plotted as radioactivity bound (cpm) against concentration of cyclic AMP or as B/B_0 against concentration of cyclic AMP where B and B_0 represent the amount of [^{125}I] - cyclic AMP bound in the presence or absence, respectively, of unlabelled cyclic AMP. In the absence of unlabelled cyclic AMP, approximately 30 % of the total added [^{125}I] - cyclic AMP was bound. Amounts of cyclic AMP in unknown samples were determined by reference to the calibration curve. 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), 10^4 fold (AMP, ADP, ATP) and 10^3 fold (adenosine) higher concentrations of the other nucleotides required to produce 50 % displacement of the [^{125}I]-cyclic AMP (Berry and Skett , 1988).

2.9 PHOSPHOLIPID STUDIES

2.9.1 Lipid Extraction

Hepatocytes (5 x 10^7 cells/ml) suspended in a phosphate free buffer were incubated (37^0 C; 90 minutes) with carrier-free [^{32}P]-orthophosphate (30 μCi/ml) and 0.025 % bovine serum albumin. At the end of the incubation time the suspension was centrifuged at 200*g* for 2 minutes and the supernatant layer removed. The pellet was washed once with phosphate free buffer to remove excess carrier-free [^{32}P] - orthophosphate and the cells (4 x 10^7 cells/ml) resuspended in 10 ml of the same buffer and 1.0 ml aliquots were dispensed into glass tubes containing nutrient medium Ham's F-10 supplemented with 0.1 % bovine serum albumin (final volume 10 ml) at 37^0 C. The cells were allowed to equilibrate for 10 minutes before being challenged with insulin. After the appropriate time, the reaction was terminated and the lipids extracted essentially by the method of Schacht, (1981) as described below.

Briefly, the treated-cell suspension was centrifuged at 200*g* for 2 minutes and the supernatant layer removed. The reaction was terminated by adding ice-cold 10 % trichloroacetic acid (w/v) to the lipid soluble pellet fraction. After thorough mixing of their contents, the tubes were centrifuged (1000*g*; 10 min) and the supernatant layer discarded. A solvent mixture (consisting of 1.5 ml of chloroform-methanol 1: 2; 0.5 ml of chloroform; 1 ml of 1 M hydrochloric acid) was added to each sample, vortex - mixed, centrifuged (500*g*; 5 min) and the lower phase was transferred into new tubes. The interface and upper phase were extracted twice with 1 ml of chloroform. The combined lower phases were washed with 2 ml of methanol-1M HCl, 1:1 (v/v), and the upper phase was discarded after centrifugation (500g; 5 min). The lower organic phase was then dried at 40^0 C under oxygen-free nitrogen, and stored at -20^0 C until used.

2.9.2 Separation by thin-layer chromatography of phosphatidic acid and phosphatidylinositol

Lipids were dissolved in 0.1 ml of chloroform-methanol, 9:1 (v/v) and spotted on silica-gel t.l.c. plates (10 cm x 10 cm, Silica Gel, Merck, Darmstadt) for two-dimensional separation of phospholipids (Yavin and Zutra, 1977). This t.l.c. system employs 3 basic solvents ; in the first dimension - chloroform : methanol : 40 % aqueous methylamine (13:6:1.5, v/v/v); diethylether : glacial acetic acid (19:1, v/v) for the intermediary run (through the second dimension) and chloroform : acetone : methanol : glacial acetic acid : distilled water (10:4:2:3:1, v/v/v/v/v) for the second direction. The chromatographic chamber was lined at both ends with Whatman I filter paper, and the tanks were equilibrated with the solvent mixtures for 1 hour before commencing the separation. All three organic mixtures were freshly prepared .

Phospholipid samples were applied to the lower left hand corner (2 cm in) of the t.l.c. plates, using a 10 µl disposable micropipette. A total of 8 plates could be run in one batch. The solvent front was allowed to run within 1 cm from the edge of the plate, they were then removed and dried in a stream of warm air for 15 minutes whilst placed on a tray. Each plate was then exposed to the fumes of a concentrated solution of HCl (36 % w/w) for approximately 5 minutes. The plates were then dried in warm air for approximately 5 minutes, then cool air for an additional 5 minutes. The plates were then placed in the second solvent running in the second dimension (origin at lower right corner) for a period of 15 -20 minutes. Following this, the plates were removed, dried in a stream of cool air for 5 minutes before placing in the third solvent in the same dimension as the second solvent. After the solvent front had run to 1 cm from the edge of the plates they were dried thoroughly in a stream of cool air. After drying, the phospholipids were visualized by placing the plates in an iodine vapour tank for

approximately 3 minutes. The spots corresponding to phosphatidic acid and phosphatidylinositol (see figure 4) were scraped into polythene scintillation vials and counted for radioactivity in a liquid-scintillation counter.

2.10 ASSAY OF SERUM GLUCOSE

Serum glucose was measured colourimetrically using an assay kit obtained from Sigma, U.K. Serum glucose was assayed by the method of Carroll et.al. (1970) and is based on the hexokinase catalysed conversion of glucose to glucose-6-phosphate coupled with the subsequent reduction of nicotinamide adenine dinucleotide phosphate [NADP] to [NADPH] by action of glucose-6-phosphate dehydrogenase. The final formation of a reduced quaternary salt was measured colourimetrically at 520 nm in a spectrophotometer. The colourimetric response is proportional to the glucose concentration and serum glucose concentration was calculated from the following formula :

$$\text{Glucose [mmol/L]} = \frac{\text{A test}}{\text{A standard}} \times 5.56$$

A = absorbance at 520 nm

2.11 CALCULATION AND STATISTICS

Results were expressed as % of the relevant control. Means and standard deviations were calculated using a custom-made computer program and statistical analysis was performed by means of Student's *t*-test. Statistical significance was set at $P < 0.05$ in all cases.

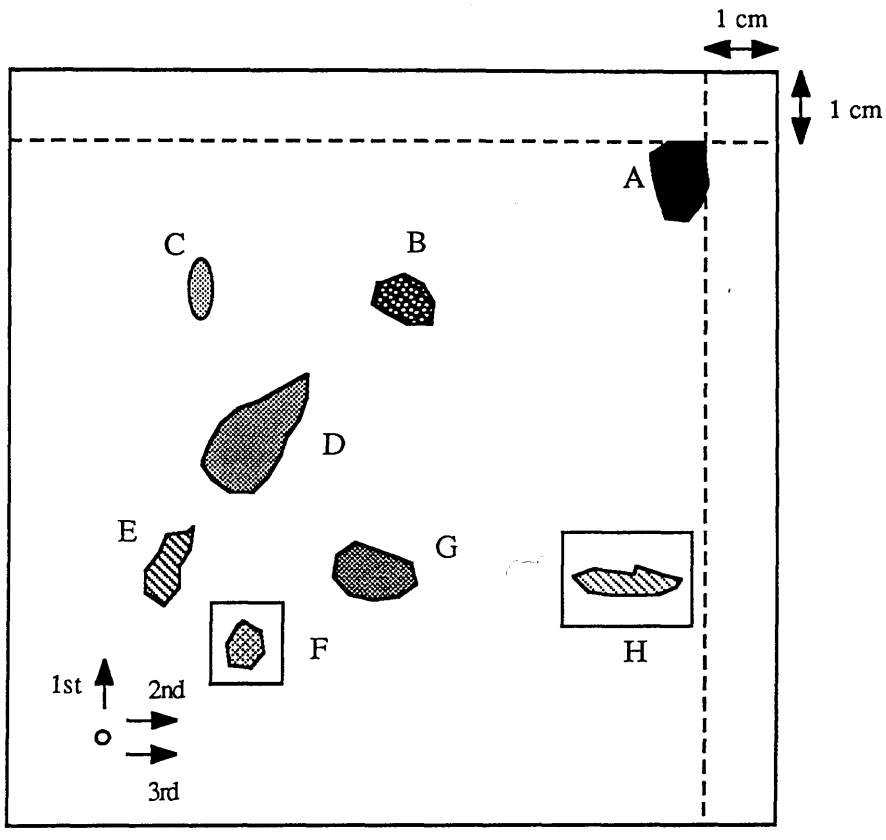


Figure 4. Schematic representation of phospholipid separation by two-dimensional thin layer chromatography

Phospholipids identified by iodine staining are indicated.

o - origin
A. Neutral lipid
B. Phosphatidylethanolamine
C. Plasmalogen lyso-phosphatidyl ethanolamine
D. Phosphatidylcholine
E. Sphyngomyelin
F. Phosphatidylinositol
G. Phosphatidylserine
H. Phosphatidic acid

2.12 SOURCES OF CHEMICALS AND HORMONES USED

Androst-4-ene-3,17-dione	Sigma Chemical Co., Poole, Dorset.
[4-^{14}C]androst-4-ene-3,17-dione	Amersham International plc. (Bucks.).
Bovine serum albumin (Fraction V)	Sigma Chemical Co., St. Louis, U.S.A.
Collagenase	Boehringer Mannheim Co. (F.R.G).
Cyclic AMP	Sigma Chemical Co., St. Louis,U.S.A.
Adenosine 3',5'-cyclic phosphoric acid 2'-0-succinyl 3-[^{125}I] iodotyrosine methyl ester. (^{125}I-cyclic AMP)	Amersham International plc. (Bucks.).
Glucagon	Sigma Chemical Co., Poole, Dorset.
Glucose assay kit	Sigma Chemical Co., Poole, Dorset.
Ham's F-10 culture medium	GIBCO BRL Limited, Paisley, Scotland.
Insulin (porcine)	NOVO Research Institute, Denmark.
K-252a	Kyowa Hakko Kogyo Co., Japan. (a gift from Dr. H. Kase).
Neulente Insulin Zinc Suspension BP	Wellcome, Welwyn Garden City, U.K.
[^{32}P] orthophosphate carrier free	Western Infirmary, Glasgow University.
Penicillin / Streptomycin	GIBCO BRL Limited, Paisley, Scotland.
Phenformin	Sterling Winthrop, Surrey, U.K.
Phosphatidic Acid	Sigma Chemical Co., St. Louis, U.S.A.
Streptozotocin	Sigma Chemical Co., St. Louis , U.S.A.
TH-Glucagon	A gift from Dr. V.J. Hruby (U.S.A)
Tolbutamide	Sigma Chemical Co., St. Louis, U.S.A.
Ultroser G	LKB Products, Sweden.
X-ray films (X-Omat S)	Kodak, France.

All other chemicals used were of analytical grade.

2.13 BUFFERS AND OTHER SOLUTIONS USED

1) Calcium-free Hank's balanced salt solution (10 % stock solution)

KCl	-	2.0 g/L
KH_2PO_4 (anhydrous)	-	0.3 g/L
NaCl	-	40.0 g/L
$NaHCO_3$ (anhydrous)	-	10.5 g/L
Na_2HPO_4 (anhydrous)	-	0.238 g/L

The above were dissolved in distilled water and the pH adjusted to 7.4 using HCl (1M).

2) Incubation medium

Glucose	-	1.0 g/L
$MgCl_2 . 6H_20$	-	0.1 g/L
$MgSO_4 .7H_20$	-	0.1 g/L
$CaCl_2 . 6H_20$	-	0.185 g/L
HBSS	-	100 ml of 10 % stock solution

The above were dissolved in 1 L of distilled water.

3) Potassium phosphate buffer (100 mM) :-

$K_2HPO_4 . 3H_20$ (200 mM)	-	66.25 ml
KH_2PO_4 (anhydrous) (200 mM)	-	183.75 ml
Distilled water	-	250 ml

The above were mixed and the pH was adjusted to 6.4 using HCl (10 M).

4) Phosphate free buffer for phospholipid studies

NaCl	-	8.18 g/L
$NaHCO_3$ (anhydrous)	-	0.24 g/L
KCl	-	0.37 g/L
$MgCl_2 . 6H_20$	-	0.20 g/L
$MgSO_4 . 7H_20$	-	0.015 g/L
Glucose	-	1.01 g/L
HEPES	-	3.60 g/L
$CaCl_2 . 6H_20$	-	1.60 mM
Bovine serum albumin	-	0.025 %

The above were dissolved in distilled water and the pH was adjusted to 7.2 using HCl (1M).

RESULTS

3.0 DEVELOPMENT AND CHARACTERIZATION OF HORMONE-FREE CULTURE MEDIUM

The assay for metabolism of androst-4-ene-3,17-dione allowed for the measurement of the 6β-, 7α- and 16α-hydroxylases, 17-oxosteroid oxidoreductase (17-OHSD) and 5α-reductase activities. Hepatocytes from adult rats were isolated and cultivated as primary monolayers in four different types of culture media.

Male rat hepatocytes cultured in Ham's F-10 medium supplemented with 2.5 % foetal calf serum and 15 % horse serum show a fall in activity of between 15 to 50 % of control depending on the enzyme studied after 3 days in culture (Table 1, top panel). After 6 days in culture, the enzyme activities were partially restored and this varied with the enzymes measured. The activity of the female-specific enzymes, 7α-hydroxylase and 5α-reductase recovered to about 90 % and 80 % of control respectively and the male-specific enzymes, 6β- and 16α-hydroxylases and 17-OHSD to 59 %, 23 % and 97 % of control respectively. With the **female** rat hepatocytes, sexual differences in androst-4-ene-3,17-dione metabolism could be detected after 3 days of culture in the same culture medium. Selective significant decreases in both the female-specific enzymes activities, 7α-hydroxylase and 5α-reductase (79 % and 21 % respectively) could be observed (Table 2, top panel). Significant increases in the male-specific enzyme activities, 6β-hydroxylase and 17-OHSD, could be seen while 16α-hydroxylase activity was unaltered when compared to freshly prepared cells. After 6 days in culture, with the exception of the 5α-reductase activity (still significantly below control level), all the enzyme activities increased and exceeded the control values.

Similar decreases in enzyme activity were seen when **male** rat hepatocytes were cultured for 3 days in Ham's F-10 supplemented with a well-defined synthetic serum substitute, Ultroser G 2 %. No sexual differences in steroid metabolism were observed

	Enzyme activities (pmoles / min / million cells)				
	7α - OHase	6β - OHase	16 α -OHase	17 - OHSD	5 α - reductase
Ham's F-10 + 2,5 % FCS + 15 % HS					
Freshly prepared (control)	174 ± 15 (100) [a]	444 ± 43 (100)	694 ± 123 (100)	536 ± 22 (100)	826 ± 65 (100)
3-day culture	60 ± 7 (34) *	80 ± 7 (18) *	108 ± 8 (16) *	258 ± 4 (48) *	362 ± 86 (44) *
6-day culture	156 ± 13 (90)	260 ± 25 (59) *	160 ± 12 (23) *	520 ± 22 (97)	660 ± 40 (80) *
Ham's F-10 + 2 % Ultroser G					
Freshly prepared (control)	333 ± 21 (100)	668 ± 17 (100)	578 ± 41 (100)	1158 ± 142 (100)	1505 ± 217 (100)
3-day culture	64 ± 5 (19) *	70 ± 7 (10) *	128 ± 4 (22) *	226 ± 5 (20) *	240 ± 12 (16) *
6-day culture	82 ± 8 (25) *	100 ± 12 (15) *	266 ± 5 (46) *	438 ± 22 (38) *	532 ± 23 (35) *

Table 1. Metabolism of 4-[4-^{14} C] androstene-3,17-dione by isolated hepatocytes derived from adult male rats cultured in (i) Ham's F-10 culture medium supplemented with 2.5 % foetal calf serum (FCS) and 15 % horse serum (HS) and (ii) Ham's F-10 medium supplemented with 2 % Ultroser G. Results expressed as mean ± S.D (N = 5); * $P < 0.05$ as compared to respective controls.

[a] Numbers in the parentheses are percentage of enzyme activity with values from freshly prepared cells taken as 100 %.

	Enzyme activities (pmoles / min / million cells)				
	7α - OHase	6β - OHase	16 α -OHase	17 - OHSD	5 α - reductase
Ham's F-10 + 2.5 % FCS + 15 % HS					
Freshly prepared (control)	224 ± 15 (100)[a]	370 ± 25 (100)	346 ±30 (100)	396 ± 21 (100)	3158 ± 448 (100)
3-day culture	178 ± 22 (79) *	402 ± 8 (109) *	300 ± 37 (87)	626 ± 34 (158) *	660 ± 46 (21) *
6-day culture	492 ± 85 (220) *	704 ± 55 (190) *	824 ± 138 (238) *	1406 ± 245 (355) *	1436 ± 198 (45) *
Ham's F-10 + 2 % Ultroser G					
Freshly prepared (control)	30 ± 3 (100)	48 ± 3 (100)	45 ± 6 (100)	73 ± 2 (100)	143 ± 15 (100)
3-day culture	19 ± 2 (63) *	39 ± 2 (81) *	35 ± 4 (78) *	69 ± 2 (95) *	85 ± 2 (59) *
6-day culture	39 ± 2 (130) *	49 ± 4 (102)	68 ± 3 (151) *	118 ± 4 (162) *	141 ± 11 (99)

Table 2 . Metabolism of 4-[4-^{14} C] androstene-3,17-dione by isolated hepatocytes derived from adult female rats cultured in (i) Ham's F-10 culture medium supplemented with 2.5 % foetal calf serum (FCS) and 15 % horse serum (HS) and (ii) Ham's F-10 medium supplemented with 2 % Ultroser G. Results expressed as mean ± S.D (N = 5); * $P < 0.05$ as compared to respective controls.

[a] The numbers in the parentheses are percentage of enzyme activity with values from freshly prepared cells taken as 100 %.

(Table 1, bottom panel). The enzyme activities declined to about 10 to 20 % of control. Only slight improvements in activity were seen after 6 days in culture. After 3 days, a significant fall in all the enzyme activities was observed when hepatocytes derived from **female** rats were cultured in Ham's F-10 medium plus 2 % Ultroser G. Activity declined to 63 % and 59 % of control for the 7α-hydroxylase and 5α-reductase respectively while 6β- and 16α-hydroxylases and 17-OHSD activities fell to 81 % , 78 % and 95 % of control respectively (Table 2, bottom panel). After 6 days, the 6β-hydroxylase and 5α-reductase activity were restored to control level and the 7α- and 16α-hydroxylases and 17-OHSD activity rose significantly above the control.

In a shorter time scale experiment, **only male** rat hepatocytes were cultured in Ham's F-10 culture medium supplemented with 2 % Ultroser G. After 24 hours the medium was removed and washed once with Ham's F-10 medium supplemented with 0.1 % bovine serum albumin. The cultures were incubated in the latter medium for a further 24, 48 and 72 hours. After 1 day in culture, the enzyme activities decreased to between 25 and 80 % of control (Table 3, top panel). After 2 days in culture, the cytochrome P-450 dependent enzyme activities (7α- , 6β- and 16α-hydroxylases) were partially restored. Both the non-cytochrome P-450 dependent enzyme activities (17-OHSD and 5α-reductase) rose above the control level though the changes were not statistically significant. After 3 days, all the enzyme activities had exceeded the basal control level.

In another similar time scale experiment, **male** rat hepatocytes were directly cultured in Ham's F-10 medium supplemented with 0.1 % bovine serum albumin (Table 3, bottom panel). After 1 day, a similar fall in all of the enzyme activities was seen (to between 30 and 60 % of control). This was followed by restoration of all the enzyme activities to control level (except 7α-hydroxylase) after 2 days in culture. After 3 days in culture, all the enzyme activities rose significantly above the control level.

	Enzyme activities (pmoles / min / million cells)				
	7α - OHase	6β - OHase	16 α -OHase	17 - OHSD	5 α - reductase
Ham's F-10 + 2 % Ultroser G ⟶ 0.1 % BSA					
Freshly prepared (control)	112 ± 35 (100) [a]	136 ± 23 (100)	250 ± 61 (100)	136 ± 27 (100)	180 ± 42 (100)
1-day culture	53 ± 5 (47) *	53 ± 5 (39) *	63 ± 5 (25) *	105 ± 6 (77) *	133 ± 5 (74) *
2-day culture	83 ± 5 (74)	93 ± 15 (68)	155 ± 21 (62) *	185 ± 24 (136)	200 ± 35 (111)
3-day culture	310 ± 8 (277) *	248 ± 30 (182) *	448 ± 41 (179) *	510 ± 94 (375) *	490 ± 34 (272) *
Ham's F-10 + 0.1 % BSA					
Freshly prepared (control)	83 ± 5 (100)	118 ± 9 (100)	113 ± 5 (100)	208 ± 22 (100)	195 ± 10 (100)
1-day culture	47 ± 6 (57) *	40 ± 2 (34) *	59 ± 4 (52) *	83 ± 6 (40) *	76 ± 3 (39) *
2-day culture	121 ± 4 (146) *	124 ± 2 (105)	121 ± 6 (107)	210 ± 5 (101)	193 ± 9 (99)
3-day culture	168 ± 7 (202) *	161 ± 7 (136) *	220 ± 7 (195) *	305 ± 1 (147) *	283 ± 10 (145) *

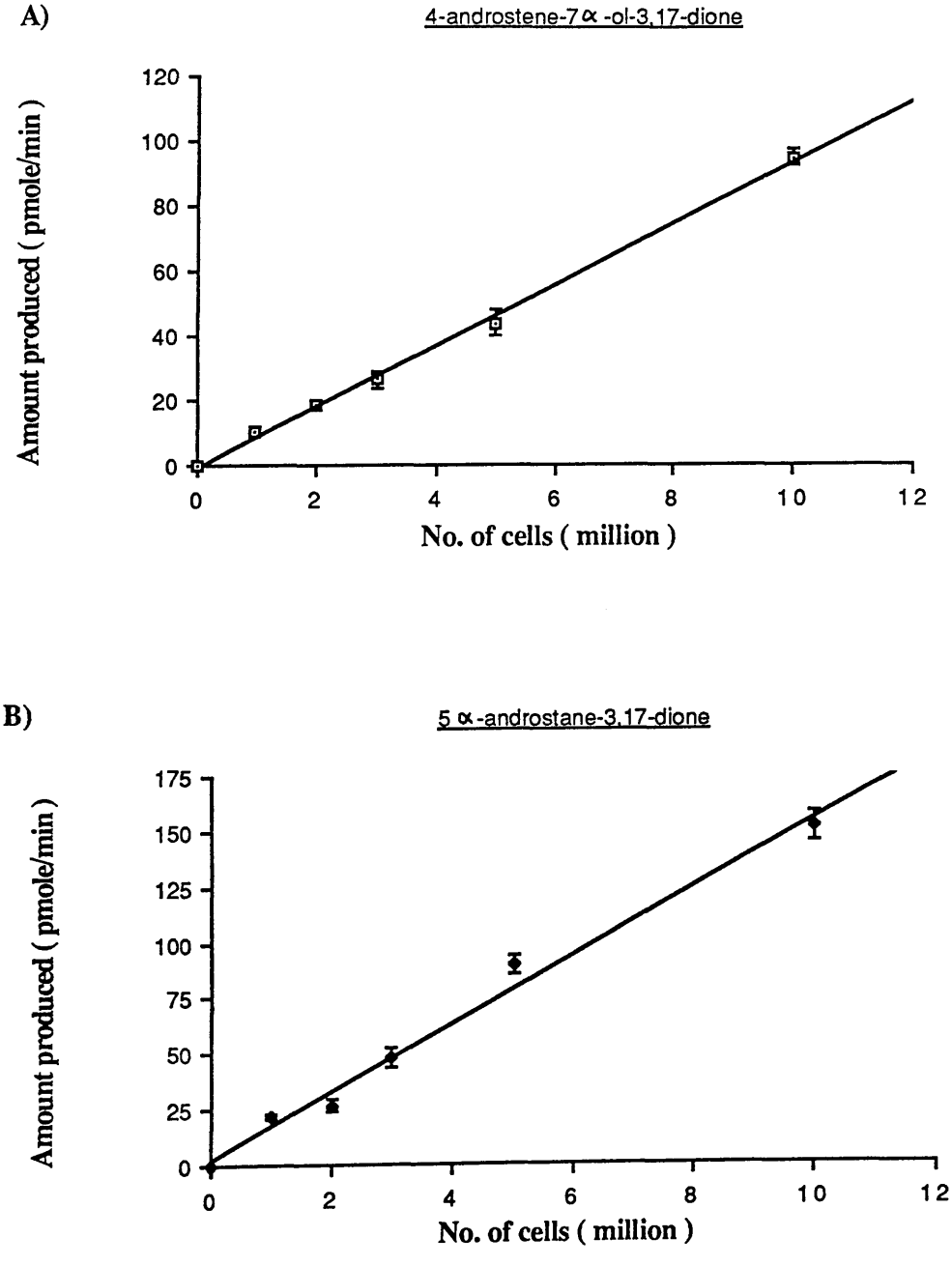
Table 3. Metabolism of 4-[4-^{14} C] androstene-3,17-dione by isolated hepatocytes derived from adult male rats cultured in (i) Ham's F-10 culture medium supplemented with 2 % Ultroser G. 24 hours later the medium was replaced with Ham's F-10 plus 0.1 % bovine serum albumin (BSA) and (ii) Ham's F-10 medium supplemented with 0.1 % (BSA). Results expressed as mean ± S.D (N = 5); * $P < 0.05$ as compared to respective controls.

[a] The numbers in the parentheses are percentage of enzyme activity with values from freshly prepared cells taken as 100 %.

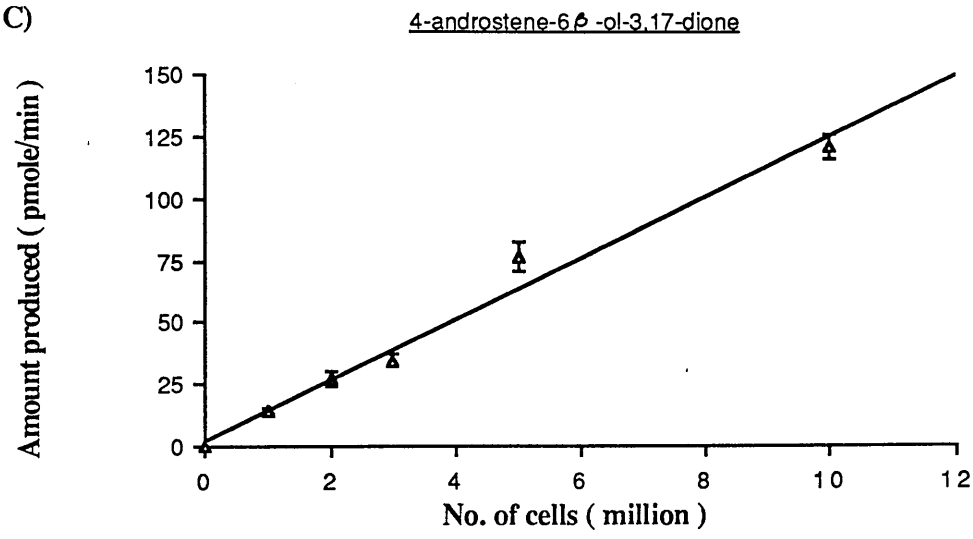
The metabolism of androst-4-ene-3,17-dione was also studied using different numbers of liver cells per incubation. In this study it was observed that the androst-4-ene-3,17-dione metabolism as a function of cell number showed a linear relationship (Figure 5). The amount of all the metabolites produced increased with increasing number of liver cells used implying that all the five enzyme activities also increased with increasing cell numbers.

A similar linear relationship was found with increasing period of incubation. The conversion to all the five metabolites increases with time of incubation (Figure 6). No lag phase in the metabolism of androst-4-ene-3,17-dione was observed at any time.

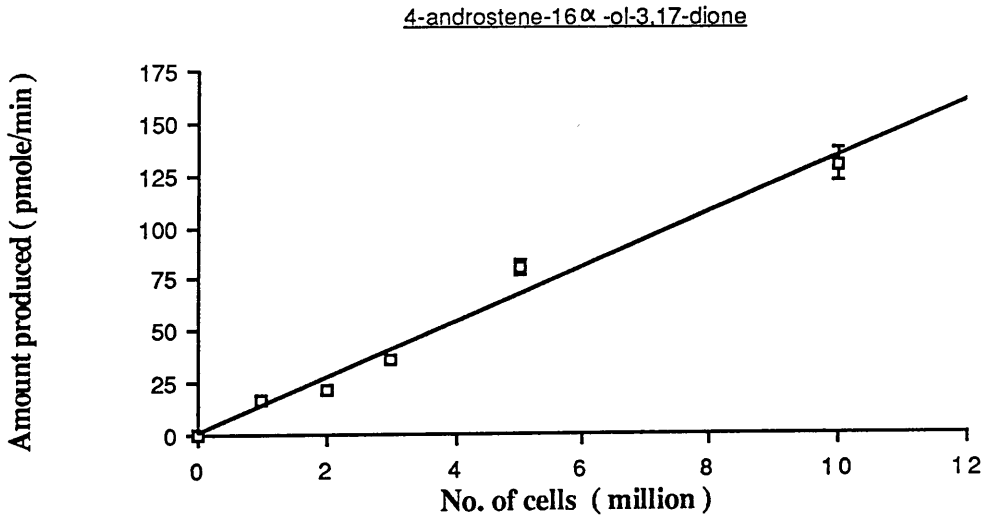
Figure 5 . Androst-4-ene-3,17-dione metabolism by isolated liver cells from normal male rat as a function of increasing of number of cells per incubation. The liver cells were incubated and the reaction was terminated at the indicated time period as described in the Methods section and the amount of metabolites (pmole/min) was calculated. The conversion of androst-4-ene-3,17-dione to the 5 different metabolites are produced by 5 different respective enzymes namely, (A) 7 α - hydroxylase (B) 5 α -reductase (C) 6 β -hydroxylase (D) 16 α -hydroxylase and (E) 17-oxosteroid oxidoreductase. Results are expressed as mean ± S.D (N = 3).



C)


4-androstene-6β -ol-3,17-dione
Amount produced (pmole/min)
150
125
100
75
50
25
0
0
2
4
6
8
10
12
No. of cells (million)

D)


4-androstene-16α -ol-3,17-dione
Amount produced (pmole/min)
175
150
125
100
75
50
25
0
0
2
4
6
8
10
12
No. of cells (million)

E)

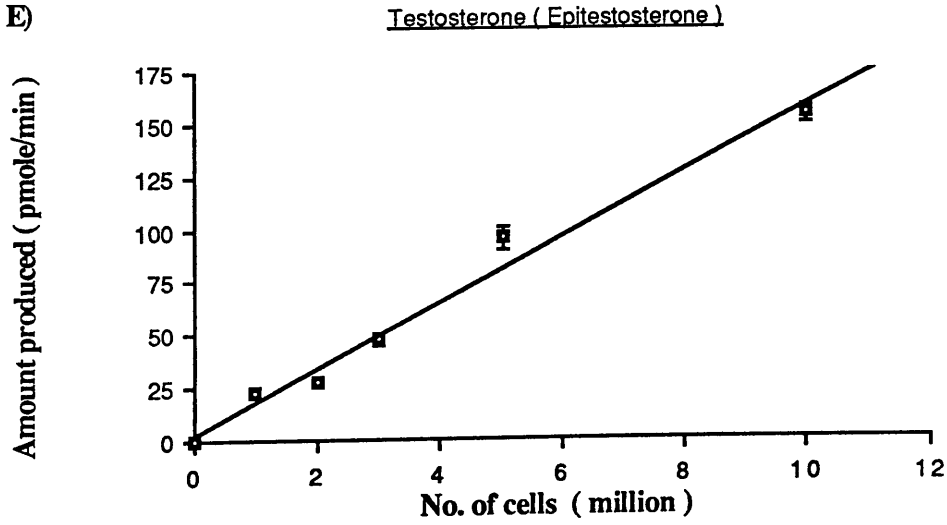
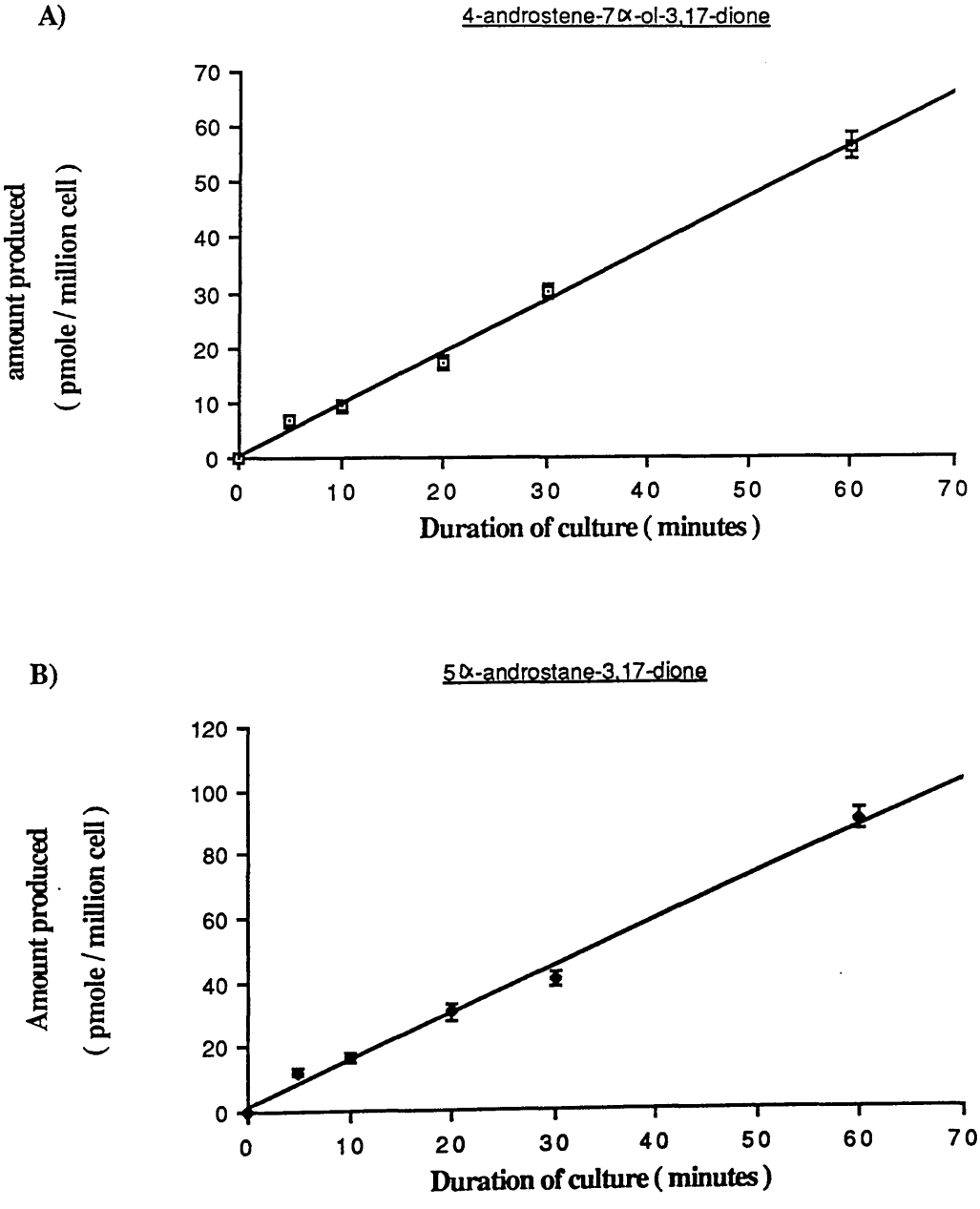
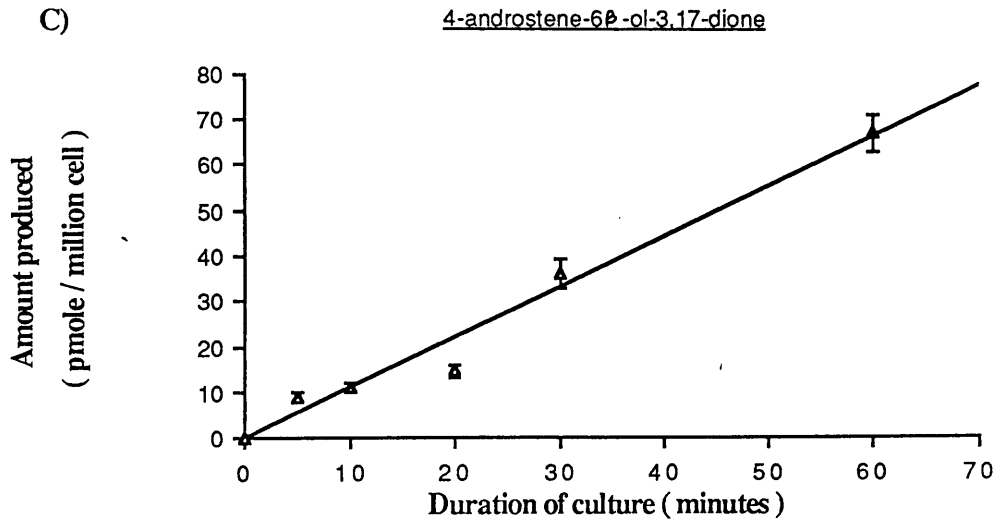

Testosterone (Epitestosterone)
Amount produced (pmole/min)
175
150
125
100
75
50
25
0
0
2
4
6
8
10
12
No. of cells (million)

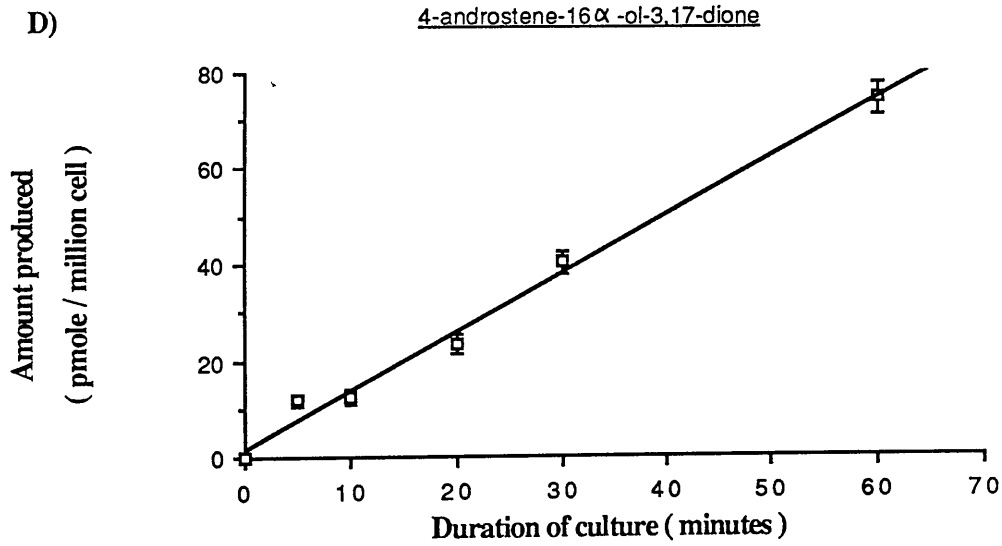
Figure 6 . Androst-4-ene-3,17-dione metabolism by isolated liver cells from normal male rat as a function of time of incubation. The liver cells were incubated and the reaction was terminated at the indicated time periods as described in the Methods section and the amount of metabolites produced (pmole/ million cells) was calculated. The conversion of androst-4-ene-3,17-dione to the 5 different metabolites are produced by 5 different respective enzymes namely, (A) 7 α - hydroxylase (B) 5 α -reductase (C) 6 β -hydroxylase (D) 16 α -hydroxylase and (E) 17-oxosteroid oxidoreductase. Results are expressed as mean ± S.D (N = 3).



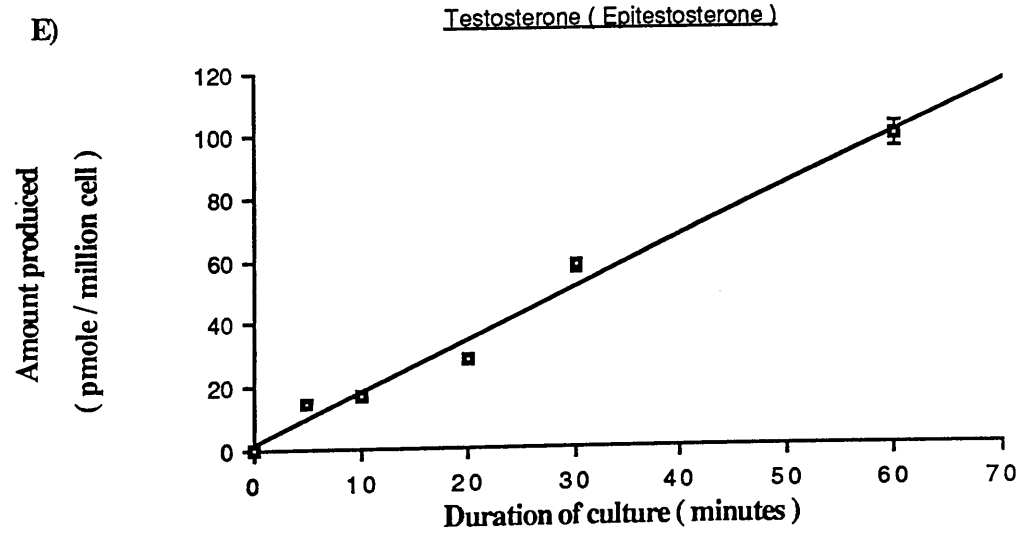
C)


4-androstene-6β-ol-3,17-dione
Amount produced
(pmole / million cell)
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
70
Duration of culture (minutes)

D)


4-androstene-16α-ol-3,17-dione
Amount produced
(pmole / million cell)
80
60
40
20
0
0
10
20
30
40
50
60
70
Duration of culture (minutes)

E)


Testosterone (Epitestosterone)
Amount produced
(pmole / million cell)
120
100
80
60
40
20
0
0
10
20
30
40
50
60
70
Duration of culture (minutes)

4.0 INSULIN

4.1 ACTION OF INSULIN ON THE METABOLISM OF ANDROST-4-ENE-3,17-DIONE

For convenience and space purposes, most of the graphic presentation for the five enzymes are divided into two graphs, the 7α-hydroxylase (OHase) and 5α-reductase activities (both are female-specific enzymes) are presented in one graph and the other three, male-specific enzymes [17-oxosteroid oxidoreductase (OHSD), 6β- and 16α-hydroxylases] in a second graph. Error bars and stars to indicate the level of significance are omitted from the graphs (except in figures 5, 6, 40 and 41) for reason of clarity but the respective tables give the complete results (mean ± S.D). Tables are expressed in absolute terms whereas the graphs are expressed as percentage of control values.

4.1.1 HEPATOCYTES FROM NORMAL RAT

4.1.1.1 Preincubation with insulin for 1/2, 1 and 2 hours

After **1/2 hour** preincubation with insulin, all the enzymes showed a dose-related increase in activity, significant ($P < 0.05$) at concentrations as low as 10^{-10} M insulin, which had not reached a maximum at 10^{-6} M (Table 4). When compared to control, insulin at 10^{-6} M, increased the activity of 7α-hydroxylase and 5α-reductase to 305 % and 214 % respectively (Figure 7A) and 6β- and 16α-hydroxylases, and 17-OHSD to 211%, 279% and 210 % respectively (Figure 7B). Evidently, there is no sex-difference in the effect of insulin on the various enzyme activities as the female-specific

Insulin concentration	Enzyme activities (pmoles / min / million cells)				
	7α - OHase	6β - OHase	16 α -OHase	17 - OHSD	5α - reductase
Control	37 ± 6	53 ± 7	43 ± 6	113 ± 6	117 ± 3
10^{-10} M	57 ± 12 *	63 ± 13	50 ± 10	150 ± 1 *	170 ± 2 *
10^{-9} M	56 ± 6 *	67 ± 5 *	53 ± 6 *	140 ± 6 *	163 ± 5 *
10^{-8} M	73 ± 12 *	93 ± 12 *	90 ± 21 *	193 ± 15 *	240 ± 3 *
10^{-7} M	77 ± 6 *	105 ± 12 *	107 ± 10 *	193 ± 6 *	257 ± 4 *
10^{-6} M	113 ± 15 *	112 ± 17 *	120 ± 31 *	237 ± 21 *	250 ± 4 *

Table 4 . Dose-response effects of 7 α -, 6 β -, 16 α -hydroxylases (OHases), 17-oxosteroid oxidoreductase (OHSD) and 5 α -reductase activities to insulin after 1/2 hour preincubation in hepatocytes obtained from normal male rat. Results expressed as mean ± S.D (N = 3); * $P < 0.05$ as compared to respective controls.

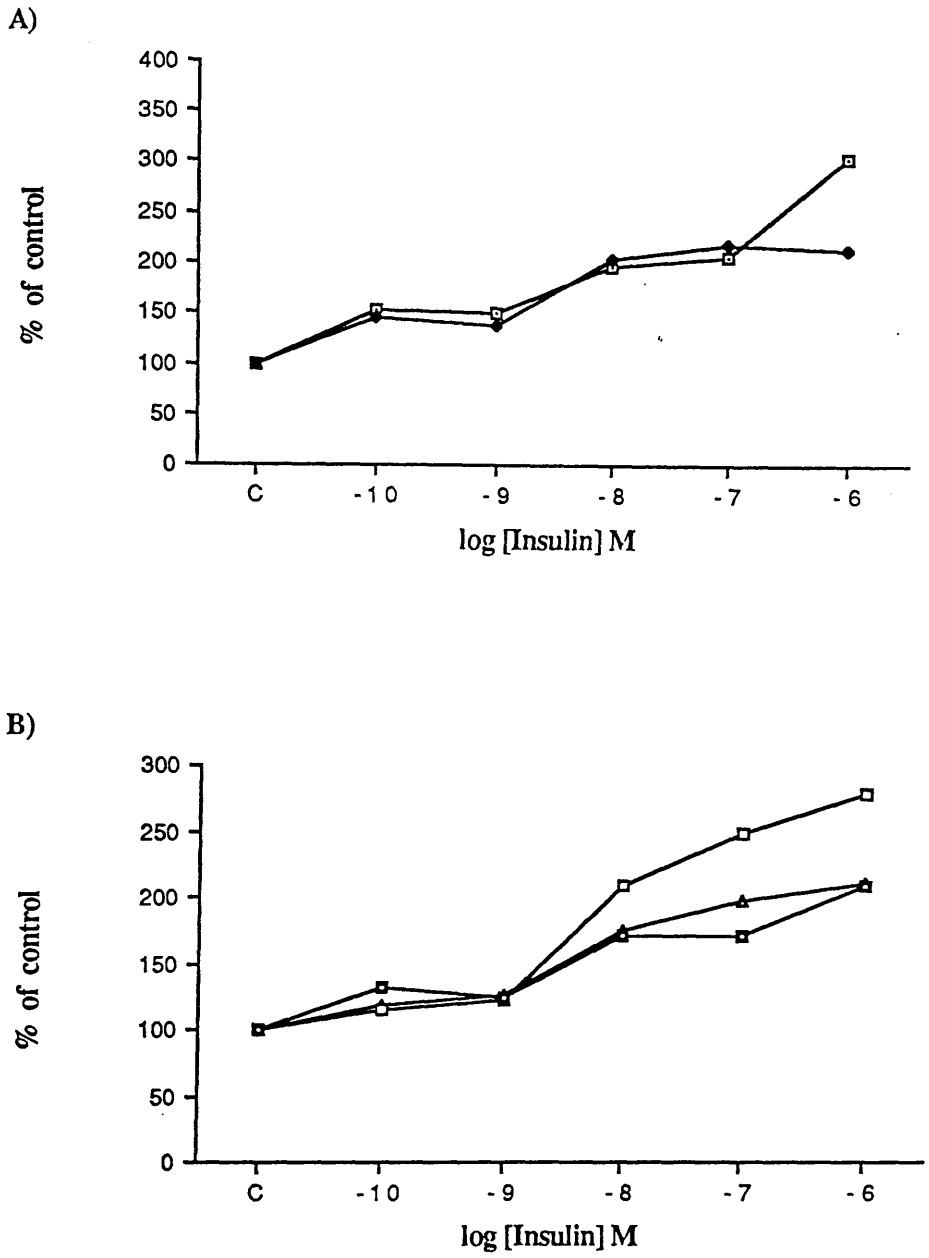


Figure 7. Dose-response curves of (A) 7α -hydroxylase [■] and 5α -reductase [◆] and (B) 17-OHSD [■], 6β - [▲] and 16α -hydroxylases [▫] to insulin after 1/2 hour of preincubation in hepatocytes obtained from normal male rat.
Results are expressed as percentage of the relevant control and mean ± S.D (N = 3). Absolute data is given in Table 4 .
C = control

enzyme activities (7α-OHase and 5α-reductase) are affected to the same extent as the male-specific activities (6β- and 16α-OHases and 17-OHSD).

This stimulation of activity virtually disappears after **1 hour** of insulin preincubation (Table 5). The effect of insulin (10^{-10} M to 10^{-6} M) on all the five enzymes measured was found to display a bell-shape dose-response curve with significant increase in enzymes activity seen at physiological insulin concentration (10^{-9} and 10^{-8} M) [although 5α-reductase exhibited a reduction in activity at higher insulin concentrations (10^{-7} and 10^{-6} M) which were statistically significant (Figure 8)].

Exposure of the primary culture of hepatocytes to insulin for **2 hours** resulted in the return of all the enzyme activities to almost basal level (Table 6). Although insulin at 10^{-10} M concentration increased the activity of 7α-hydroxylase, 5α-reductase and 16α-hydroxylase (145 %, 138 % and 116 % of control respectively), they are not statistically significant ($P > 0.05$)(Figure 9). However, at high (10^{-6} M) concentration, insulin significantly increased all the enzyme activities.

4.1.1.2 Preincubation with insulin for 24, 48 and 72 hours

An increase in all of the enzyme activities is seen at **24 hour** insulin preincubation (Table 7). Maximum response could be observed at 10^{-9} or 10^{-8} M concentration (to about 140 - 150 % of control). Unlike the dose-related effect of insulin seen at 1/2 hour (see Figure 7), the percentage maximal response against control seemed to fall as the concentration of insulin is increased above the physiological level (Figure 10) with activity declining significantly below the control at 10^{-6} M insulin concentration.

At **48 hours,** insulin (10^{-9} M) elicited a significant increase in activity of

Insulin concentration	Enzyme activities (pmoles / min / million cells)				
	7α - OHase	6β - OHase	16α -OHase	17 - OHSD	5 α - reductase
Control	53 ± 6	57 ± 6	73 ± 6	83 ± 6	140 ± 17
10^{-10} M	50 ± 6	67 ± 6 *	87 ± 6 *	87 ± 6	117 ± 6 *
10^{-9} M	62 ± 4 *	67 ± 6 *	86 ± 10	98 ± 6 *	150 ± 10
10^{-8} M	63 ± 6 *	67 ± 15 *	87 ± 6 *	93 ± 6 *	133 ± 15
10^{-7} M	50 ± 6	60 ± 10	73 ± 6	77 ± 6	107 ± 12 *
10^{-6} M	50 ± 6	67 ± 6 *	73 ± 6	87 ± 6	97 ± 6 *

Table 5 . Dose-response effects of 7 α -, 6 β -, 16 α -hydroxylases (OHases), 17-oxosteroid oxidoreductase (OHSD) and 5 α -reductase activities to insulin after 1 hour preincubation in hepatocytes obtained from normal male rat. Results expressed as mean ± S.D (N = 3); * P < 0.05 as compared to respective controls.

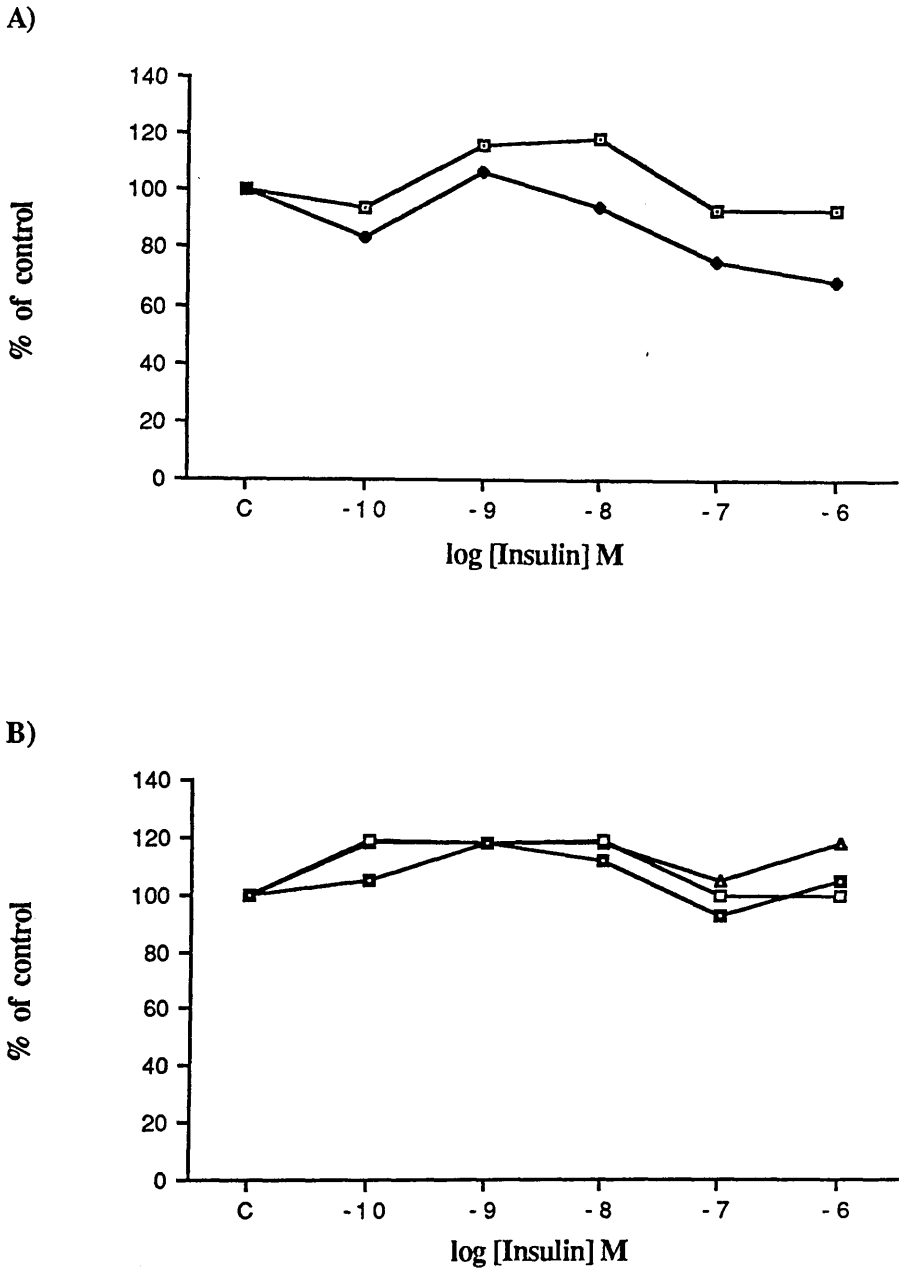


Figure 8. Dose-response curves of (A) 7 α -hydroxylase [▪] and 5 α -reductase [•] and (B) 17-OHSD [▪], 6 β -[▲] and 16 α -hydroxylases [▫] to insulin after 1 hour of preincubation in hepatocytes obtained from normal male rat.
Results are expressed as percentage of the relevant control and mean ± S.D (N = 3). Absolute data is given in Table 5 .
C = control

Insulin concentration	Enzyme activities (pmoles / min / million cells)				
	7α - OHase	6β - OHase	16α -OHase	17 - OHSD	5 α-reductase
Control	53 ± 6	83 ± 3	80 ± 3	147 ± 12	147 ± 21
10^{-10} M	77 ± 21	87 ± 12	110 ± 35	147 ± 6	170 ± 20
10^{-9} M	53 ± 5	77 ± 9	76 ± 7	153 ± 6	133 ± 12
10^{-8} M	53 ± 6	70 ± 10	60 ± 10 *	143 ± 6	133 ± 35
10^{-7} M	60 ± 1	83 ± 6	80 ± 17	173 ± 16	137 ± 15
10^{-6} M	83 ± 6 *	120 ± 20 *	133 ± 31 *	223 ± 6 *	217 ± 21 *

Table 6 . Dose-response effects of 7α -, 6 β -, 16 α -hydroxylases (OHases), 17-oxosteroid oxidoreductase (OHSD) and 5 α -reductase activities to insulin after 2 hour preincubation in hepatocytes obtained from normal male rat. Results expressed as mean ± S.D (N = 3); * P < 0.05 as compared to respective controls.

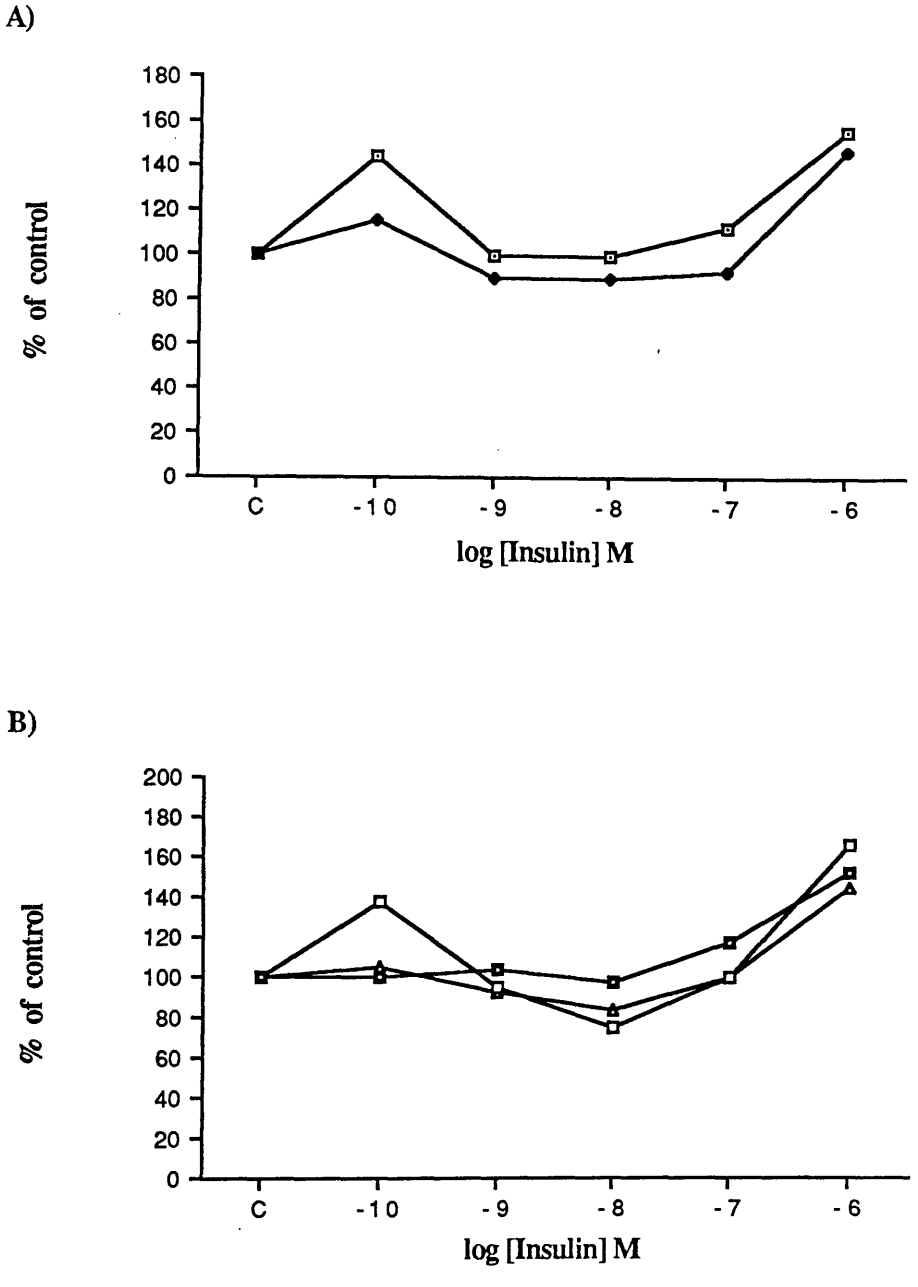


Figure 9. Dose-response curves of (A) 7 α -hydroxylase [▫] and 5 α -reductase [◆] and (B) 17-OHSD [▪], 6 β -[▲] and 16 α -hydroxylases [▫] to insulin after 2 hour of preincubation in hepatocytes obtained from normal male rat.
Results are expressed as percentage of the relevant control and mean ± S.D (N = 3). Absolute data is given in Table 6 .
C = control

Insulin concentration	Enzyme activities (pmoles / min / million cells)				
	7α - OHase	6β - OHase	16 α -OHase	17 - OHSD	5 α - reductase
Control	47 ± 6	40 ± 2	59 ± 4	83 ± 6	76 ± 3
10^{-10} M	68 ± 3 *	50 ± 2 *	54 ± 1	89 ± 3	84 ± 2
10^{-9} M	74 ± 5 *	57 ± 2 *	80 ± 3 *	124 ± 8 *	111 ± 7 *
10^{-8} M	77 ± 5 *	61 ± 1 *	81 ± 7 *	121 ± 8 *	119 ± 4 *
10^{-7} M	39 ± 6	46 ± 1 *	52 ± 3	61 ± 3 *	59 ± 2 *
10^{-6} M	51 ± 4	33 ± 1 *	33 ± 1 *	61 ± 4 *	60 ± 3 *

Table 7 . Dose-response effects of 7 α -, 6 β -, 16 α -hydroxylases (OHases), 17-oxosteroid oxidoreductase (OHSD) and 5 α -reductase activities to insulin after 24 hour preincubation in hepatocytes obtained from normal male rat.Results expressed as mean ± S.D (N = 3); * $P < 0.05$ as compared to respective controls.

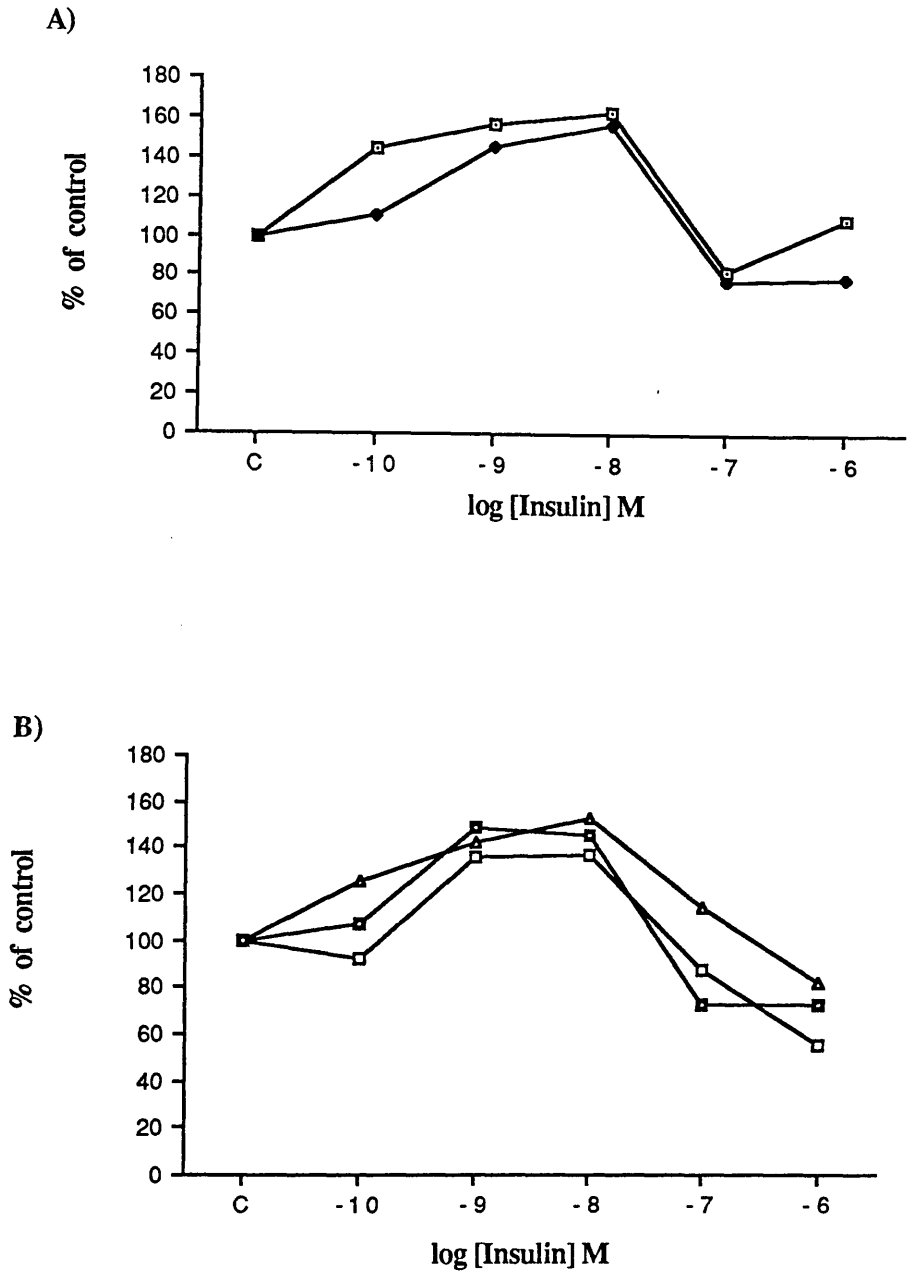


Figure 10. Dose-response curves of (A) 7 α -hydroxylase [▪] and 5 α -reductase [•] and (B) 17-OHSD [▪], 6 β - [▲] and 16 α -hydroxylases [▫] to insulin after 24 hour of preincubation in hepatocytes obtained from normal male rat. Results are expressed as percentage of the relevant control and mean ± S.D (N = 3). Absolute data is given in Table 7 .
C = control

all of the enzymes with the exception of the 6β-hydroxylase (Table 8). The activity began to decline sharply and significantly below control to about 50 % of control for both 7α-hydroxylase and 5α-reductase (Figure 11A) and about 40 - 70 % of control for 6β- and 16α-hydroxylases and 17-OHSD (Figure 11B) at higher concentrations of insulin. Maximal decrease in activity was seen at 10^{-8} M insulin concentration after which it began to plateau.

After **72 hours** of insulin preincubation, all of the enzymes exhibited a U-shaped dose-response curve with significant reductions in enzyme activity at concentration as low as 10^{-9} M, reaching maximal reduction at 10^{-8} M insulin concentration (Table 9 and Figure 12). The enzyme activities increased again at 10^{-7} and 10^{-6} M although they were still significantly below control levels. It is interesting to note that the basal level of the five enzymes measured increased with time as illustrated in Table 7, 8 and 9.

From the data from Tables 4 to 9, we are able to construct a graph illustrating the time-course of events following the addition of insulin at physiological concentration (10^{-9} M) over the time period studied (1/2 hour to 72 hours preincubation) for all the five enzyme activities in the normal rat hepatocytes.

Figure 13 shows the response of the cultured hepatocytes to various periods of preincubation with insulin (at 10^{-9} M) on all the enzyme activities studied. Evidently, the effect of insulin on 7α-hydroxylase and 5α-reductase (female specific) and 17-OHSD, 6β- and 16α-hydroxylases (male specific) was non-selective with respect to sex differences in steroid metabolism. Maximal increase in enzyme activities are observed at 1/2 hour and 24 hour of insulin preincubation. Enzyme activities diminished, though significantly above control level, at 1 hour followed by a return of activity to basal level at 2 hours of insulin preincubation. Reduced enzyme activities were observed at 48 hours and continued to fall below basal level (70-80 % of control) at 72 hours.

In order to verify whether the disappearance in effect of insulin at 48 and 72 hour

Insulin concentration	Enzyme activities (pmoles / min / million cells)				
	7α - OHase	6β - OHase	16α -OHase	17 - OHSD	5 α- reductase
Control	121 ± 4	124 ± 2	121 ± 6	210 ± 5	193 ± 9
10^{-10} M	129 ± 4	134 ± 5 *	129 ± 5	216 ± 2	203 ± 12
10^{-9} M	144 ± 3 *	103 ± 6 *	160 ± 4 *	254 ± 5 *	216 ± 6 *
10^{-8} M	62 ± 2 *	47 ± 2 *	75 ± 6 *	108 ± 2 *	95 ± 10 *
10^{-7} M	69 ± 3 *	53 ± 2 *	85 ± 7 *	123 ± 3 *	101 ± 5 *
10^{-6} M	73 ± 1 *	61 ± 2 *	83 ± 10 *	120 ± 3 *	103 ± 10 *

Table 8 . Dose-response effects of 7α -, 6β -, 16α -hydroxylases (OHases), 17-oxosteroid oxidoreductase (OHSD) and 5 α -reductase activities to insulin after 48 hour preincubation in hepatocytes obtained from normal male rat. Results expressed as mean ± S.D (N = 3); * $P < 0.05$ as compared to respective controls.

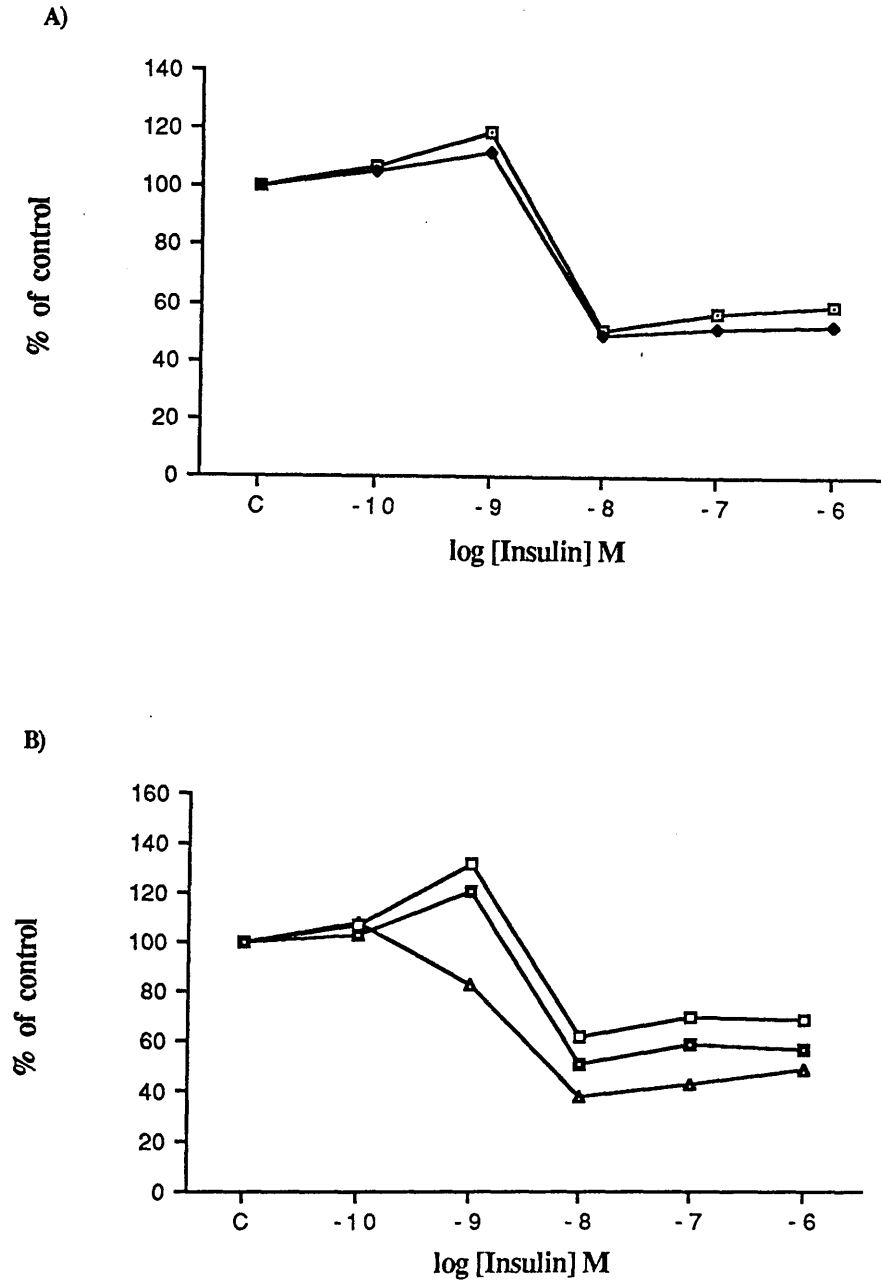


Figure 11. Dose-response curves of (A) 7α -hydroxylase [▫] and 5α -reductase [•] and (B) 17-OHSD [▪], 6β - [▲] and 16α -hydroxylases [▫] to insulin after 48 hour of preincubation in hepatocytes obtained from normal male rat.
Results are expressed as percentage of the relevant control and mean ± S.D (N = 3). Absolute data is given in Table 8 .
C = control

Insulin concentration	Enzyme activities (pmoles / min / million cells)				
	7α - OHase	6β - OHase	16 α -OHase	17 - OHSD	5 α - reductase
Control	168 ± 7	161 ± 7	220 ± 7	305 ± 1	283 ± 10
10^{-10} M	179 ± 4	173 ± 5	225 ± 6	315 ± 5 *	246 ± 12 *
10^{-9} M	124 ± 4 *	119 ± 5 *	150 ± 8 *	210 ± 5 *	243 ± 7 *
10^{-8} M	91 ± 4 *	90 ± 7 *	119 ± 1 *	150 ± 3 *	140 ± 8 *
10^{-7} M	138 ± 4 *	123 ± 3 *	168 ± 12 *	215 ± 7 *	207 ± 11 *
10^{-6} M	129 ± 7 *	115 ± 4 *	147 ± 7 *	197 ± 6 *	217 ± 9 *

Table 9 . Dose-response effects of 7 α -, 6 β -, 16 α -hydroxylases (OHases), 17-oxosteroid oxidoreductase (OHSD) and 5 α -reductase activities to insulin after 72 hour preincubation in hepatocytes obtained from normal male rat. Results expressed as mean ± S.D (N = 3); * P < 0.05 as compared to respective controls.

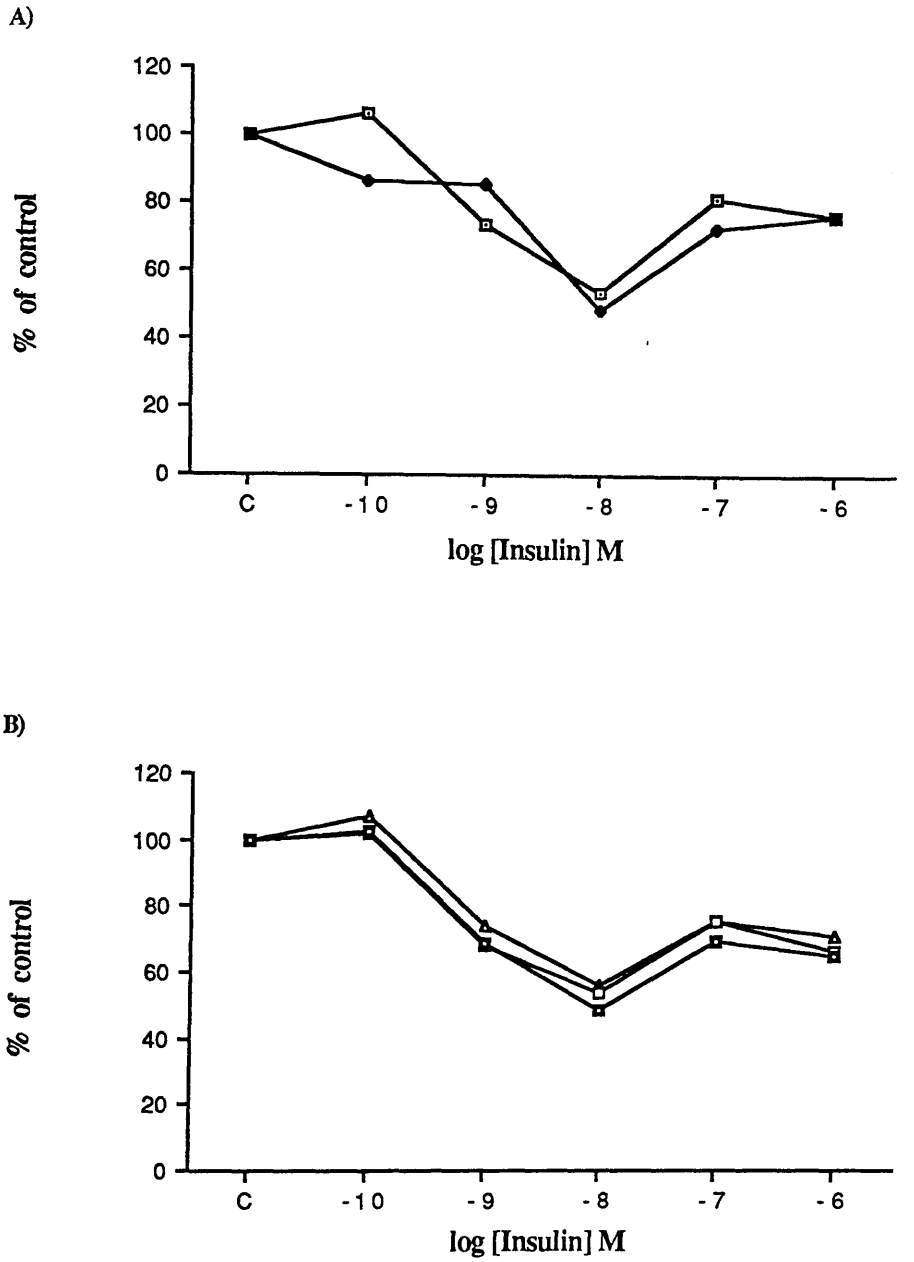


Figure 12. Dose-response curves of (A) 7α -hydroxylase [▪] and 5α -reductase [•] and (B) 17-OHSD [▪], 6β - [▲] and 16α -hydroxylases [▫] to insulin after 72 hour of preincubation in hepatocytes obtained from normal male rat.
Results are expressed as percentage of the relevant control and mean ± S.D (N = 3). Absolute data is given in Table 9 .
C = control

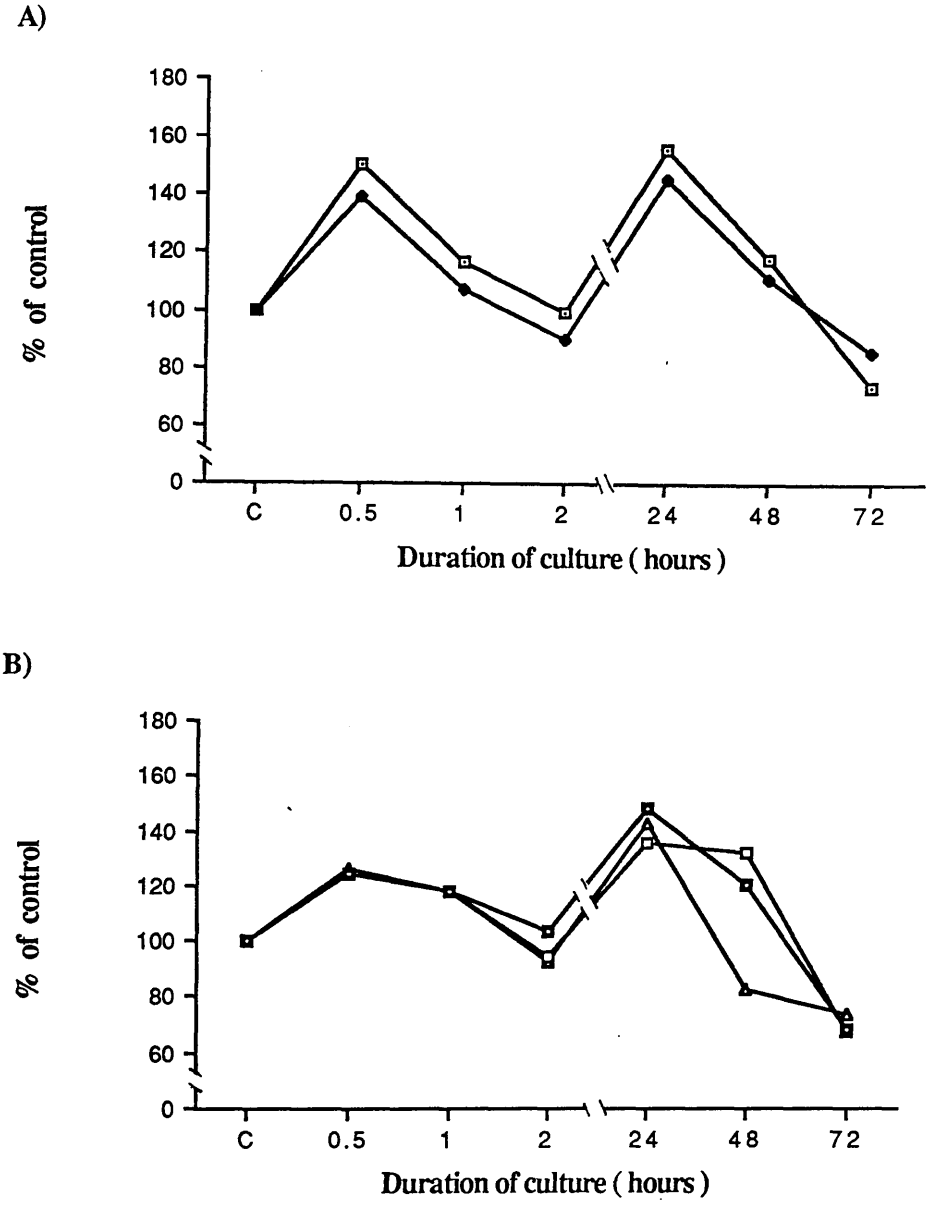


Figure 13. Time course of the effect of insulin (10^{-9} M concentration) on (A) 7 α -hydroxylase [▪] and 5 α -reductase [•] and (B) 17-OHSD [▪], 6 β - [▲] and 16 α -hydroxylases [▫] activities in hepatocytes obtained from normal male rat.

Results are expressed as percentage of the relevant control and mean ± S.D (N = 3). Absolute data is given in Table 4 to 9
C = control

(as shown in Figure 13) was due to insulin degradation or to ligand-induced insulin receptor down-regulation, insulin (10^{-10} to 10^{-6} M) was added at 24 hour intervals from 0 to 72 hour (Table 10). As shown in table 10A, a 24 hour insulin preincubation gave a bell-shaped response, consistent with our previous result (Table 7). Accumulated addition of insulin at 24 and 48 hours resulted in no significant increase in any of the enzymes (Table 10B and C; see Figure 14).

4.1.2 HEPATOCYTES FROM *3-DAYS* STZ-TREATED DIABETIC RAT

4.1.2.1 Preincubation with insulin for 1/2 and 1 hour

Unlike the hepatocytes from normal rats, after **1/2 hour** preincubation, the enzyme activities in hepatocytes from 3-days STZ-induced diabetic male rat were not responsive to insulin over the range of concentration used (Table 11). Lack of insulin responsiveness is evident with all the enzymes irrespective of their sex specificity. Although 17-OHSD activity rose to about 20 % above control , this result was not statistically significant (Figure 15). As demonstrated in Table 12, serum glucose levels in the 3-days STZ-diabetic rats were grossly elevated (about 3-fold increase) establishing that the rats were diabetic. Whilst the normal rats gained weight, the 3-days diabetic rats experienced about 20 g of weight loss. When compared to control data (Table 12), 3-days diabetic rats exhibited a decrease in the male-specific enzyme activities (6β-hydroxylase and 17-OHSD) while the female-specific enzymes activities (7α-hydroxylase and 5α-reductase) were increased.

Similarly, **1 hour** insulin preincubation in hepatocytes obtained from 3-days STZ- diabetic rat elicited no change in enzyme activities from control (Table 13). When expressed in percentage of control, again no significant change in any of

Table 10 . Time course effect of cumulative insulin addition on 7 α -, 6 β - and 16 α - hydroxylases (OHases),17-OHSD and 5 α -reductase activities in hepatocytes obtained from normal male rat. Insulin was added at 24 hour intervals at (A) 0 hr , (B) 24 hr and (C) 48 hr of cells preincubation. Results expressed as mean ± SD (N = 3); * $P < 0.05$ as compared to the respective control.

(A)

Insulin concentration	Enzyme activities (pmoles / min / million cells)				
	7 α - OHase	6 β - OHase	16 α - OHase	17 - OHSD	5 α - reductase
Control	33 ± 6	53 ± 4	63 ± 4	73 ± 6	93 ± 6
10^{-10} M	36 ± 6	64 ± 3	67 ± 5	77 ± 6	106 ± 10
10^{-9} M	53 ± 6 *	81 ± 3 *	91 ± 7 *	95 ± 4 *	135 ± 7 *
10^{-8} M	52 ± 6 *	85 ± 2 *	92 ± 6 *	96 ± 7 *	143 ± 5 *
10^{-7} M	43 ± 1 *	40 ± 1 *	50 ± 7 *	60 ± 5 *	78 ± 6 *
10^{-6} M	35 ± 6	46 ± 1 *	53 ± 4 *	72 ± 5	93 ± 6

(B)

Insulin concentration	Enzyme activities (pmoles / min / million cells)				
	7 α - OHase	6 β - OHase	16 α - OHase	17 - OHSD	5 α - reductase
Control	47 ± 3	63 ± 10	77 ± 8	83 ± 3	137 ± 6
10^{-10} M	50 ± 3	53 ± 6	63 ± 8	83 ± 4	142 ± 6
10^{-9} M	48 ± 2	57 ± 6	77 ± 7	81 ± 9	129 ± 6
10^{-8} M	50 ± 1	67 ± 7	67 ± 4	85 ± 3	143 ± 7
10^{-7} M	46 ± 4	57 ± 9	67 ± 5	81 ± 4	132 ± 5
10^{-6} M	47 ± 2	69 ± 6	73 ± 6	78 ± 5	147 ± 3

(C)

Insulin concentration	Enzyme activities (pmoles / min / million cells)				
	7 α-OHase	6 β-OHase	16 α-OHase	17 - OHSD	5 α-reductase
Control	47± 6	63 ± 6	89 ± 4	87±15	127 ±5
10^{-10} M	46 ± 6	67 ± 6	87 ± 3	83 ± 9	130 ± 10
10^{-9} M	47 ± 6	73 ± 6*	96 ± 7	87 ± 11	120 ± 10
10^{-8} M	46 ± 6	60 ± 10	83 ± 6	83 ± 6	119 ± 14
10^{-7} M	45 ± 6	60 ± 10	87 ± 1	87 ± 7	119 ± 10
10^{-6} M	44 ± 6	70 ± 10	93 ± 1	85 ± 6	133 ± 6

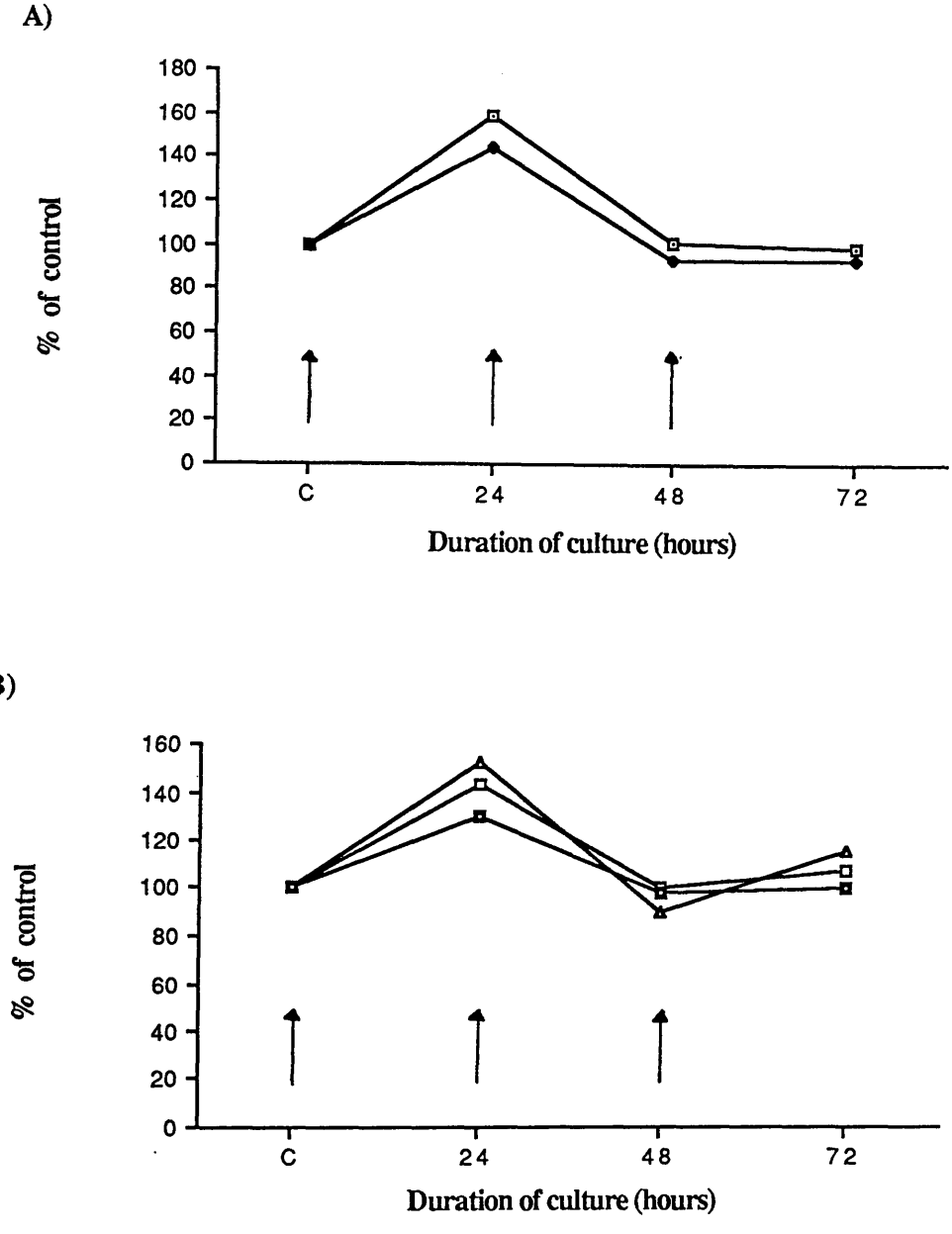


Figure 14. Time course effect of cumulative insulin (10^{-9} M) addition on (A) 7 α -hydroxylase [▪] and 5 α -reductase [◆] and (B) 17-OHSD [▪], 6 β - [▲] and 16 α -hydroxylases [▫] activities compared to respective control. The arrow (↑) denotes the point where insulin (10^{-9} M) was added during the preincubating phase.
Results are expressed as percentage of the relevant control and mean ± S.D (N = 3).
Absolute data is given in Table 10 . C = control

Insulin concentration	Enzyme activities (pmoles / min / million cells)				
	7α - OHase	6β - OHase	16 α -OHase	17 - OHSD	5 α - reductase
Control	60 ± 9	38 ± 7	80 ± 10	85 ± 2	130 ± 9
10^{-10} M	59 ± 5	53 ± 10 *	82 ± 4	130 ± 6 *	128 ± 4
10^{-9} M	48 ± 9	37 ± 2	75 ± 5	101 ± 10	114 ± 9
10^{-8} M	46 ± 11	34 ± 4	87 ± 10	107 ± 15	142 ± 13
10^{-7} M	54 ± 3	39 ± 2	73 ± 4	97 ± 8	158 ± 16
10^{-6} M	57 ± 6	47 ± 6	80 ± 1	104 ± 12	148 ± 9

Table 11 . Dose-response effects of 7 α -, 6 β -, 16 α -hydroxylases (OHases), 17-oxosteroid oxidoreductase (OHSD) and 5 α -reductase activities to insulin after 1/2 hour preincubation in hepatocytes obtained from 3 days STZ-treated diabetic male rat. Results expressed as mean ± S.D (N = 3); * $P < 0.05$ as compared to respective controls.

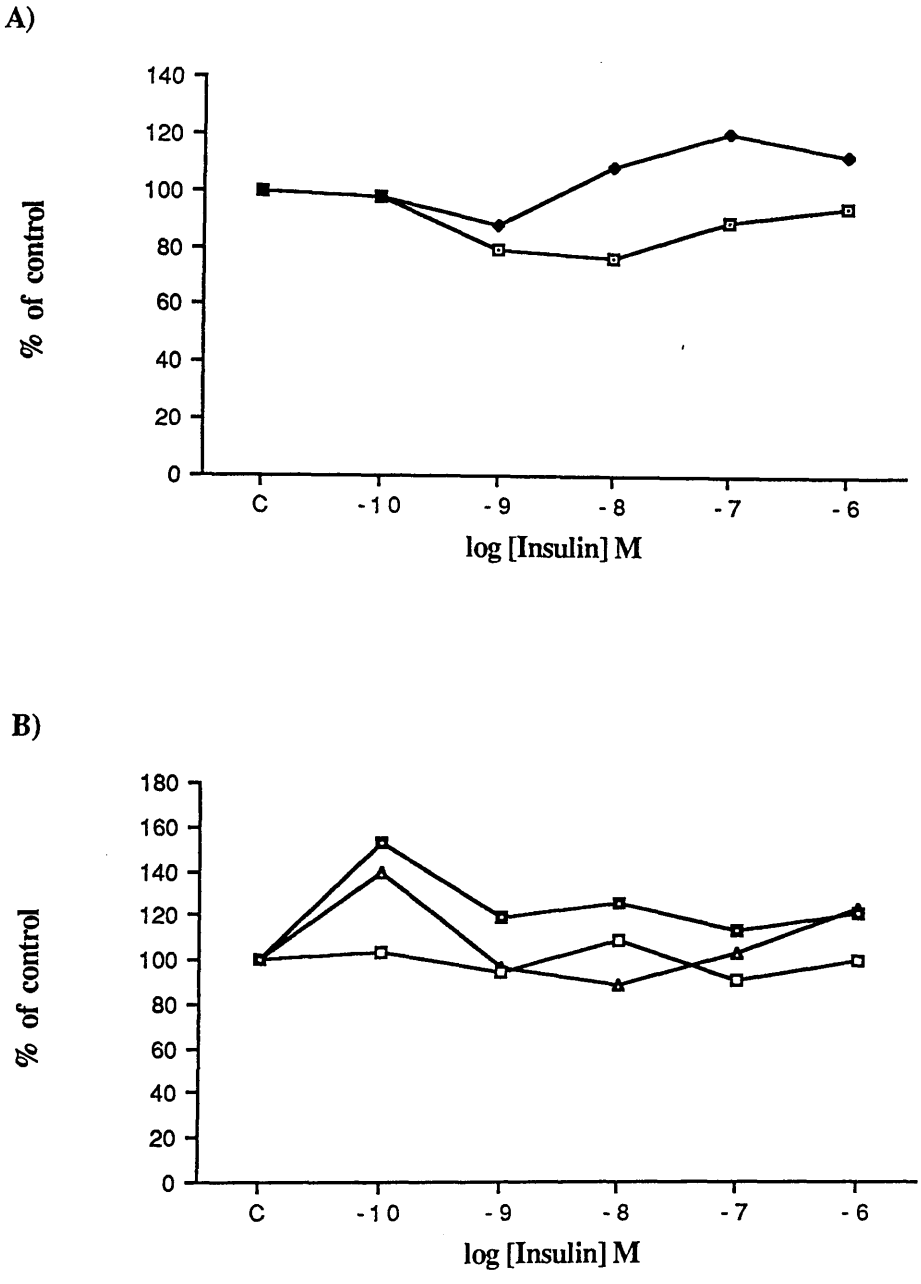


Figure 15. Dose-response curves of (A) 7 α -hydroxylase [▪] and 5 α -reductase [•] and (B)17-OHSD [▪], 6 β - [▲] and 16 α -hydroxylases [▫] to insulin after 1/2 hour preincubation in hepatocytes obtained from 3 days STZ-treated diabetic male rat. Results are expressed as percentage of the relevant control and mean ± S.D (N = 3). Absolute data is given in Table 11 .
C = control

Table 12. The effect of streptozotocin and insulin treatment on the metabolism of androst-4-ene-3,17-dione in isolated hepatocytes from male rat and serum glucose concentration and changes in body weight of intact animal.

Parameter	Control [b]	3 days STZ diabetic	21 days STZ diabetic	3 days STZ +Insulin [c] (*In-vivo*)
7α-OHase	37 ± 6	60 ± 9 [a]	40 ± 7	49 ± 9 [a]
6β-OHase	53 ± 7	38 ± 7 [a]	34 ± 2 [a]	81 ± 3 [a]
16α-OHase	43 ± 6	80 ± 10 [a]	44 ± 3	75 ± 7 [a]
17-OHSD	113 ± 6	85 ± 2 [a]	123 ± 2 [a]	116 ± 5
5α-reductase	117 ± 3	130 ± 9 [a]	86 ± 9 [a]	112 ± 5
Serum glucose (mM)	5.9 ± 0.7	19.7 ± 2.5 [a]	16.3 ± 3.3 [a]	7.3 ± 1.2
Initial wt. (g)	225 ± 8	230 ± 10	220 ± 8	225 ± 9
Change in weight (g)	5 ± 2	-20 ± 3 [a]	-25 ± 4 [a]	-2 ± 5

[a] Significantly different from the relevant control ; $p < 0.05$

[b] Hepatocytes were preincubated with insulin in serum-free medium as described in the Methods Section and all enzyme activities expressed as *p* moles product/min/10^6 cells and as mean ± S.D. of 4 animals.

[c] Animals were given 12 U of insulin as described in the Methods Section.

Abbreviations : OHase = hydroxylase ; OHSD = oxosteroid oxidoreductase

Insulin concentration	Enzyme activities (pmoles / min / million cells)				
	7α - OHase	6β - OHase	16α -OHase	17 - OHSD	5 α-reductase
Control	42 ± 4	37 ± 2	76 ± 4	62 ± 7	185 ± 12
10^{-10} M	46 ± 3	37 ± 2	73 ± 5	74 ± 8	188 ± 16
10^{-9} M	48 ± 5	41 ± 5	79 ± 4	71 ± 16	185 ± 12
10^{-8} M	44 ± 7	36 ± 7	81 ± 8	68 ± 7	187 ± 5
10^{-7} M	37 ± 4	32 ± 4	76 ± 7	61 ± 9	196 ± 4
10^{-6} M	36 ± 3	34 ± 4	80 ± 7	61 ± 8	186 ± 3

Table 13 . Dose-response effects of 7 α -, 6 β -, 16 α -hydroxylases (OHases), 17-oxosteroid oxidoreductase (OHSD) and 5 α -reductase activities to insulin after 1 hour preincubation in hepatocytes obtained from 3 days STZ-treated diabetic male rat. Results expressed as mean ± S.D (N = 3); * $P < 0.05$ as compared to respective controls.

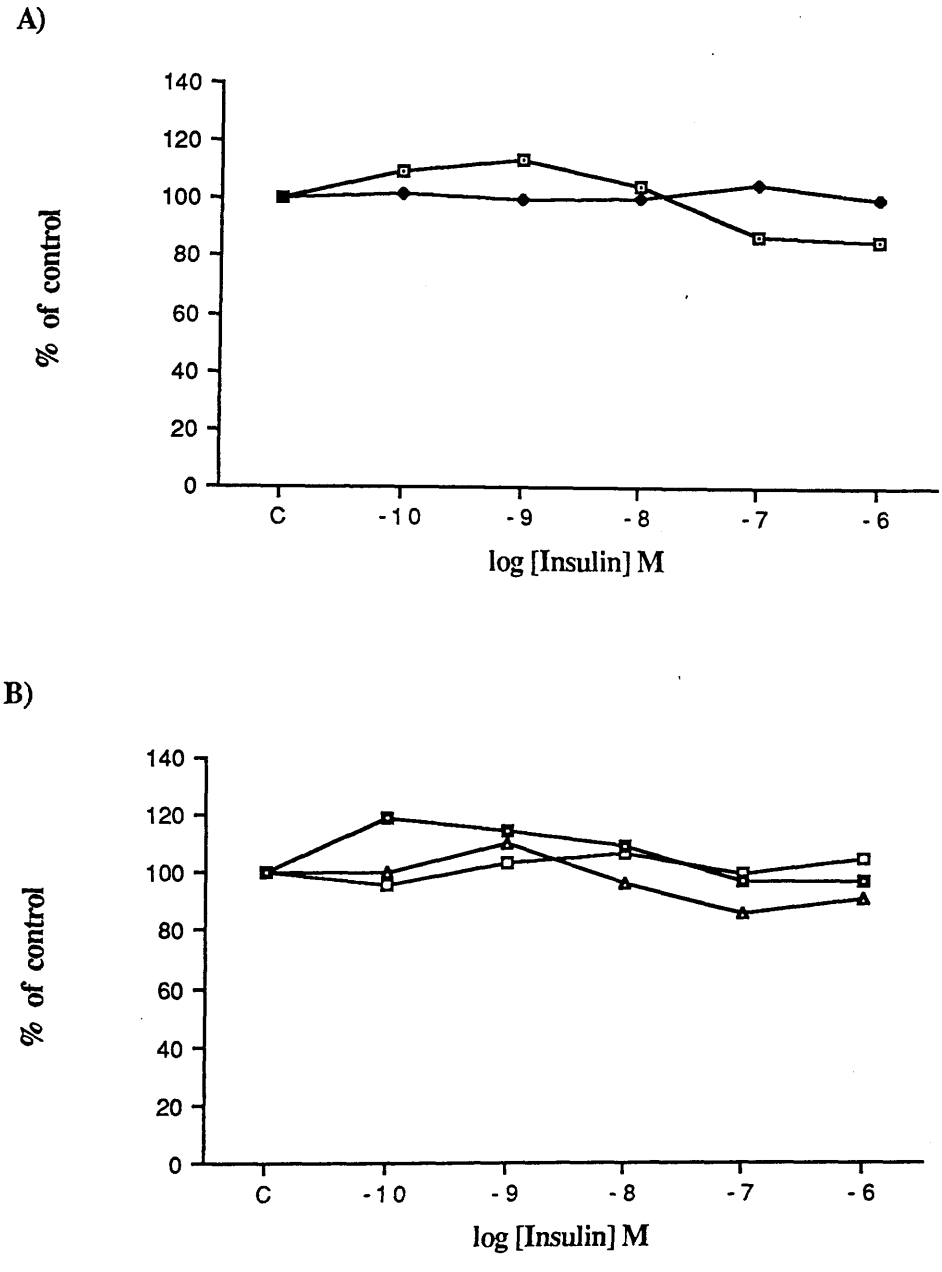


Figure 16 · Dose-response curves of (A) 7 α -hydroxylase [■] and 5 α -reductase [◆] and (B)17-OHSD [▩], 6 β - [▲] and 16 α -hydroxylases [□] to insulin after 1 hour preincubation in hepatocytes obtained from 3 days STZ-treated diabetic male rat. Results are expressed as percentage of the relevant control and mean ± S.D (N = 3). Absolute data is given in Table 13 .
C = control

the enzyme activities was seen (Figure 16).

4.1.3 HEPATOCYTES FROM *21-DAYS* STZ-TREATED DIABETIC RAT

4.1.3.1 Preincubation with insulin for 1/2, 1 and 2 hours

At **1/2 hour**, hepatocytes from 21-days STZ-diabetic rats were resistant to the effect of insulin (Table 14) showing no significant change for all the enzyme activities measured throughout the range of insulin concentration used (Figure 17). Serum glucose level rose 2.5 times and the animals underwent a weight loss (Table 12). Changes in the basal level of enzyme activity varied in the chronic diabetic rats. No significant changes were seen on 7α- and 16α-hydroxylases activity but lowering of activities of 6β-hydroxylase and 5α-reductase were observed.

At **1 hour**, low insulin concentrations (10^{-10} to 10^{-8} M) increased the enzyme activities in a dose - dependent manner (Table 15) but at higher concentrations (10^{-7} and 10^{-6} M) there was less effect. Figure 18 illustrates the dose-response effects on the female-specific enzymes (top panel) and male-specific enzymes (bottom panel) of insulin after 1 hour preincubation. It is interesting to note that hepatocytes from chronic-diabetic rat (21-days) exhibited higher activities for the female-specific enzymes (Figure 18A) than the male-specific enzymes (Figure 18B).

Hepatocytes subjected to **2 hours** of insulin preincubation displayed a small but significant dose-dependent increase in enzymes activities with maximum activity achieved at 10^{-8} M insulin concentration (Table 16 and Figure 19).

Insulin concentration	Enzyme activities (pmoles / min / million cells)				
	7α - OHase	6β - OHase	16α -OHase	17 - OHSD	5α - reductase
Control	40 ± 7	34 ± 2	44 ± 3	123 ± 2	86 ± 9
10^{-10} M	42 ± 4	38 ± 8	45 ± 2	131 ± 8	108 ± 6
10^{-9} M	43 ± 3	32 ± 3	45 ± 3	121 ± 6	92 ± 2
10^{-8} M	41 ± 2	34 ± 5	44 ± 5	125 ± 10	84 ± 2
10^{-7} M	36 ± 2	30 ± 5	34 ± 10	110 ± 5	88 ± 8
10^{-6} M	33 ± 3	36 ± 5	35 ± 10	113 ± 8	71 ± 3

Table 14 . Dose-response effects of 7 α -, 6 β -, 16 α -hydroxylases (OHases), 17-oxosteroid oxidoreductase (OHSD) and 5 α -reductase activities to insulin after 1/2 hour preincubation in hepatocytes obtained from 21 days STZ-treated diabetic male rat. Results expressed as mean ± S.D (N = 3); * $P < 0.05$ as compared to respective controls.

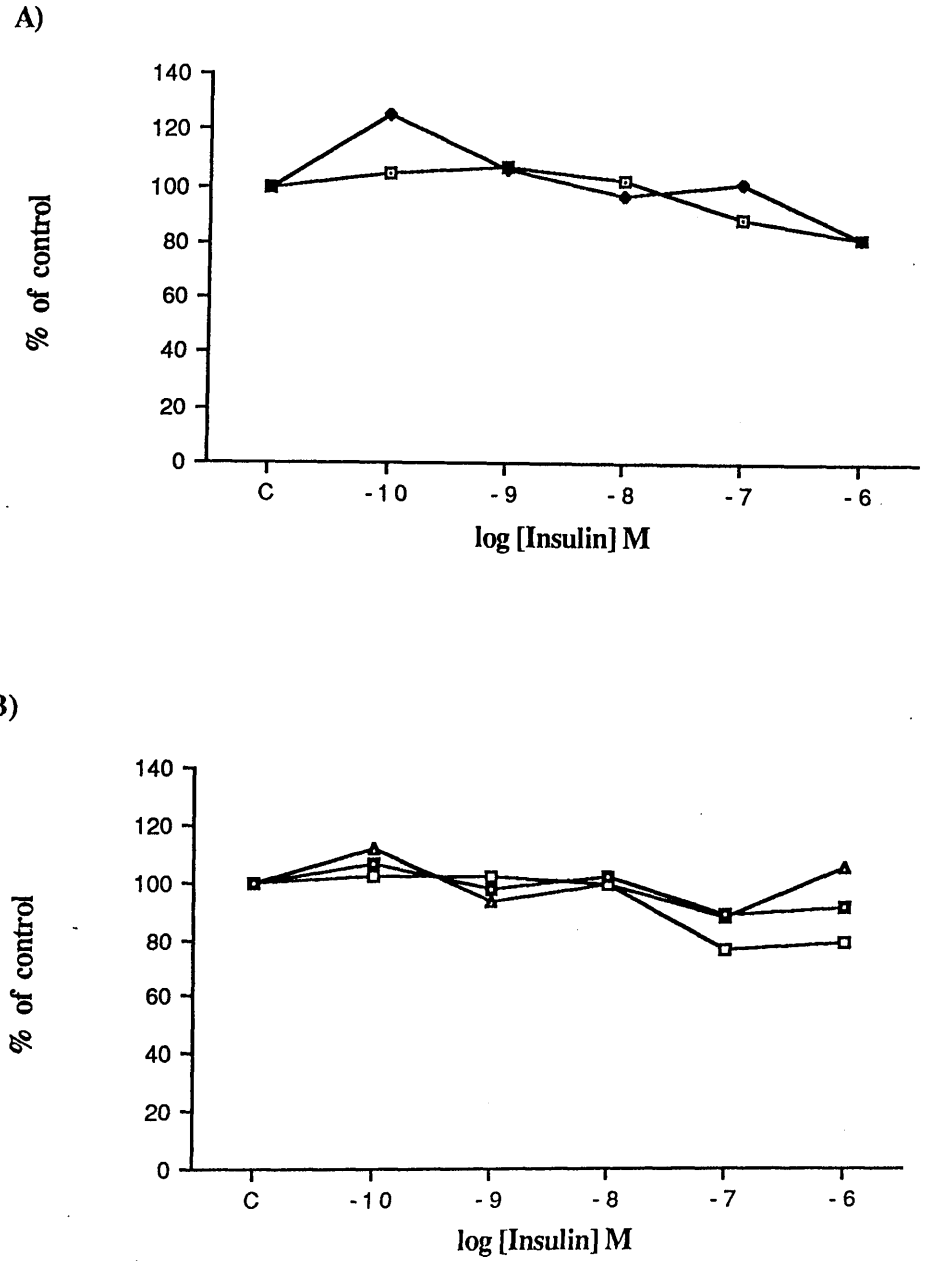


Figure 17 . Dose-response curves of (A) 7 α -hydroxylase [▫] and 5 α -reductase [•] and (B) 17-OHSD [▫], 6 β - [▲] and 16 α -hydroxylases [▫] to insulin after 1/2 hour preincubation in hepatocytes obtained from 21 days STZ-treated diabetic male rat. Results are expressed as percentage of the relevant control and mean ± S.D (N = 3). Absolute data is given in Table 14 .
C = control

Insulin concentration	Enzyme activities (pmoles / min / million cells)				
	7α - OHase	6β - OHase	16α -OHase	17 - OHSD	5α - reductase
Control	31 ± 2	30 ± 2	37 ± 1	118 ± 3	74 ± 10
10^{-10} M	34 ± 6	32 ± 3	36 ± 3	130 ± 9 *	122 ± 2 *
10^{-9} M	40 ± 3 *	35 ± 2 *	47 ± 3 *	143 ± 7 *	127 ± 7 *
10^{-8} M	.50 ± 5 *	41 ± 3 *	50 ± 4 *	140 ± 8 *	112 ± 10 *
10^{-7} M	38 ± 3 *	36 ± 2 *	43 ± 3 *	126 ± 3 *	104 ± 3 *
10^{-6} M	39 ± 3 *	34 ± 2 *	45 ± 1 *	126 ± 3 *	86 ± 3 *

Table 15 . Dose-response effects of 7 α -, 6 β -, 16 α -hydroxylases (OHases), 17-oxosteroid oxidoreductase (OHSD) and 5 α -reductase activities to insulin after 1 hour preincubation in hepatocytes obtained from 21 days STZ-treated diabetic male rat. Results expressed as mean ± S.D (N = 3); * $P < 0.05$ as compared to respective controls.

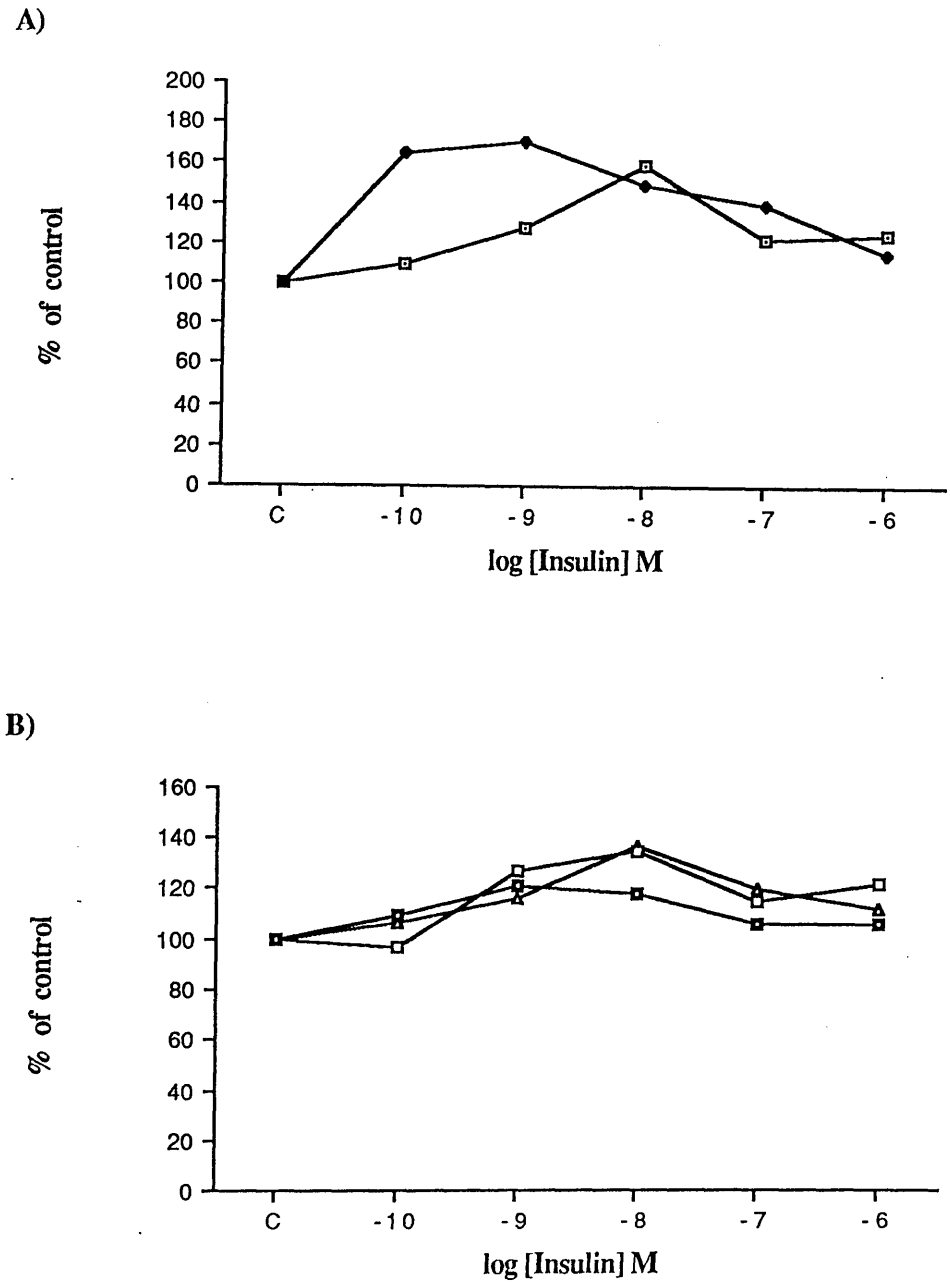


Figure 18. Dose-response curves of (A) 7 α -hydroxylase [▫] and 5 α -reductase [•] and (B) 17-OHSD [▫], 6 β - [▲] and 16 α -hydroxylases [▫] to insulin after 1 hour preincubation in hepatocytes obtained from 21 days STZ-treated diabetic male rat. Results are expressed as percentage of the relevant control and mean ± S.D (N = 3). Absolute data is given in Table 15 .
C = control

Insulin concentration	Enzyme activities (pmoles / min / million cells)				
	7α - OHase	6β - OHase	16α -OHase	17 - OHSD	5α - reductase
Control	42 ± 6	37 ± 1	52 ± 3	135 ± 3	85 ± 2
10^{-10} M	41 ± 4	39 ± 2	50 ± 2	135 ± 3	86 ± 20
10^{-9} M	41 ± 4	43 ± 2 *	58 ± 3 *	150 ± 8 *	94 ± 5 *
10^{-8} M	.45 ± 2	44 ± 3 *	57 ± 3 *	134 ± 2	111 ± 10 *
10^{-7} M	49 ± 1 *	40 ± 2 *	58 ± 4 *	150 ± 1 *	110 ± 1 *
10^{-6} M	49 ± 1 *	43 ± 8	54 ± 4	156 ± 7 *	114 ± 10 *

Table 16 . Dose-response effects of 7 α -, 6 β -, 16 α -hydroxylases (OHases), 17-oxosteroid oxidoreductase (OHSD) and 5 α -reductase activities to insulin after 2 hour preincubation in hepatocytes obtained from 21 days STZ-treated diabetic male rat. Results expressed as mean ± S.D (N = 3); * $P < 0.05$ as compared to respective controls.

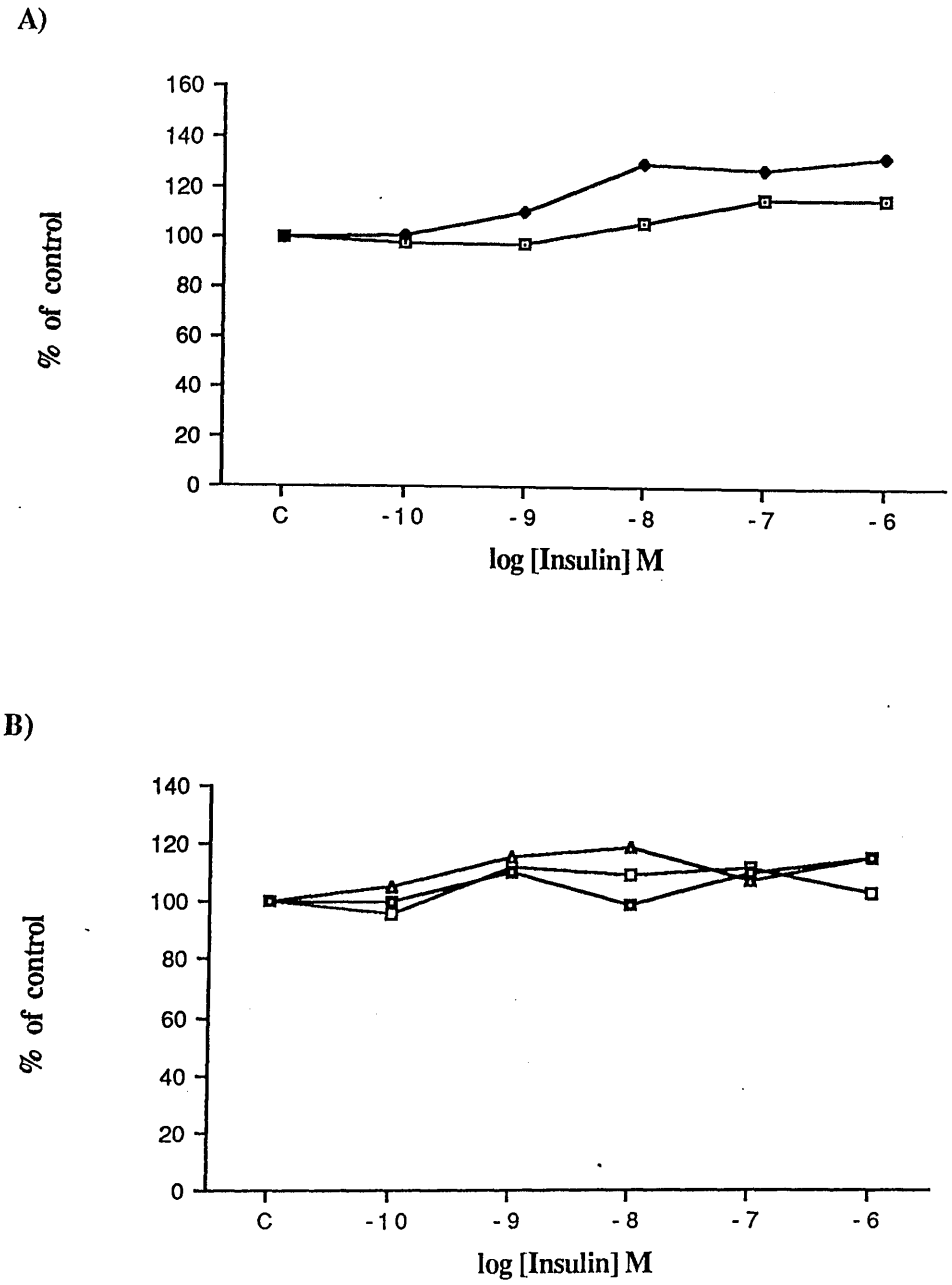


Figure 19. Dose-response curves of (A) 7 α -hydroxylase [▪] and 5 α -reductase [•] and (B) 17-OHSD [▫], 6 β - [▲] and 16 α -hydroxylases [▫] to insulin after 2 hour preincubation in hepatocytes obtained from 21 days STZ-treated diabetic male rat. Results are expressed as percentage of the relevant control and mean ± S.D (N = 3). Absolute data is given in Table 16 .
C = control

4.1.4 HEPATOCYTES FROM *INSULIN TREATED* 3-DAYS DIABETIC RAT

Different doses of insulin (2 , 12 and 16 units) were administered into the acutely STZ-treated diabetic rats (3-days) to investigate their efficacy to stimulate *in-vitro* insulin effect with respect to steroid enzymes activity (see Methods Section **2.2** for detail). In these experiments, hepatocytes were preincubated with insulin for 1/2 hour and then assayed for androst-4-ene-3,17-dione metabolism.

In diabetic rats treated with **2 units** insulin, *in-vitro* insulin addition elicited a significant dose-related increase in all of the enzyme activities (Table 17A). At 10^{-6} M insulin, enzyme activities were restored to about 50 to 75 % of that in normal hepatocytes. The enzyme activities increased significantly at concentration as low as 10^{-10} M insulin and the maximum effect (about 140 to 160 % of control) was seen at 10^{-8} M concentration for all of the enzymes (Figure 20).

Similar results were obtained with diabetic rats treated with **12 units** insulin. Again, partial restoration of the normal rat enzyme activities were noticed (Table 17B). Generally, statistically significant increases in enzyme activities were seen at 10^{-10} M insulin and above. As shown in Figure 20, all of the enzymes displayed a dose-dependent increase in activity reaching maximal responses at 10^{-8} M (except 6β-hydroxylase whose maximal response occurred at 10^{-7} M insulin concentration - Figure 20C). Thus the restoration of activity was about the same as that seen with diabetic rats treated with 2 units of insulin. With regard to the serum glucose level and change in body weight, they were not statistically significant from the control (Table 12). The 7α- , 6β- and 16α-hydroxylase activities were significantly greater than controls.

Hepatocytes from diabetic rats treated with **16 units** insulin however were unresponsive to *in-vitro* insulin addition throughout the concentration range used

Table 17. Dose-response effects of 7 α -, 6 β - and 16 α - hydroxylases, 17- oxosteroid oxidoreductase (OHSD) and 5 α -reductase activities to insulin after 1/2 hour preincubation in hepatocytes obtained from (A) 2 units (B) 12 units (C) 16 units of insulin - treated 3 -days STZ diabetic male rat. Results expressed as mean ± SD (N = 3); * P < 0.05 as compared to the respective control..

(A)

Insulin concentration	Enzyme activities (pmoles / min / million cells)				
	7 α-OHase	6 β-OHase	16 α-OHase	17 - OHSD	5 α-reductase
Control	31± 2	79 ± 4	79 ± 3	106±2	54 ±2
10^{-10} M	38 ± 2 *	88 ± 7	94 ± 3 *	118 ± 2 *	63 ± 2 *
10^{-9} M	40 ± 2 *	100 ± 5 *	102 ± 2 *	148 ± 4 *	72 ± 3 *
10^{-8} M	47 ± 3 *	117 ± 3 *	116 ± 3 *	164 ± 4 *	87 ± 4 *
10^{-7} M	50 ± 3 *	114 ± 3 *	121 ± 3 *	161 ± 6 *	90 ± 3 *
10^{-6} M	51 ± 3 *	110 ± 2 *	112 ± 3 *	151 ± 4 *	71 ± 3 *

(B)

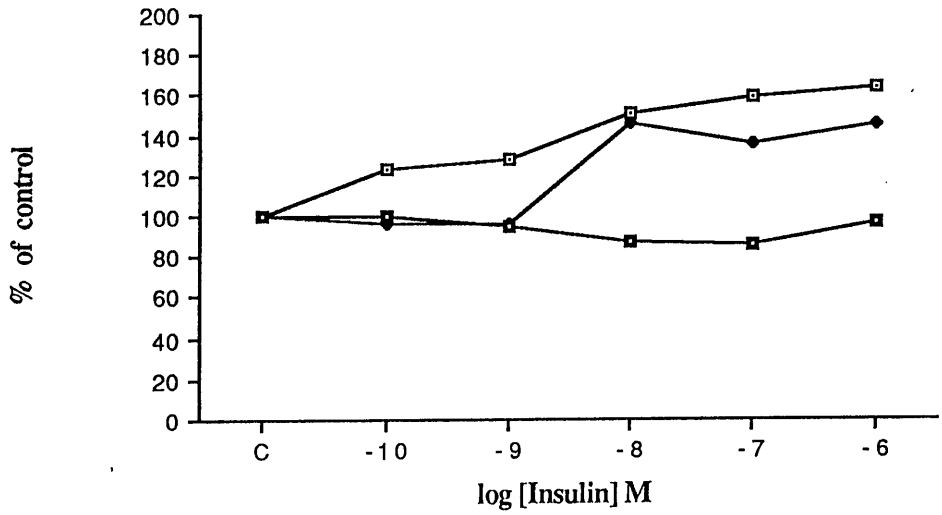
Insulin concentration	Enzyme activities (pmoles / min / million cells)				
	7 α-OHase	6 β-OHase	16 α-OHase	17 - OHSD	5 α-reductase
Control	49± 9	81 ± 3	75 ± 7	116 ±5	112 ±5
10^{-10} M	47 ± 5	94 ± 5 *	63 ± 4 *	123 ± 6	159 ± 4 *
10^{-9} M	47 ± 5	93 ± 10 *	79 ± 15	148 ± 10 *	172 ± 5 *
10^{-8} M	72 ± 1 *	108 ± 5 *	95 ± 9 *	168 ± 4 *	184 ± 4 *
10^{-7} M	67 ± 4 *	129 ± 4 *	91 ± 12 *	161 ± 8 *	189 ± 3 *
10^{-6} M	72 ± 6 *	118 ± 3 *	102 ± 6 *	169 ± 2 *	185 ± 4 *

(C)

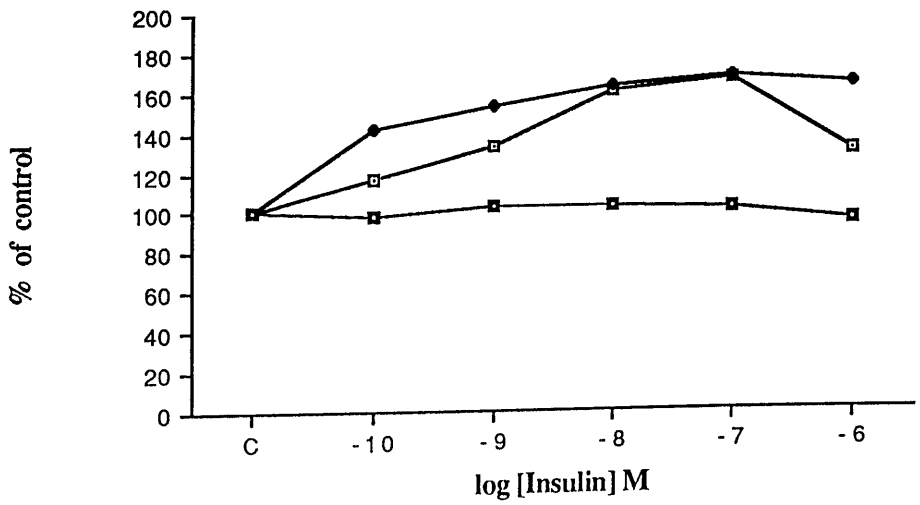
Insulin concentration	Enzyme activities (pmoles / min / million cells)				
	7 α -OHase	6 β-OHase	16 α-OHase	17 - OHSD	5 α-reductase
Control	59± 4	105 ± 5	109 ± 7	145± 4	114 ±4
10^{-10} M	59 ± 3	106 ± 1	106 ± 1	147 ± 4	112 ± 2
10^{-9} M	56 ± 3	107 ± 2	108 ± 6	145 ± 8	117 ± 4
10^{-8} M	52 ± 2 *	105 ± 6	100 ± 6	140 ± 6	117 ± 1
10^{-7} M	51 ± 2 *	100 ± 6	97 ± 7 *	139 ± 6	116 ± 2
10^{-6} M	58 ± 4	105 ± 3	101 ± 3	143 ± 5	110 ± 6

Figure 20. Dose-response curves of (A) 7 α -hydroxylase (B) 5 α -reductase (C) 6 β -hydroxylase (D)16 α -hydroxylase and (E) 17-OHSD activities to insulin after 1/2 hour preincubation in hepatocytes obtained from 3 days STZ-induced diabetic male rat treated with 2 units [▫] 12 units [•] and 16 units [▪] of Neulente insulin.
Results are expressed as percentage of the relevant control and mean ± S.D (N = 3).
Absolute data is given in Table 17 .
C = control

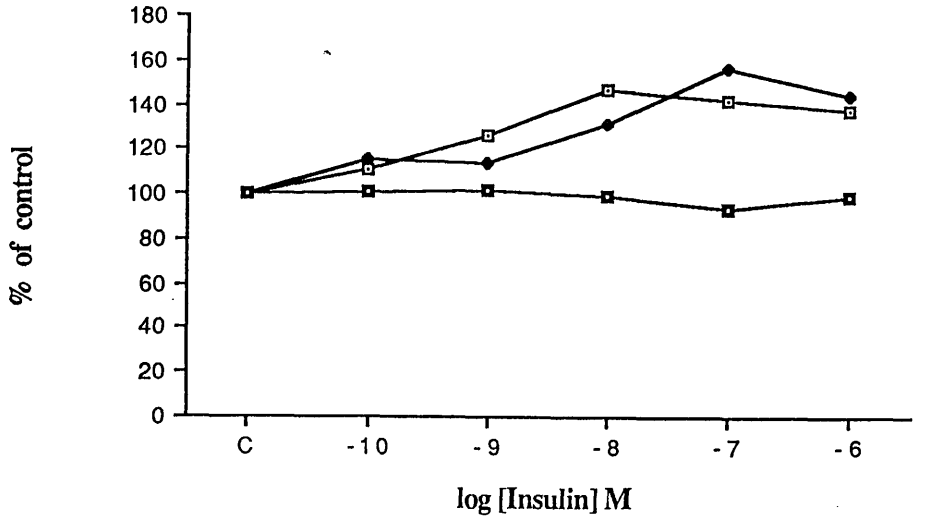
A)



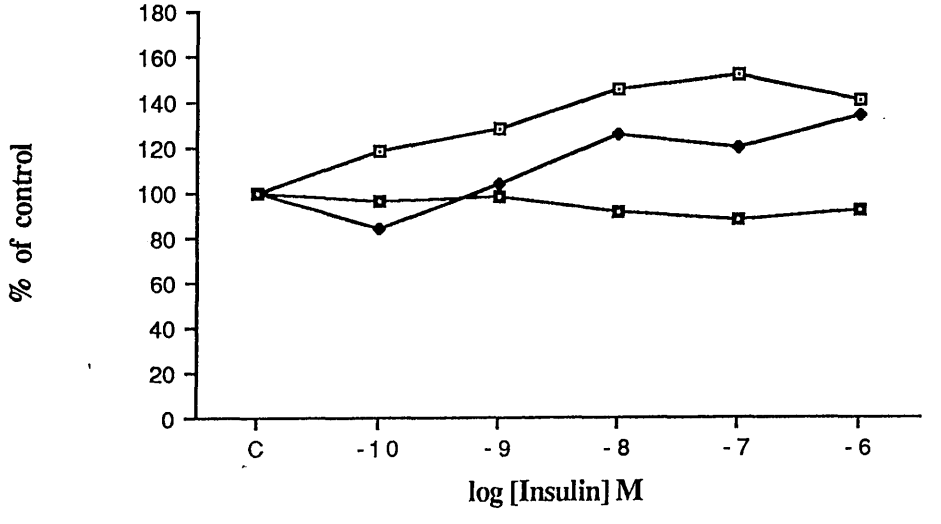
B)



C)


180
160
140
120
100
80
60
40
20
0
% of control
C
-10
-9
-8
-7
-6
log [Insulin] M

D)


180
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% of control
C
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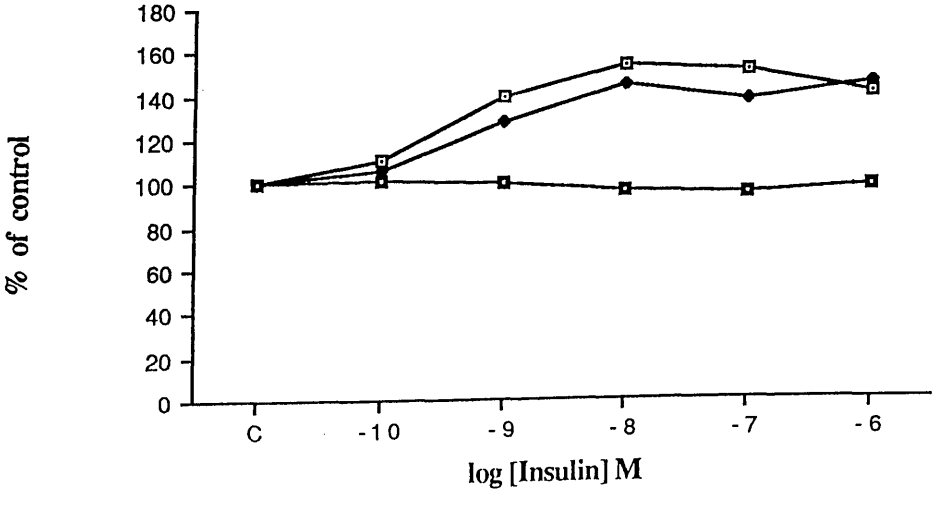
-8
-7
-6
log [Insulin] M

E)


180
160
140
120
100
80
60
40
20
0
% of control
C
-10
-9
-8
-7
-6
log [Insulin] M

(Table 17). All enzyme activities were not significantly different from control (Figure 20).

4.2 EFFECT OF INSULIN ON CYTOCHROME P-450 CONCENTRATION IN ISOLATED MALE RAT HEPATOCYTES

As cytochrome P-450 is one of the major component of the steroid-metabolising enzyme system, we decided to investigate the relationship between cytochrome P-450 content and insulin with respect to steroid metabolism. The cytochrome P-450 content was measured after 1/2, 1 , 2 and 24 hours of insulin *in-vitro* addition in normal male rat hepatocytes. As illustrated in Table 18, insulin at 10^{-9} and 10^{-6} M gave no significant changes in the hepatic level of cytochrome P-450 in the acutely (1/2, 1 and 2 hours) or chronically (24 hours) treated hepatocytes. Small amount of cytochrome P-420 was noted in culture but were ignored.

4.3 EFFECT OF INSULIN ON CYCLIC AMP CONCENTRATION IN ISOLATED MALE RAT HEPATOCYTES

The effect of insulin on cyclic AMP concentration in hepatocytes from normal male rat was investigated. Primary cultures of hepatocytes were preincubated with insulin (10^{-10} to 10^{-6} M) for 1/2 , 1 and 2 hours and then cyclic AMP content was determined as described in Methods Section 2.8.

Table 19 shows that no significant changes in the cyclic AMP concentration was seen in hepatocytes previously exposed to insulin for 1/2, 1 and 2 hours. There was a slight reduction in cyclic AMP concentration (to about 83 % of control) by 10^{-6} M insulin at 1 hour preincubation but this effect was not statistically significant (Figure 21).

Table 18 . Time course assay of cytochrome P-450 content after 1/2, 1, 2 and 24 hour preincubation with insulin (10^{-6} M and 10^{-9} M) in hepatocytes obtained from normal male rat. Cytochrome P-450 content was determined as described in Methods Section. The results are expressed as *p* moles million cells^{-1}.

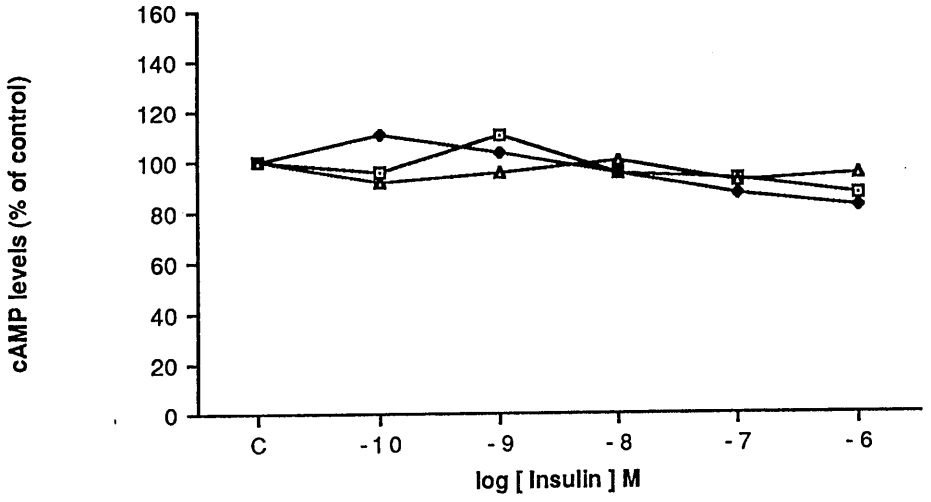
The data are the mean and standard deviations from a single experiment (representative of six similar experiments). § denotes no significant difference against control.

	Preincubation Period			
	1/2 hr.	1 hr.	2 hr.	24hr.
1) Control	58 ± 4	51 ± 3	53 ± 4	20 ± 3
2) Insulin (10^{-9} M)	57 ± 8 §	49 ± 4 §	49 ± 2 §	22 ± 5 §
3) Insulin (10^{-6} M)	56 ± 4 §	52 ± 2 §	51 ± 3 §	24 ± 6 §

Table 19. Effect of insulin on cyclic AMP content in hepatocytes from normal male rat. Hepatocytes were incubated with insulin for 1/2, 1 and 2 hours at the concentrations indicated and cyclic AMP content was determined as described in Methods Section 2.8. The data are the mean and standard deviations from a single experiment (representative of three similar experiments). None are significantly different from their respective controls.

Insulin Concentration (M)	cyclic AMP content (nmol / million cells)		
	1/2 hour	1 hour	2 hour
Control	11.25 ± 1.35	9.00 ± 0.27	9.00 ± 0.72
10^{-10} M	10.80 ± 0.22	9.99 ± 1.19	8.28 ± 0.66
10^{-9} M	12.49 ± 0.87	9.36 ± 1.40	8.64 ± 0.52
10^{-8} M	10.80 ± 0.64	8.64 ± 0.34	9.09 ± 0.27
10^{-7} M	10.58 ± 0.53	7.92 ± 0.39	8.37 ± 0.41
10^{-6} M	9.90 ± 0.39	7.47 ± 0.37	8.64 ± 0.60

Figure 21. Effect of insulin on cyclic AMP content in hepatocytes obtained from normal male rat. Hepatocytes were incubated with insulin for 1/2 [▫], 1 [•] and 2 [▲] hours and cyclic AMP content was determined as described in Methods Section 2.8. The results are mean values ± S.D (N = 3). None are significantly different from their respective controls (C).



4.4 EFFECT OF INSULIN ON PHOSPHATIDIC ACID AND PHOSPHATIDYLINOSITOL FORMATION IN ISOLATED MALE RAT HEPATOCYTES

4.4.1 Hepatocytes from normal male rat

The effect of insulin on $^{32}P_i$ incorporation into phospholipid in normal male rat hepatocytes was investigated. Figure 22 shows that insulin elicited no significant changes in the formation of phosphatidic acid and phosphatidylinositol. There was no significant difference in the ^{32}P labelling of phosphatidic acid and phosphatidylinositol between control- and insulin-treated cells. As illustrated in Figure 22,the ratio of formed phosphatidic acid to phosphatidylinositol was about 3 to 1 (6000 c.p.m : 2000 c.p.m).

4.4.2 Hepatocytes from 3-days STZ-treated male rat

Insulin-treated cells gave no significant increase in the ^{32}P labelling of phosphatidic acid and phosphatidylinositol when compared to control cells (Figure 23). However, the amount of ^{32}P labelling in the control from these diabetic animals was dramatically lower than in hepatocytes from normal rats. The amount of labelled [^{32}P]-phosphatidic acid formed in normal rat hepatocytes was 46-fold higher than in the hepatocytes from the diabetic rats (6000 c.p.m versus 130 c.p.m respectively) and about 17-fold higher for [^{32}P]-phosphatidylinositol in the normal rats compared to diabetic rats (2000 c.p.m versus 120 c.p.m respectively). The ratio of formed phosphatidic acid to phosphatidylinositol was about 1: 1 (Figure 23).

Figure 22. Concentration-response curves for insulin-induced (A) [^{32}-P] phosphatidic acid and (B) [^{32}-P] phosphatidylinositol formation in hepatocytes obtained from normal male rat.
The formation of both labelled-lipids was monitored as described in the Methods section. The results are mean values ± S.D for 2 experiments performed in triplicate. All points were not significantly different from control.

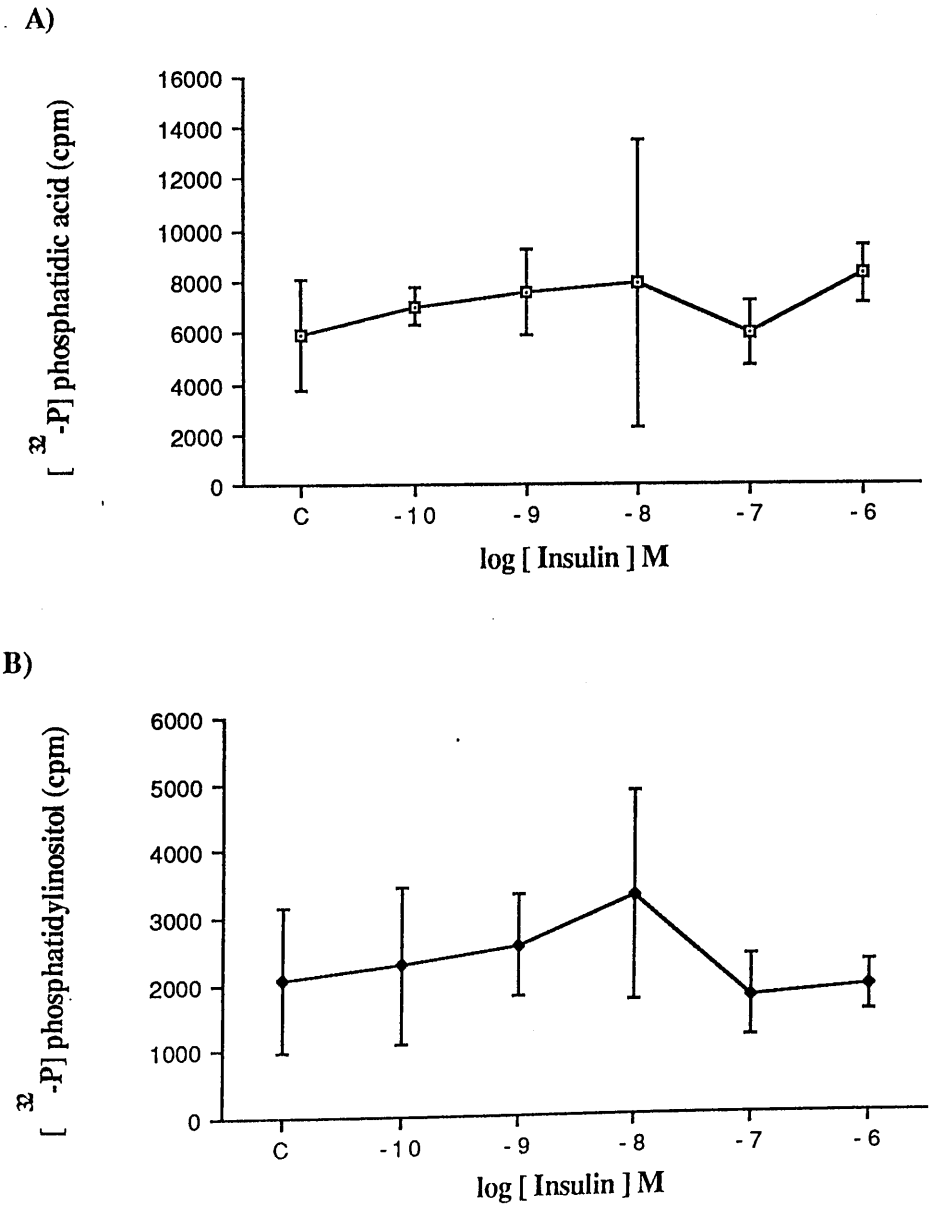
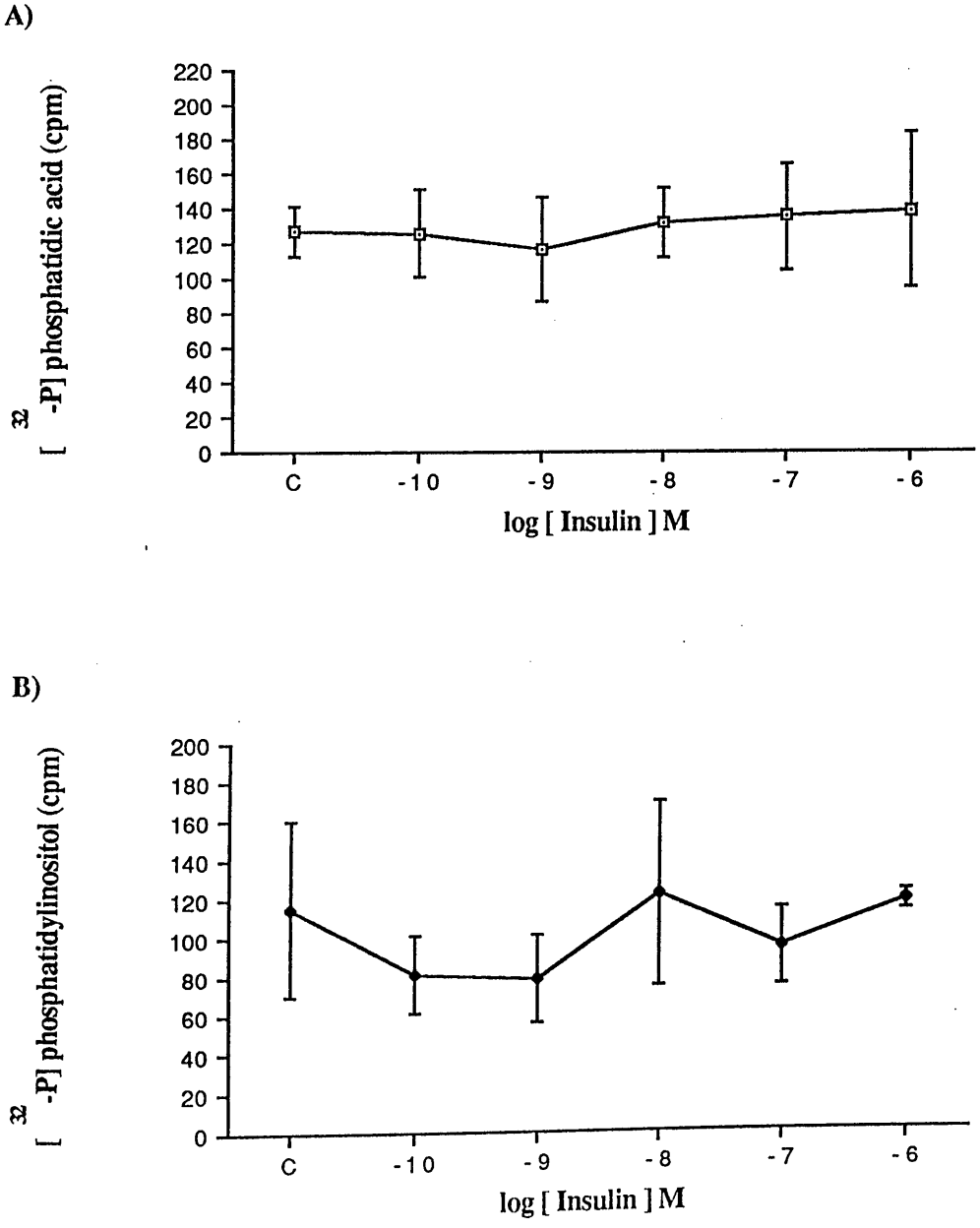


Figure 23 . Concentration-response curves for insulin-induced (A) [^{32} -P] phosphatidic acid and (B) [^{32} -P] phosphatidylinositol formation in hepatocytes obtained from 3-days STZ-treated diabetic male rat.
The formation of both labelled-lipids was monitored as described in the Methods section. The results are mean values ± S.D for 2 experiments performed in triplicate. All points were not significantly different from control.



4.5 PREINCUBATION OF MALE RAT HEPATOCYTES WITH INSULIN IN THE PRESENCE OF THE PROTEIN KINASE INHIBITOR K-252a

4.5.1 Preincubation with insulin for 1/2 hour in the presence of K-252a (20 nM)

Since it is well recognized that the second messengers of hormone action are proposed to be mediated through activation of protein kinases and phosphorylation of proteins plays a key role in regulating cellular functions, we decided to investigate the role of protein kinases in insulin action with respect to steroid metabolism. In this study, K-252a, a non-selective inhibitor of the protein kinases was used. K-252a was found to inhibit cyclic nucleotide-dependent protein kinases and protein kinase C to various extents with K_i values at 20 nM (Kase et.al. 1987).

Table 20 shows that K-252a alone had no effect on androst-4-ene-3,17-one metabolism. None of the enzyme activities showed any significant changes when compared to control. In the presence of K-252a (20 nM), increasing doses of insulin (10^{-10} - 10^{-6} M) did not produce any statistically significant changes in the enzyme activities (Figure 24). This should be compared to Table 4 and Figure 7 .

4.5.2 Preincubation with insulin for 24 hours in the presence of K-252a (20 nM)

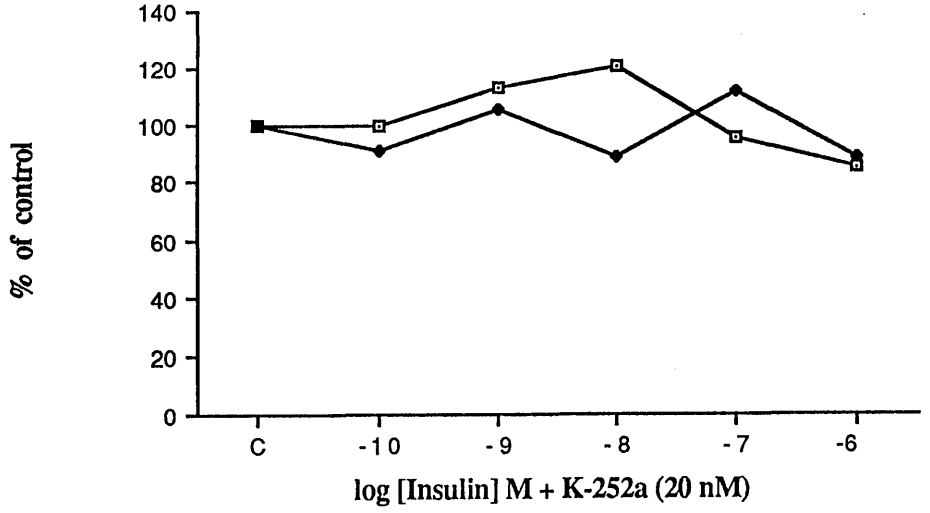
Table 21 shows that K-252a alone had no effect on androst-4-ene-3,17-dione when compared to control. Similar to preincubation with insulin for 1/2 hour, the addition of K-252a (20 nM) to the culture medium after 24 hour insulin preincubation resulted in no significant changes in all the enzyme activities (Figure 25). The basal activity for all of the enzymes was reduced when compared to the activity in 1/2 hour preincubation with insulin in the presence of K-252a (see **section 4.5.1**).

Drug concentration	Enzyme activities (pmoles / min / million cells)				
	7α - OHase	6β - OHase	16 α-OHase	17 - OHSD	5α - reductase
Control (DMSO)	37 ± 4	48 ± 3	74 ± 3	68 ± 7	48 ± 3
K (20 nM) [a] alone	36 ± 4	48 ± 6	68 ± 10	72 ± 3	48 ± 2
K + I (0.1 nM)	36 ± 4	44 ± 6	63 ± 16	63 ± 6	44 ± 3
K + I (1 nM)	41 ± 5	55 ± 11	63 ± 3	64 ± 7	51 ± 6
K + I (10 nM)	44 ± 6	57 ± 6 *	67 ± 11	68 ± 7	43 ± 5
K + I (100 nM)	35 ± 8	49 ± 14	72 ± 17	60 ± 16	54 ± 16
K + I (1 μM)	31 ± 4	46 ± 6	72 ± 14	66 ± 7	43 ± 2

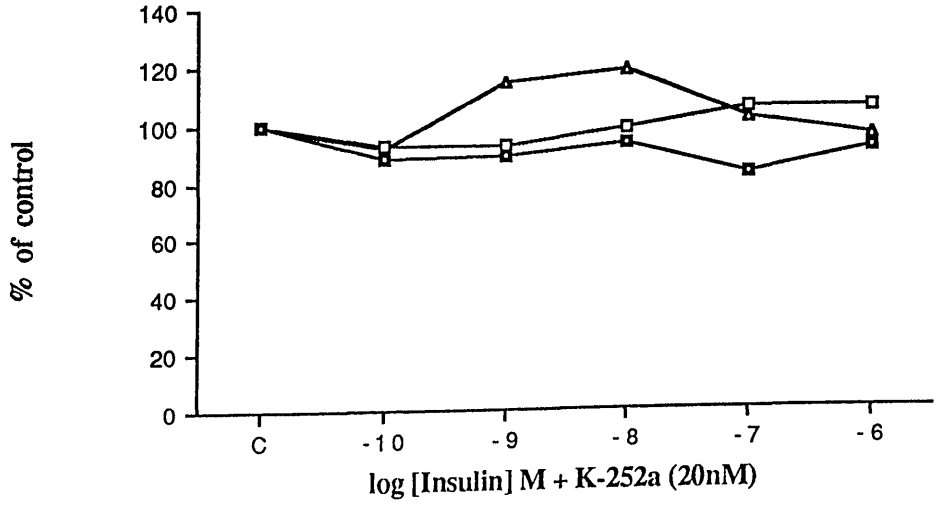
Table 20. Dose-response effects of 7 α -, 6 β -, 16 α -hydroxylases (OHases), 17-oxosteroid dehydrogenase (OHSD) and 5 α -reductase activities to insulin (I) after 1/2 hour preincubation in the presence of 20 nM K-252a (K) in hepatocytes obtained from normal male rat. K-252a was dissolved in dimethyl-sulphoxide (DMSO). Results expressed as mean ± S.D (N = 3); * $P < 0.05$ with respect to [a] .

Figure 24 . Dose-response curves of (A) 7 α -hydroxylase [◘] and 5 α -reductase [•] and (B) 17-OHSD [▫], 6 β - [▲] and 16 α -hydroxylases [▫] activities to insulin after 1/2 hour preincubation in the presence of 20 nM of K-252a in hepatocytes obtained from normal male rat as described in the Methods section. Results are expressed as percentage of the relevant control and mean ± S.D (N = 3). Absolute data is given in Table 20 .
C = control (20 nM K-252a dissolved in the vehicle)

A)



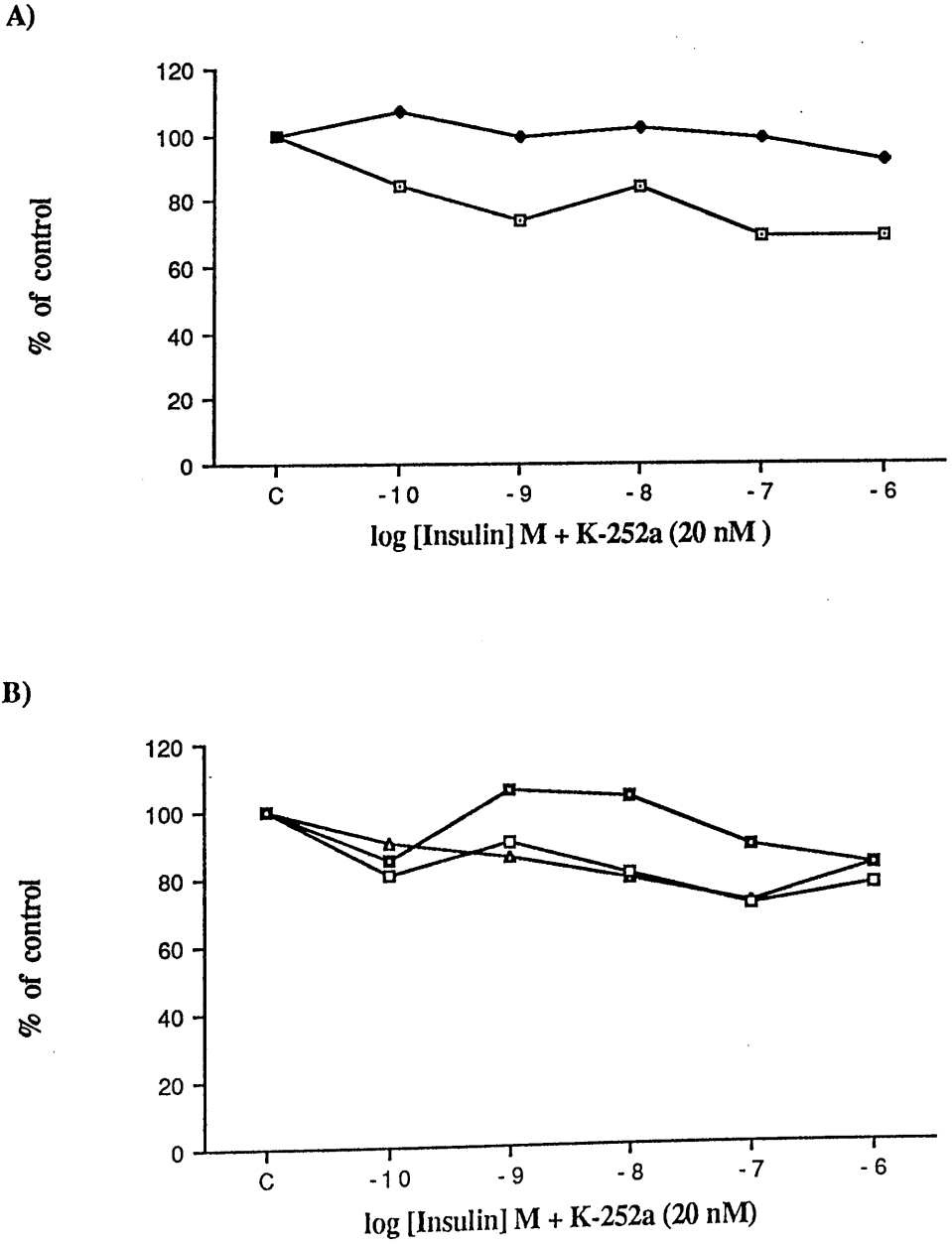
B)



Drug concentration	Enzyme activities (pmoles / min / million cells)				
	7α - OHase	6β - OHase	16 α-OHase	17 - OHSD	5 α-reductase
Control (DMSO)	25 ± 2	27 ± 2	33 ± 2	44 ± 2	32 ± 3
K (20 nM) [a] alone	27 ± 7	29 ± 6	31 ± 5	47 ± 6	29 ± 3
K + I (0.1 nM)	23 ± 2	26 ± 1	25 ± 3	40 ± 5	31 ± 3
K + I (1 nM)	20 ± 3	25 ± 2	28 ± 3	50 ± 3	29 ± 1
K + I (10 nM)	23 ± 2	23 ± 2	25 ± 1	49 ± 4	30 ± 1
K + I (100 nM)	19 ± 3	21 ± 2	22 ± 2	42 ± 1	29 ± 2
K + I (1 μM)	19 ± 3	24 ± 4	24 ± 2	39 ± 5	27 ± 1

Table 21. Dose-response effects of 7 α -, 6 β -, 16 α -hydroxylases (OHases), 17-oxosteroid dehydrogenase (OHSD) and 5 α -reductase activities to insulin (I) after 24 hour preincubation in the presence of 20 nM K-252a (K) in hepatocytes obtained from normal male rat. K-252a was dissolved in dimethyl-sulphoxide (DMSO). Results expressed as mean ± S.D (N = 3); * $P < 0.05$ with respect to [a] .

Figure 25 . Dose-response curves of (A) 7 α -hydroxylase [▫] and 5 α -reductase [•] and (B) 17-OHSD [▫], 6 β - [▲] and 16 α -hydroxylases [▫] activities to insulin after 24 hour preincubation in the presence of 20 nM of K-252a in hepatocytes obtained from normal male rat as described in the Methods section. Results are expressed as percentage of the relevant control and mean ± S.D (N = 3). Absolute data is given in Table 21 .
C = control (20 nM K-252a dissolved in the vehicle)



5.0 GLUCAGON

5.1 ACTION OF GLUCAGON ON THE METABOLISM OF ANDROST-4-ENE-3,17-DIONE

5.1.1 HEPATOCYTES FROM NORMAL RAT

5.1.1.1 Preincubation with glucagon for 1/2, 1, 2, 5 and 10 minutes

After **1/2 minute** preincubation with glucagon, no changes in the activity of any of the enzymes were observed (Table 22). Lack of effect could be seen at physiological and supraphysiological glucagon concentrations (Figure 26).

Similar observations were made when the cells were preincubated with glucagon for **1 minute** (Table 23). When comparing to control, no alteration in the enzyme activities was evident. Though all of the five enzyme activities measured were shown to increased above their respective controls at 10^{-8} M glucagon concentration, they were found to be not statistically significant (Figure 27).

At **2, 5 and 10 minutes** after glucagon addition, no effect on any of the enzyme activities was seen when compared to their respective controls (Tables 24, 25 and 26; Figures 28, 29 and 30). It is noteworthy to point out that the basal activity of 5α-reductase (female-specific enzyme) was seen to increase with glucagon preincubation time (from 1/2 minute to 10 minutes).

5.1.1.2 Preincubation with glucagon for 1/2, 1 and 2 hours

After exposing the hepatocytes to glucagon for **1/2 hour**, significant changes in

Glucagon concentration	Enzyme activities (pmoles / min / million cells)				
	7α - OHase	6β - OHase	16 α -OHase	17 - OHSD	5 α - reductase
Control	18 ± 1	47 ± 1	75 ± 3	50 ± 1	77 ± 13
10^{-10} M	16 ± 2 *	51 ± 4	70 ± 7	53 ± 6	75 ± 6
10^{-9} M	17 ± 4	50 ± 2	84 ± 21	56 ± 8	84 ± 23
10^{-8} M	25 ± 9	48 ± 3	72 ± 7	53 ± 3	70 ± 7
10^{-7} M	16 ± 5	49 ± 2	76 ± 4	55 ± 6	81 ± 3
10^{-6} M	15 ± 3	47 ± 5	74 ± 2	50 ± 5	60 ± 5

Table 22 . Dose-response effects of 7 α -, 6 β -, 16 α -hydroxylases (OHases), 17-oxosteroid oxidoreductase (OHSD) and 5 α -reductase activities to glucagon after 1/2 minute preincubation in hepatocytes obtained from normal male rat. Results expressed as mean ± S.D (N = 3); * $P < 0.05$ as compared to respective controls.

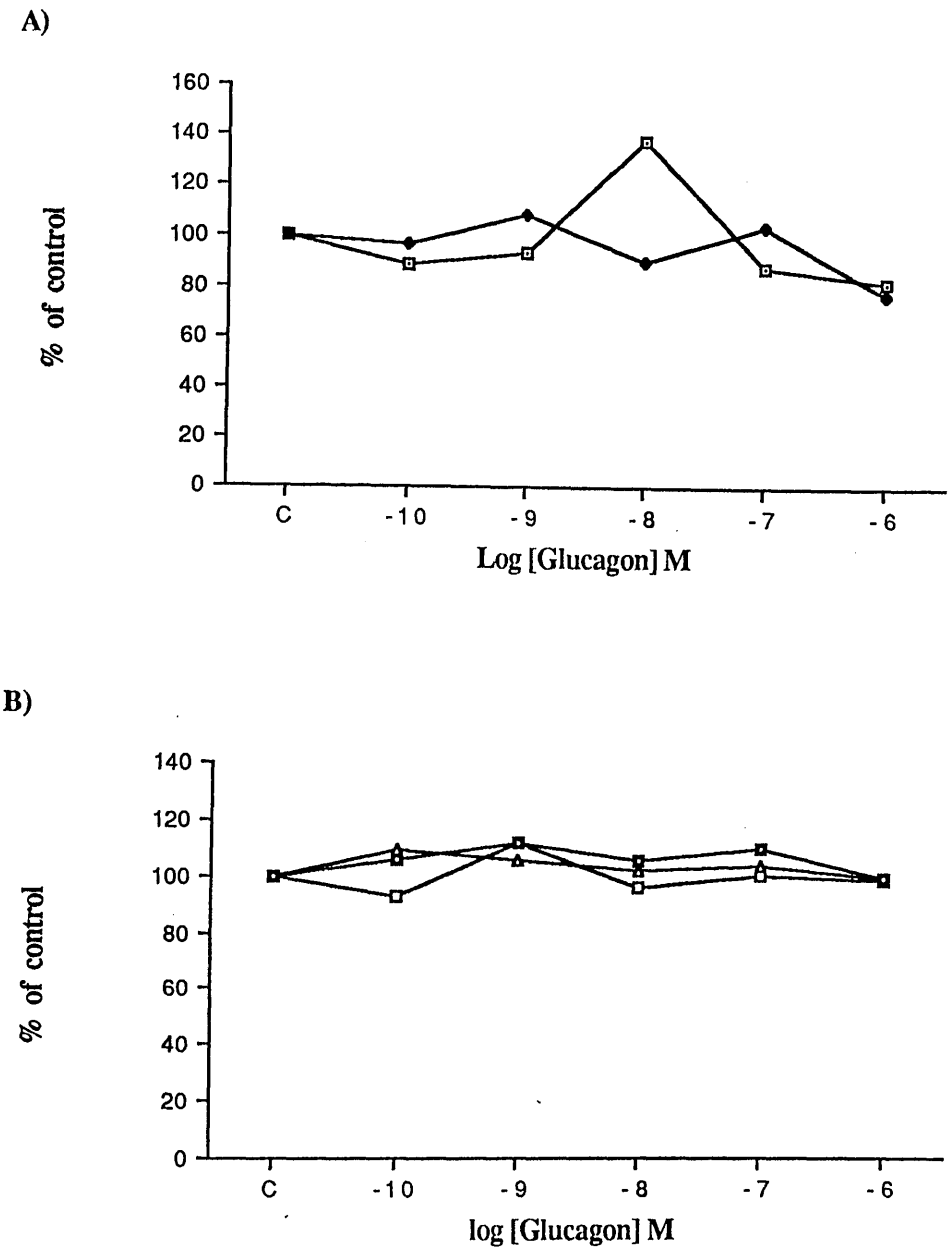
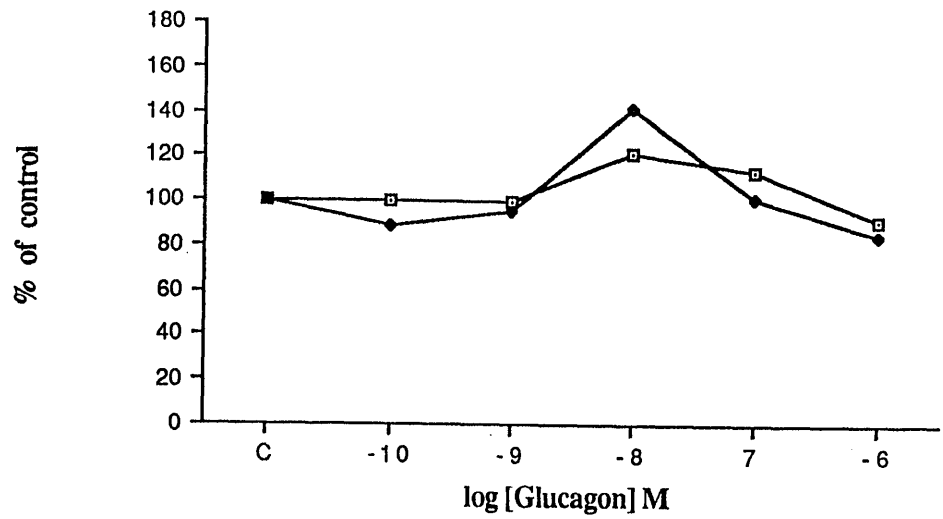


Figure 26 . Dose-response curves of (A) 7 α -hydroxylase [▫] and 5 α -reductase [♦] and (B) 17-OHSD [▫], 6 β - [▲] and 16 α -hydroxylases [▫] to glucagon after 1/2 minute preincubation in hepatocytes obtained from normal male rat.
Results are expressed as percentage of the relevant control and mean ± S.D (N = 3). Absolute data is given in Table 22 .
C = control

Glucagon concentration	Enzyme activities (pmoles / min / million cells)				
	7α - OHase	6β - OHase	16 α -OHase	17 - OHSD	5 α - reductase
Control	13 ± 3	32 ± 12	48 ± 26	48 ± 9	54 ± 12
10^{-10} M	13 ± 10	33 ± 2	46 ± 3	49 ± 2	48 ± 1
10^{-9} M	13 ± 2	33 ± 9	48 ± 16	51 ± 4	52 ± 6
10^{-8} M	16 ± 1	47 ± 5	77 ± 8	58 ± 3	77 ± 11 *
10^{-7} M	15 ± 2	38 ± 4	58 ± 10	51 ± 3	55 ± 7
10^{-6} M	12 ± 1	29 ± 4	38 ± 5	48 ± 2	46 ± 2

Table 23 . Dose-response effects of 7 α -, 6 β -, 16 α -hydroxylases (OHases), 17-oxosteroid oxidoreductase (OHSD) and 5 α -reductase activities to glucagon after 1 minute preincubation in hepatocytes obtained from normal male rat. Results expressed as mean ± S.D (N = 3); * $P < 0.05$ as compared to respective controls.

A)



B)

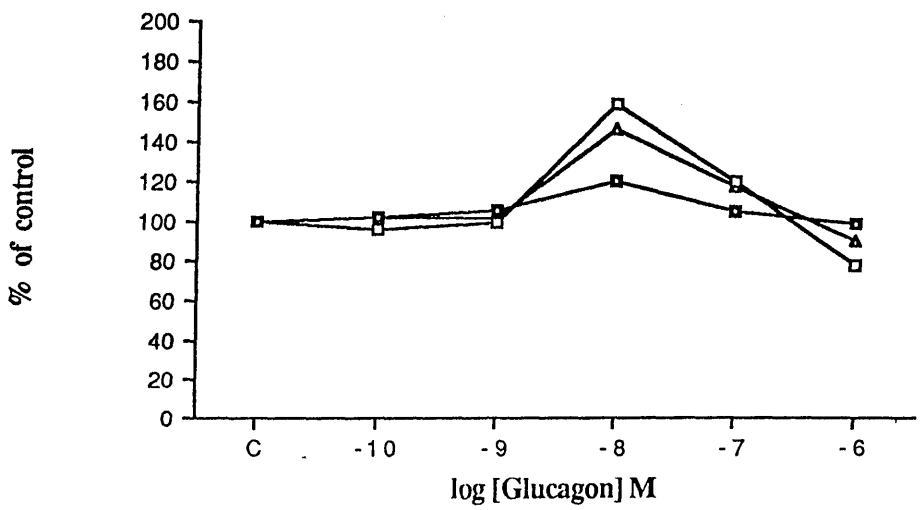


Figure 27 . Dose-response curves of (A) 7 α -hydroxylase [▫] and 5 α -reductase [•] and (B) 17-OHSD [▫], 6 β - [▲] and 16 α -hydroxylases [▫] to glucagon after 1 minute preincubation in hepatocytes obtained from normal male rat.
Results are expressed as percentage of the relevant control and mean ± S.D (N = 3). Absolute data is given in Table 23 .
C = control

Glucagon concentration	Enzyme activities (pmoles / min / million cells)				
	7 α - OHase	6 β - OHase	16 α -OHase	17 - OHSD	5 α - reductase
Control	24 ± 2	64 ± 7	59 ± 11	99 ± 21	76 ± 8
10^{-10} M	29 ± 5	84 ± 18	85 ± 19	102 ± 4	99 ± 27
10^{-9} M	30 ± 6	83 ± 18	74 ± 22	104 ± 3	84 ± 12
10^{-8} M	21 ± 2	65 ± 4	60 ± 1	107 ± 8	76 ± 4
10^{-7} M	23 ± 2	58 ± 9	53 ± 12	80 ± 13	91 ± 17
10^{-6} M	22 ± 0	58 ± 14	53 ± 13	90 ± 3	73 ± 3

Table 24 . Dose-response effects of 7 α -, 6 β -, 16 α -hydroxylases (OHases), 17-oxosteroid oxidoreductase (OHSD) and 5 α -reductase activities to glucagon after 2 minutes preincubation in hepatocytes obtained from normal male rat. Results expressed as mean ± S.D (N = 3); * $P < 0.05$ as compared to respective controls.

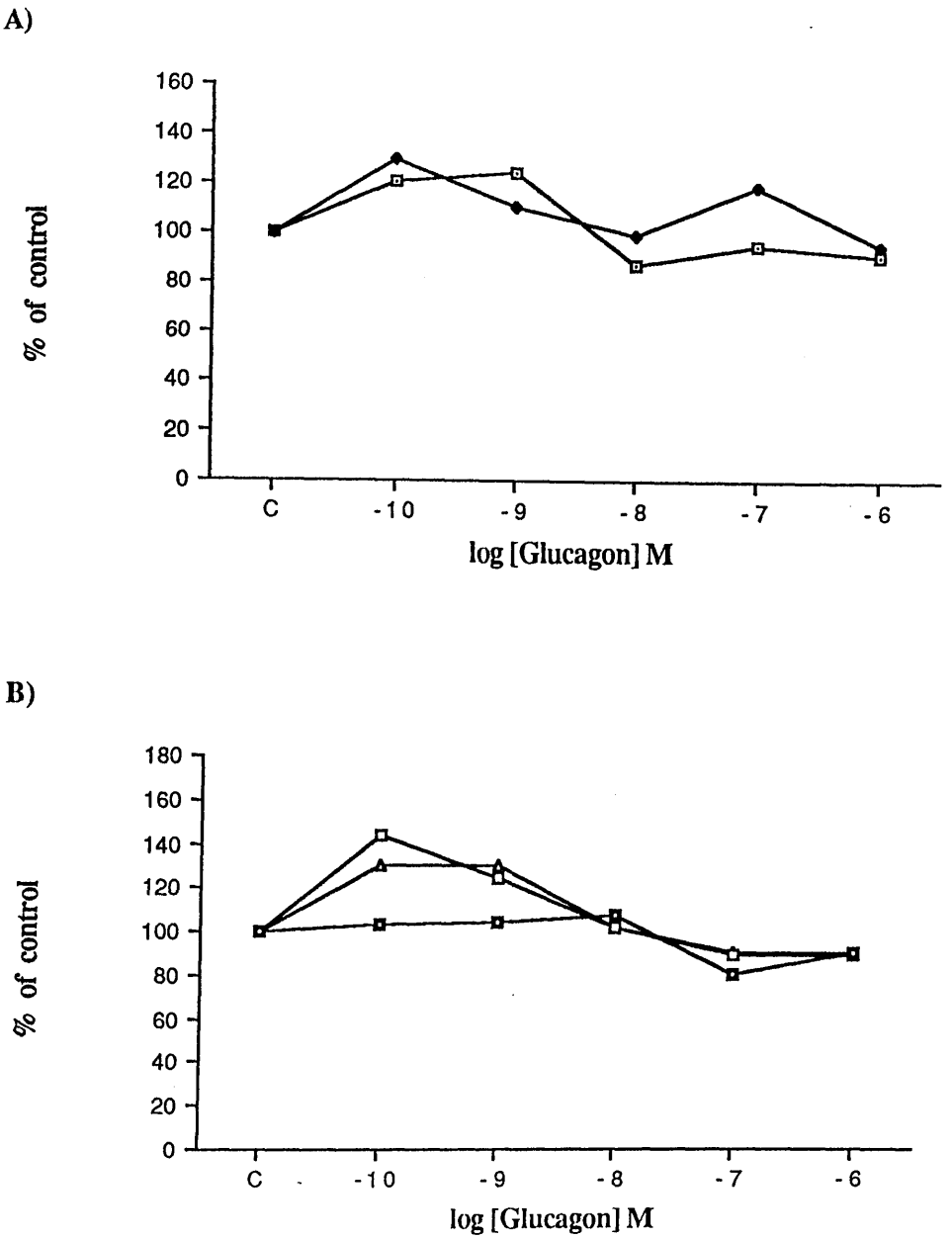


Figure 28 . Dose-response curves of (A) 7 α -hydroxylase [▫] and 5 α -reductase [•] and (B) 17-OHSD [▫], 6 β - [▲] and 16 α -hydroxylases [▫] to glucagon after 2 minutes preincubation in hepatocytes obtained from normal male rat.
Results are expressed as percentage of the relevant control and mean ± S.D (N = 3). Absolute data is given in Table 24 .
C = control

Glucagon concentration	Enzyme activities (pmoles / min / million cells)				
	7 α - OHase	6 β - OHase	16 α -OHase	17 - OHSD	5 α - reductase
Control	23 ± 1	69 ± 2	62 ± 8	91 ± 14	80 ± 13
10^{-10} M	20 ± 4	65 ± 8	51 ± 24	83 ± 9	68 ± 8
10^{-9} M	21 ± 1 *	59 ± 3	57 ± 3	97 ± 9	73 ± 19
10^{-8} M	.23 ± 3	63 ± 6	63 ± 15	94 ± 16	85 ± 6
10^{-7} M	21 ± 2	69 ± 6	61 ± 8	85 ± 9	70 ± 3
10^{-6} M	20 ± 4	71 ± 3	44 ± 7 *	87 ± 13	77 ± 4

Table 25 . Dose-response effects of 7 α -, 6 β -, 16 α -hydroxylases (OHases), 17-oxosteroid oxidoreductase (OHSD) and 5 α -reductase activities to glucagon after 5 minutes preincubation in hepatocytes obtained from normal male rat. Results expressed as mean ± S.D (N = 3); * $P < 0.05$ as compared to respective controls.

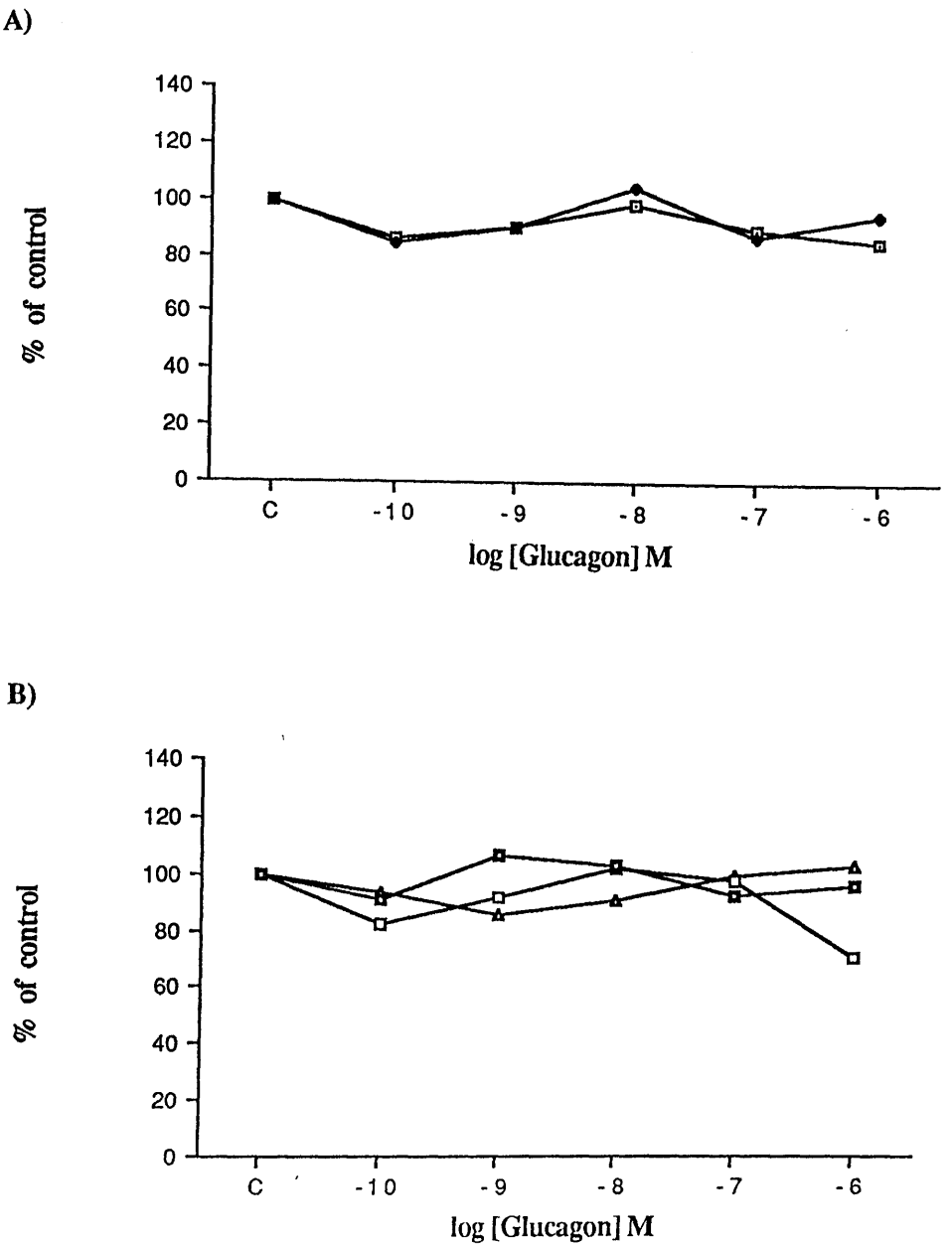


Figure 29 . Dose-response curves of (A) 7 α -hydroxylase [▫] and 5 α -reductase [♦] and (B) 17-OHSD [▫], 6 β - [▲] and 16 α -hydroxylases [▫] to glucagon after 5 minutes preincubation in hepatocytes obtained from normal male rat.
Results are expressed as percentage of the relevant control and mean ± S.D (N = 3). Absolute data is given in Table 25 .
C = control

Glucagon concentration	Enzyme activities (pmoles / min / million cells)				
	7 α - OHase	6 β - OHase	16 α -OHase	17 - OHSD	5 α - reductase
Control	12 ± 1	37 ± 4	55 ± 5	48 ± 4	106 ± 28
10^{-10} M	10 ± 1 *	36 ± 1	52 ± 3	49 ± 3	101 ± 6
10^{-9} M	11 ± 1	37 ± 2	55 ± 2	50 ± 2	95 ± 12
10^{-8} M	12 ± 1	34 ± 2	50 ± 4	46 ± 4	99 ± 14
10^{-7} M	11 ± 1	37 ± 3	51 ± 3	49 ± 7	105 ± 29
10^{-6} M	11 ± 1	34 ± 3	51 ± 4	46 ± 5	100 ± 26

Table 26 . Dose-response effects of 7 α -, 6 β -, 16 α -hydroxylases (OHases), 17-oxosteroid oxidoreductase (OHSD) and 5 α -reductase activities to glucagon after 10 minutes preincubation in hepatocytes obtained from normal male rat. Results expressed as mean ± S.D (N = 3); * $P < 0.05$ as compared to respective controls.

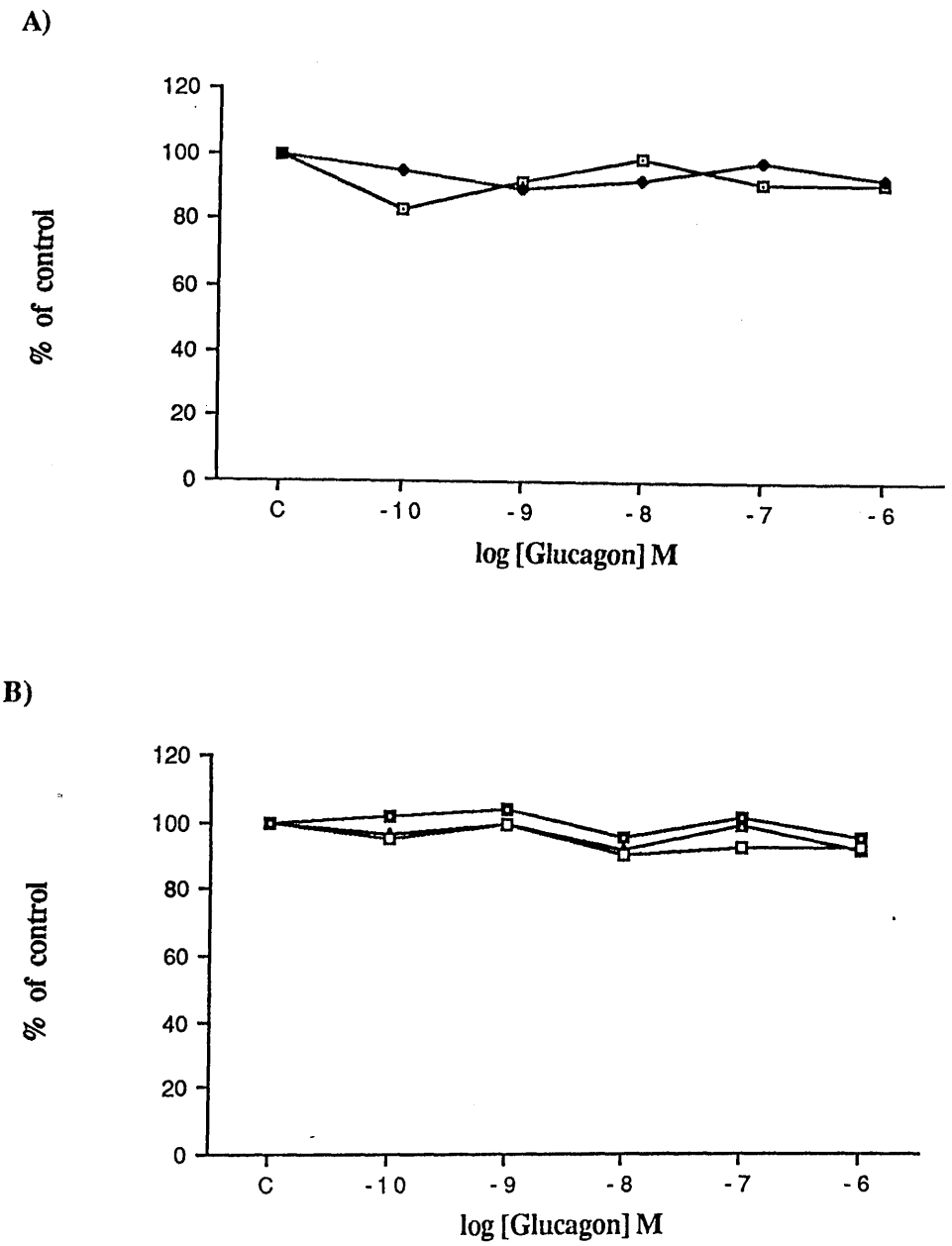


Figure 30 . Dose-response curves of (A) 7 ∝ -hydroxylase [▫] and 5 ∝ -reductase [•] and (B) 17-OHSD [▫], 6 β - [▲] and 16 ∝ -hydroxylases [▫] to glucagon after 10 minutes preincubation in hepatocytes obtained from normal male rat.

Results are expressed as percentage of the relevant control and mean ± S.D (N = 3). Absolute data is given in Table 26 .

C = control

enzyme activities could be observed which were dose-dependent (Table 27). At physiological concentrations (10^{-10} to 10^{-8} M), glucagon caused a dose-dependent decrease in all the enzyme activities showing no selective effect on the male- and female-specific enzymes in the cultured hepatocytes system (Figure 31). The maximum effect of glucagon was seen at 10^{-9} M or 10^{-8} M. Attenuation of glucagon's effect could be seen at concentrations of 10^{-7} M and above with activity returning near to control levels.

After **1 hour** preincubation glucagon gave exactly an inverse response as seen at 1/2 hour preincubation (Table 28). Glucagon, at lower concentrations (10^{-10} to 10^{-8} M) caused a dose-dependent increase in all the five enzyme activities with maximum response seen at 10^{-8} M (Figure 32). At 10^{-7} M the activity of all the enzymes diminished though still significantly higher than their respective controls. At 10^{-6} M, 7α- and 16α-hydroxylase activity returned to control levels while the other enzymes had their activity reduced significantly below control. Significant increases in activity of the female-specific enzymes (Figure 32A) were first observed at 10^{-8} M concentration and the male-specific enzymes (except 17-OHSD) at 10^{-10} M (Figure 32B).

At **2 hour** preincubation with glucagon, selective increases in enzyme activities were observed. The activity of the female-specific enzymes were not significantly altered (Table 29) but the male-specific enzymes had their activity significantly increased at concentrations as low as 10^{-9} M. The maximum response was seen at 10^{-8} M(Figure 33) after which further increase in glucagon concentration resulted in a reduction in their activity despite the fact that all were still significantly greater than control at 10^{-6} M.

Glucagon concentration	Enzyme activities (pmoles / min / million cells)				
	7 α - OHase	6 β - OHase	16 α -OHase	17 - OHSD	5 α - reductase
Control	76 ± 4	273 ± 5	440 ± 15	149 ± 4	105 ± 3
10^{-10} M	65 ± 4 *	244 ± 6 *	367 ± 41 *	157 ± 23	103 ± 15
10^{-9} M	57 ± 8 *	179 ± 6 *	280 ± 16 *	131 ± 6 *	77 ± 3 *
10^{-8} M	51 ± 1 *	179 ± 8 *	259 ± 13 *	133 ± 2 *	91 ± 7 *
10^{-7} M	57 ± 2 *	193 ± 7 *	293 ± 19 *	148 ± 8	95 ± 11
10^{-6} M	61 ± 3 *	198 ± 17 *	328 ± 25 *	150 ± 6	103 ± 5

Table 27 . Dose-response effects of 7 α -, 6 β -, 16 α -hydroxylases (OHases), 17-oxosteroid oxidoreductase (OHSD) and 5 α -reductase activities to glucagon after 1/2 hour preincubation in hepatocytes obtained from normal male rat. Results expressed as mean ± S.D (N = 3); * $P < 0.05$ as compared to respective controls.

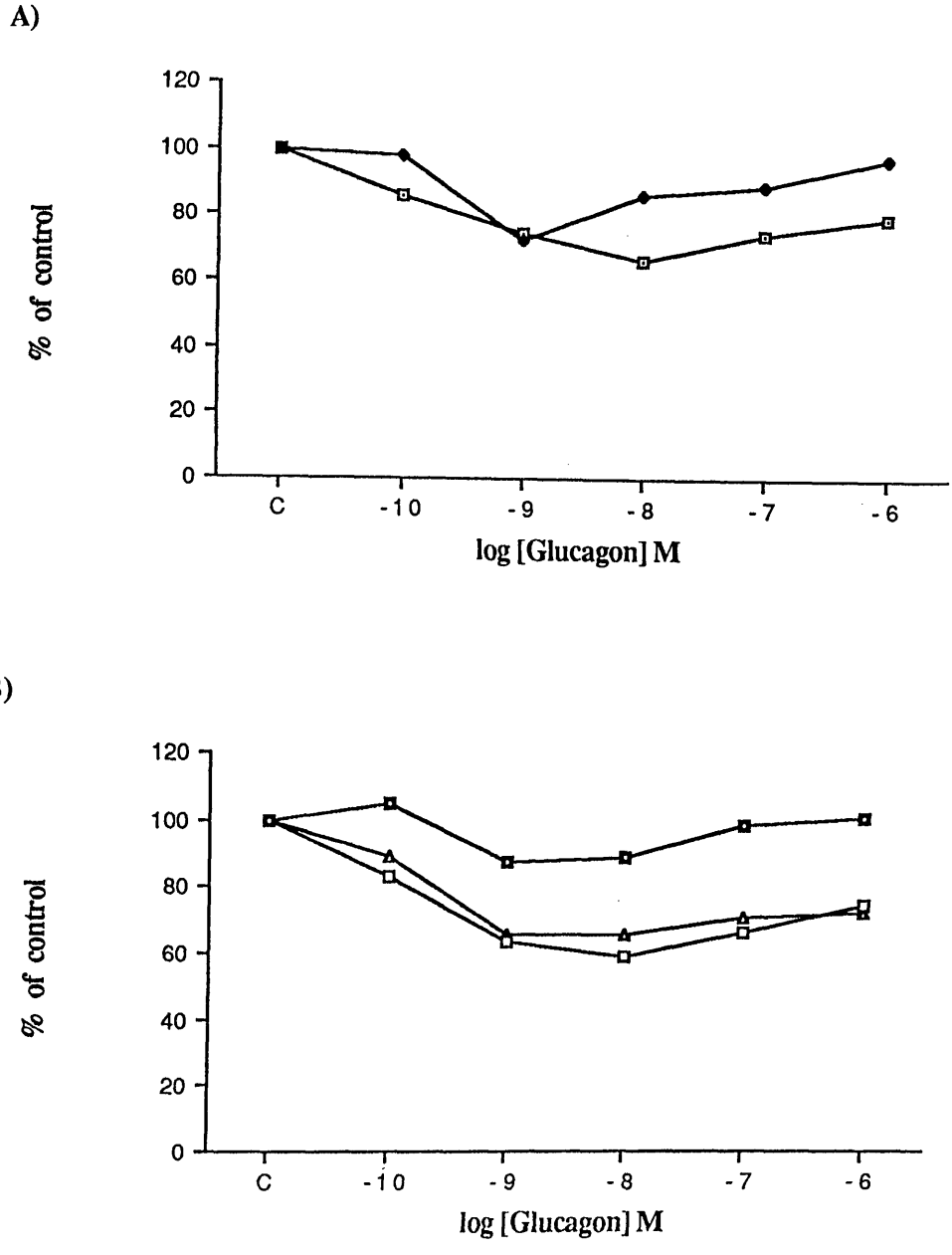


Figure 31 . Dose-response curves of (A) 7 ∝ -hydroxylase [▫] and 5 ∝ -reductase [♦] and (B) 17-OHSD [▫], 6 β - [▲] and 16 ∝ -hydroxylases [▫] to glucagon after 1/2 hour preincubation in hepatocytes obtained from normal male rat.
Results are expressed as percentage of the relevant control and mean ± S.D (N = 3). Absolute data is given in Table 27 .
C = control

Glucagon concentration	Enzyme activities (pmoles / min / million cells)				
	7 α - OHase	6 β - OHase	16 α -OHase	17 - OHSD	5 α - reductase
Control	43 ± 2	127 ± 6	188 ± 5	123 ± 5	69 ± 5
10^{-10} M	45 ± 4	165 ± 4 *	249 ± 6 *	121 ± 4	65 ± 14
10^{-9} M	40 ± 3	179 ± 2 *	285 ± 3 *	122 ± 4	71 ± 2
10^{-8} M	62 ± 4 *	218 ± 3 *	342 ± 5 *	146 ± 7 *	95 ± 16 *
10^{-7} M	54 ± 4 *	165 ± 16 *	268 ± 17 *	137 ± 2 *	81 ± 15
10^{-6} M	40 ± 9	102 ± 10 *	171 ± 13 *	86 ± 4 *	53 ± 4 *

Table 28 . Dose-response effects of 7 α -, 6 β -, 16 α -hydroxylases (OHases), 17-oxosteroid oxidoreductase (OHSD) and 5 α -reductase activities to glucagon after 1 hour preincubation in hepatocytes obtained from normal male rat. Results expressed as mean ± S.D (N = 3); * $P < 0.05$ as compared to respective controls.

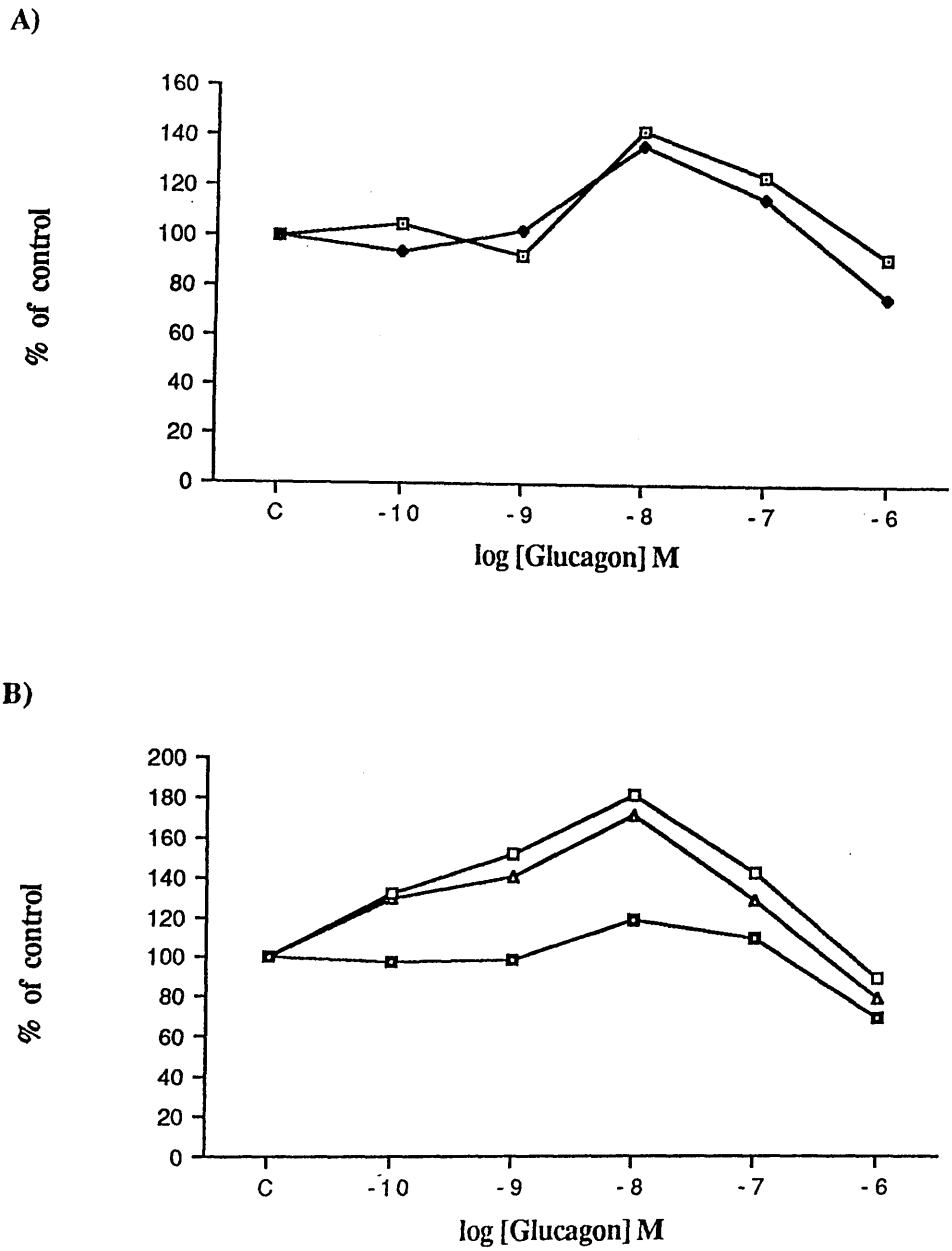


Figure 32 . Dose-response curves of (A) 7 α -hydroxylase [▫] and 5 α -reductase [◆] and (B) 17-OHSD [▫], 6 β - [▲] and 16 α -hydroxylases [▫] to glucagon after 1 hour preincubation in hepatocytes obtained from normal male rat.
Results are expressed as percentage of the relevant control and mean ± S.D (N = 3). Absolute data is given in Table 28 .
C = control

Glucagon concentration	Enzyme activities (pmoles / min / million cells)				
	7 α - OHase	6 β - OHase	16 α -OHase	17 - OHSD	5 α - reductase
Control	54 ± 5	162 ± 13	228 ± 6	139 ± 5	102 ± 21
10^{-10} M	58 ± 6	163 ± 7	261 ± 13 *	141 ± 4	90 ± 14
10^{-9} M	58 ± 2	185 ± 4 *	282 ± 10 *	161 ± 4 *	92 ± 4
10^{-8} M	79 ± 10 *	231 ± 5 *	389 ± 10 *	175 ± 11 *	111 ± 16
10^{-7} M	61 ± 10	250 ± 16 *	387 ± 28 *	169 ± 7 *	116 ± 9
10^{-6} M	52 ± 1	183 ± 4 *	276 ± 17 *	152 ± 4 *	87 ± 6

Table 29 . Dose-response effects of 7 α -, 6 β -, 16 α -hydroxylases (OHases), 17-oxosteroid oxidoreductase (OHSD) and 5 α -reductase activities to glucagon after 2 hour preincubation in hepatocytes obtained from normal male rat. Results expressed as mean ± S.D (N = 3); * $P < 0.05$ as compared to respective controls.

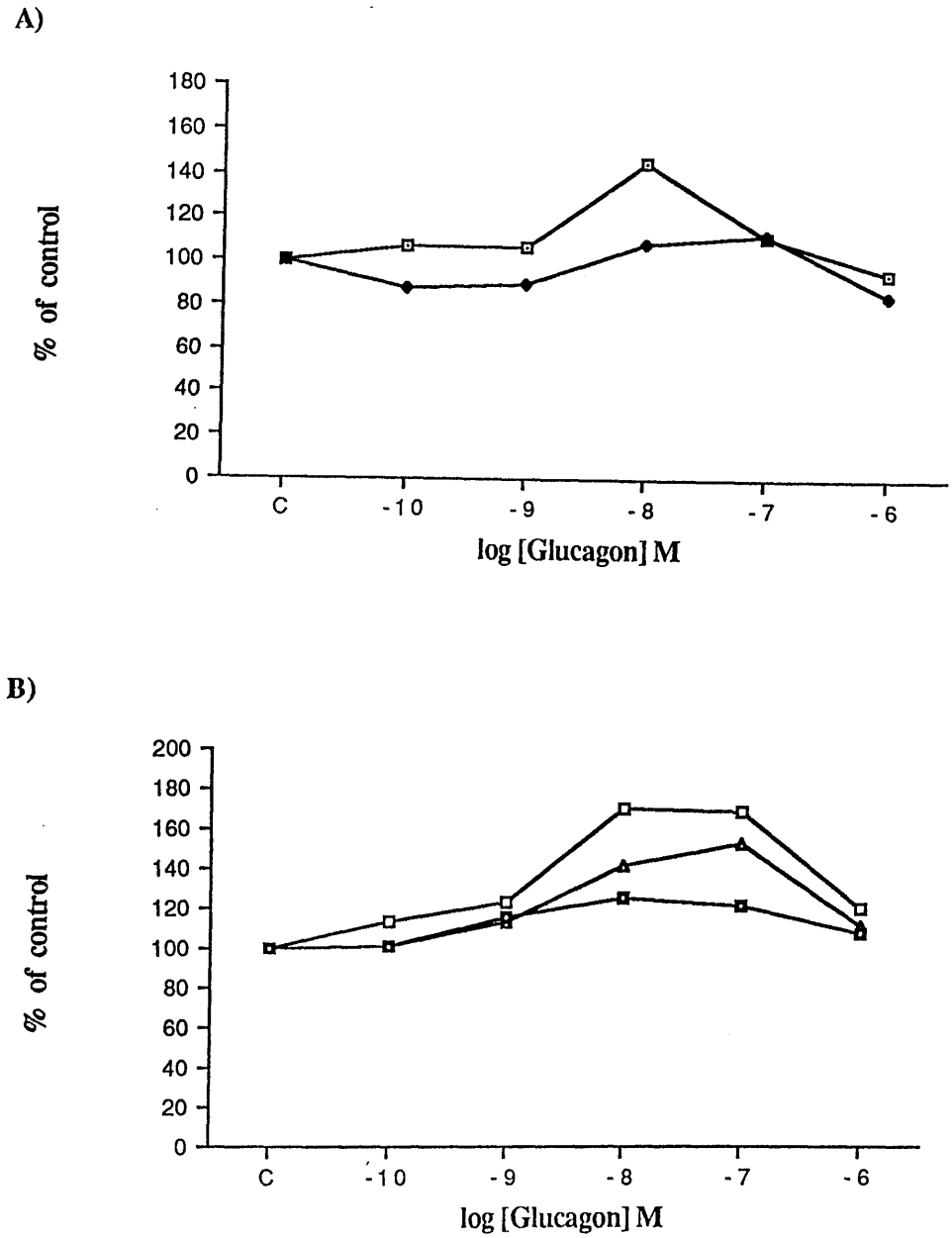


Figure 33 . Dose-response curves of (A) 7 α -hydroxylase [▫] and 5 α -reductase [•] and (B) 17-OHSD [▫], 6 β - [▲] and 16 α -hydroxylases [▫] to glucagon after 2 hour preincubation in hepatocytes obtained from normal male rat.
Results are expressed as percentage of the relevant control and mean ± S.D (N = 3). Absolute data is given in Table 29 .
C = control

5.1.1.3 Preincubation with glucagon for 24, 48 and 72 hours

A similar dose-response effect for glucagon to that observed at 1/2 hour could be seen after **24 hour** glucagon preincubation (Table 30). At lower concentrations (10^{-10} to 10^{-8} M) glucagon elicited a dose-dependent decrease in activity for all the enzymes measured reaching to about 60 to 70 % below control. The maximum effect was again seen at 10^{-8} M concentration. At higher concentrations the effect of glucagon was markedly reduced with most activities returning to control at 10^{-6} M (Figure 34).

After **48 hour** preincubation, the enzymes exhibited a triphasic response towards glucagon (Figure 35). Significant decreases in enzyme activity seen at 10^{-10} M was followed by restoration of activity to control level at 10^{-9} M (Table 31). Maximum increase of enzyme activities occurred at 10^{-8} M with a subsequent fall in all enzymes activities at higher concentrations.

At **72 hour** of glucagon preincubation, the alteration in enzyme activities was observed to be sex-dependent. No significant changes in activity could be seen with the female-specific enzymes i.e. 7α-hydroxylase and 5α-reductase (Table 32). Increases in activity seen with 6β-hydroxylase and 17-OHSD were found to be dose-dependent with maximum response not reached even at 10^{-6} M (Figure 36).

5.1.2 HEPATOCYTES FROM 3-DAYS STZ-TREATED DIABETIC RAT

5.1.2.1 Preincubation with glucagon for 1/2 hour

In contrast to hepatocytes from normal rats which exhibited U-shaped dose-response curves, preincubation with glucagon for **1/2 hour** resulted in a dose-dependent decrease in all the enzyme activities except the 17-OHSD (Table 33) with the

Glucagon concentration	Enzyme activities (pmoles / min / million cells)				
	7α - OHase	6β - OHase	16 α -OHase	17 - OHSD	5 α - reductase
Control	40 ± 5	64 ± 7	84 ± 6	91 ± 4	109 ± 5
10^{-10} M	35 ± 9	49 ± 8	75 ± 5	70 ± 2 *	77 ± 7 *
10^{-9} M	27 ± 4 *	46 ± 8 *	68 ± 7 *	56 ± 3 *	66 ± 5 *
10^{-8} M	22 ± 5 *	38 ± 9 *	59 ± 7 *	54 ± 6 *	58 ± 5 *
10^{-7} M	33 ± 7	51 ± 6	70 ± 9	70 ± 2 *	87 ± 8 *
10^{-6} M	40 ± 6	64 ± 7	84 ± 6	77 ± 8	94 ± 9

Table 30 . Dose-response effects of 7 α -, 6 β -, 16 α -hydroxylases (OHases), 17-oxosteroid oxidoreductase (OHSD) and 5 α -reductase activities to glucagon after 24 hour preincubation in hepatocytes obtained from normal male rat. Results expressed as mean ± S.D (N = 3); * $P < 0.05$ as compared to respective controls.

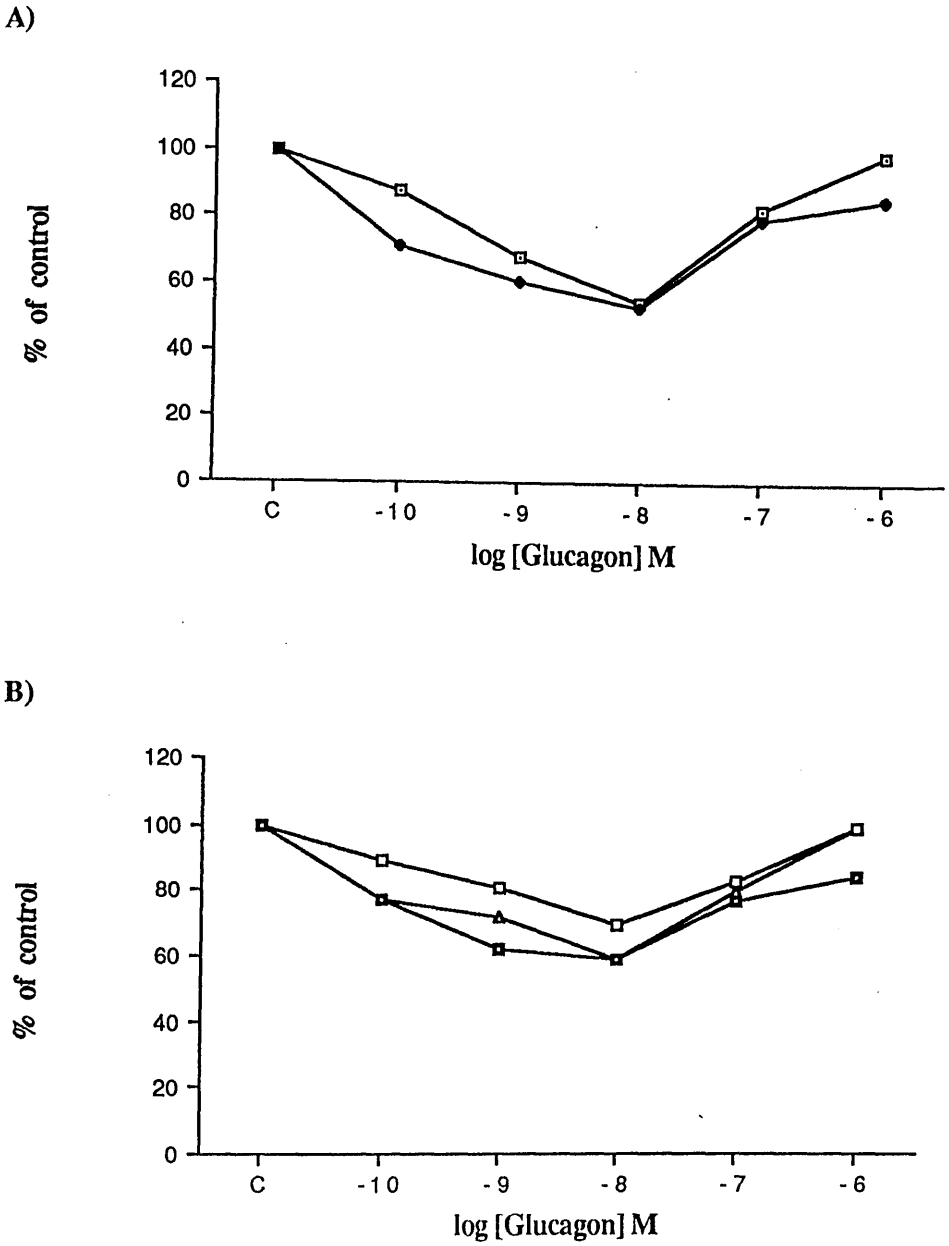


Figure 34 . Dose-response curves of (A) 7 α -hydroxylase [▪] and 5 α -reductase [•] and (B) 17-OHSD [▪], 6 β - [▲] and 16 α -hydroxylases [▫] to glucagon after 24 hour preincubation in hepatocytes obtained from normal male rat.
Results are expressed as percentage of the relevant control and mean ± S.D (N = 3). Absolute data is given in Table 30 .
C = control

Glucagon concentration	Enzyme activities (pmoles / min / million cells)				
	7 α - OHase	6 β - OHase	16 α -OHase	17 - OHSD	5 α - reductase
Control	49 ± 4	54 ± 4	91 ± 4	92 ± 6	94 ± 3
10^{-10} M	42 ± 2	44 ± 4 *	72 ± 4 *	73 ± 5 *	83 ± 3 *
10^{-9} M	47 ± 3	59 ± 2	87 ± 4	79 ± 8	96 ± 10
10^{-8} M	67 ± 5 *	90 ± 4 *	140 ± 3 *	127 ± 4 *	165 ± 10 *
10^{-7} M	54 ± 3	69 ± 4 *	111 ± 4 *	99 ± 7	103 ± 10
10^{-6} M	47 ± 4	61 ± 3	90 ± 2	78 ± 5 *	104 ± 9

Table 31 . Dose-response effects of 7 α -, 6 β -, 16 α -hydroxylases (OHases), 17-oxosteroid oxidoreductase (OHSD) and 5 α -reductase activities to glucagon after 48 hour preincubation in hepatocytes obtained from normal male rat. Results expressed as mean ± S.D (N = 3); * $P < 0.05$ as compared to respective controls.

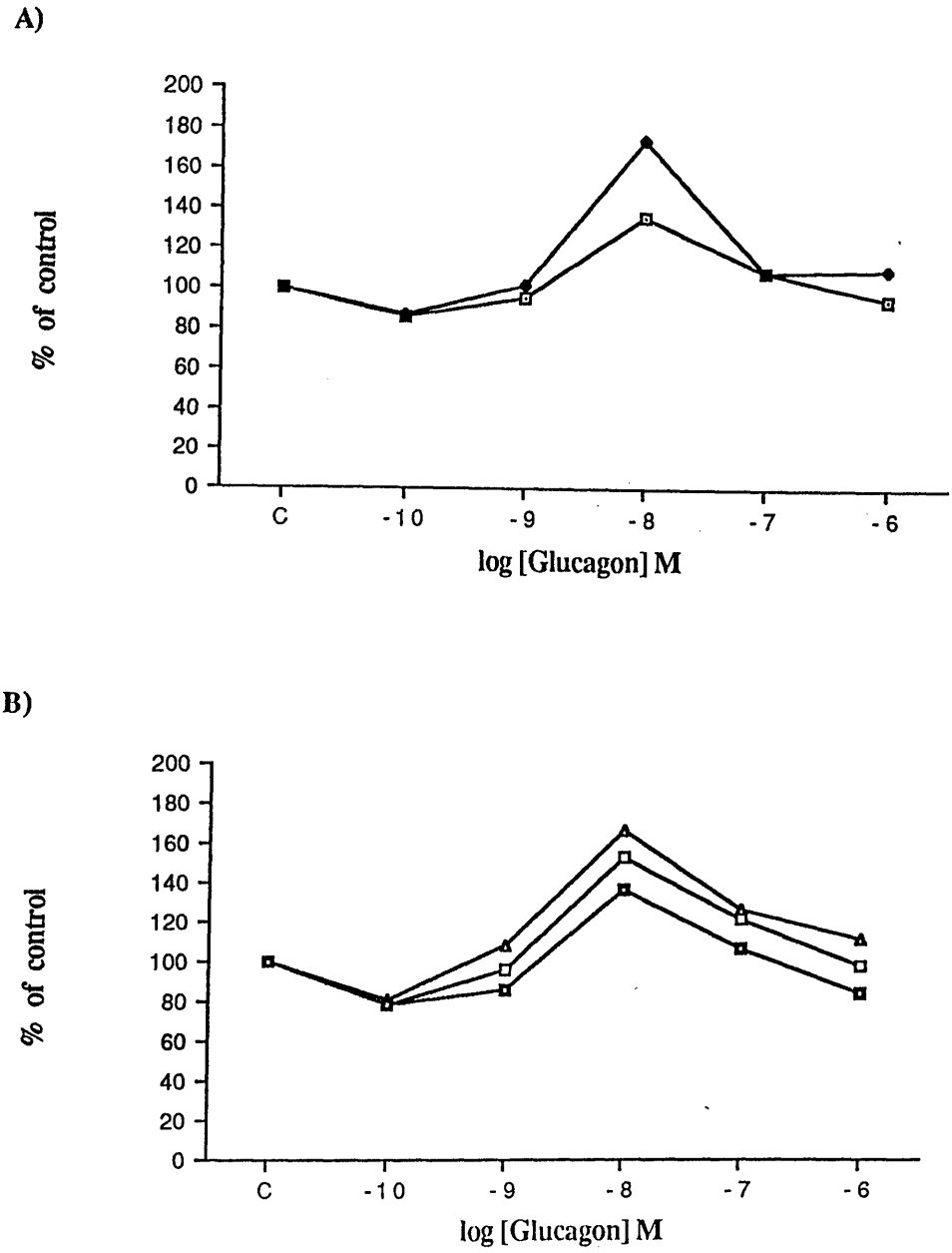


Figure 35 . Dose-response curves of (A) 7 α -hydroxylase [▫] and 5 α -reductase [•] and (B) 17-OHSD [▫], 6 β - [▲] and 16 α -hydroxylases [▫] to glucagon after 48 hour preincubation in hepatocytes obtained from normal male rat.
Results are expressed as percentage of the relevant control and mean ± S.D (N = 3). Absolute data is given in Table 31 .
C = control

Glucagon concentration	Enzyme activities (pmoles / min / million cells)				
	7α · OHase	6β · OHase	16 α ·OHase	17 - OHSD	5 α · reductase
Control	44 ± 5	53 ± 6	96 ± 5	68 ± 8	110 ± 8
10^{-10} M	45 ± 3	71 ± 5 *	94 ± 4	82 ± 7	97 ± 10
10^{-9} M	40 ± 2	52 ± 4	72 ± 1 *	62 ± 10	76 ± 6 *
10^{-8} M	52 ± 3	74 ± 9 *	97 ± 2	89 ± 7 *	104 ± 5
10^{-7} M	61 ± 8 *	75 ± 10 *	96 ± 8	91 ± 3 *	103 ± 7
10^{-6} M	59 ± 9	87 ± 9 *	109 ± 2 *	105 ± 10 *	132 ± 12

Table 32 . Dose-response effects of 7 α -, 6 β -, 16 α -hydroxylases (OHases), 17-oxosteroid oxidoreductase (OHSD) and 5 α -reductase activities to glucagon after 72 hour preincubation in hepatocytes obtained from normal male rat. Results expressed as mean ± S.D (N = 3); * $P < 0.05$ as compared to respective controls.

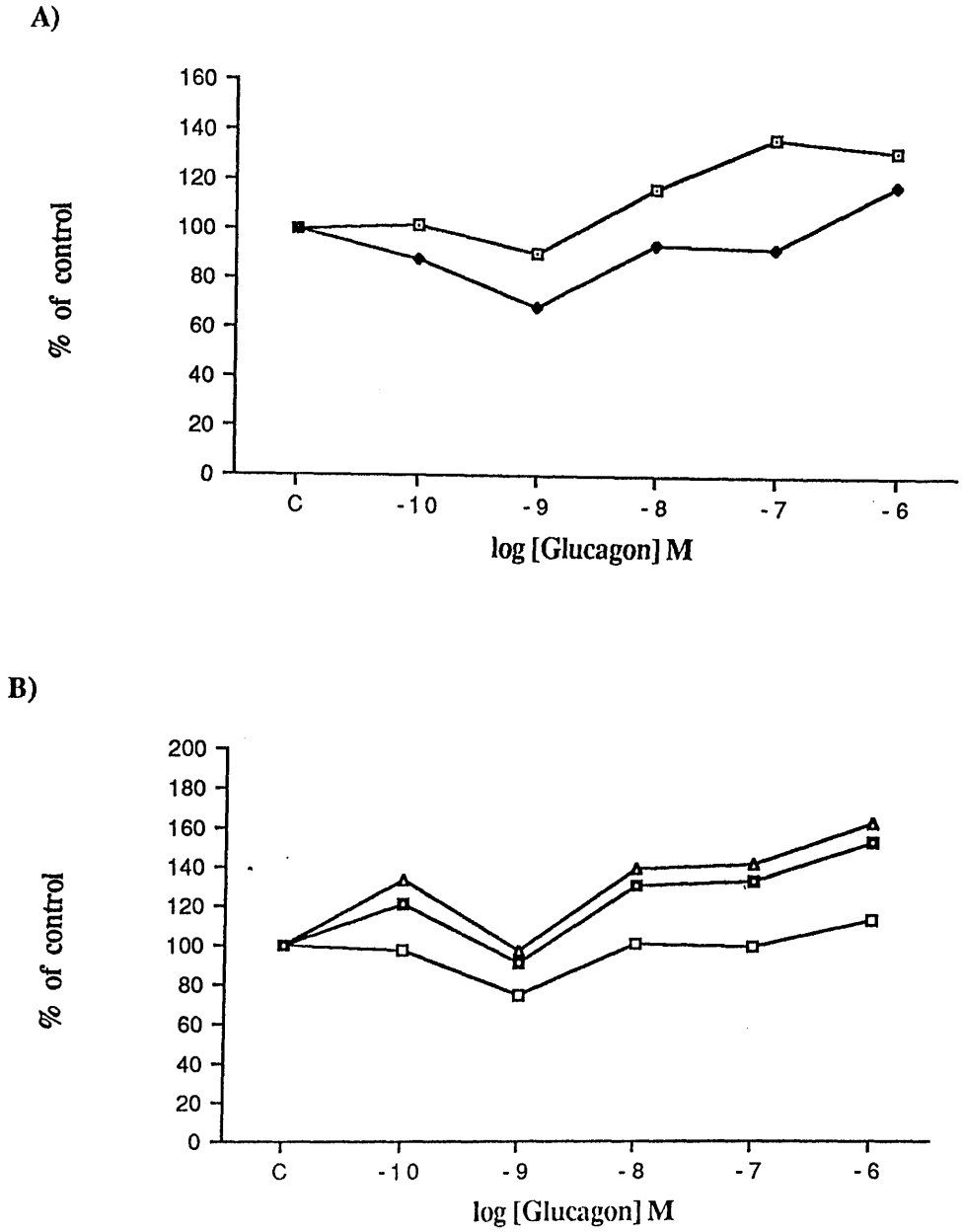


Figure 36 . Dose-response curves of (A) 7 α -hydroxylase [▫] and 5 α -reductase [•] and (B) 17-OHSD [▫], 6 β - [▲] and 16 α -hydroxylases [▫] to glucagon after 72 hour preincubation in hepatocytes obtained from normal male rat.
Results are expressed as percentage of the relevant control and mean ± S.D (N = 3). Absolute data is given in Table 32 .
C = control

Glucagon concentration	Enzyme activities (pmoles / min / million cells)				
	7 α - OHase	6 β - OHase	16 α -OHase	17 - OHSD	5 α - reductase
Control	27 ± 2	71 ± 7	87 ± 6	56 ± 4	200 ± 11
10^{-10} M	21 ± 1 *	64 ± 5	71 ± 2 *	56 ± 2	164 ± 7 *
10^{-9} M	19 ± 1 *	56 ± 6 *	65 ± 6 *	62 ± 5	184 ± 7 *
10^{-8} M	23 ± 2 *	59 ± 4 *	69 ± 4 *	57 ± 2	179 ± 8 *
10^{-7} M	21 ± 3 *	55 ± 1 *	57 ± 3 *	52 ± 3	123 ± 7 *
10^{-6} M	20 ± 1 *	55 ± 5 *	63 ± 2 *	48 ± 5 *	111 ± 3 *

Table 33 . Dose-response effects of 7 α -, 6 β -, 16 α -hydroxylases (OHases), 17-oxosteroid oxidoreductase (OHSD) and 5 α -reductase activities to glucagon after 1/2 hour preincubation in hepatocytes obtained from 3 days STZ-treated diabetic male rat. Results expressed as mean ± S.D (N = 3); * $P < 0.05$ as compared to respective controls.

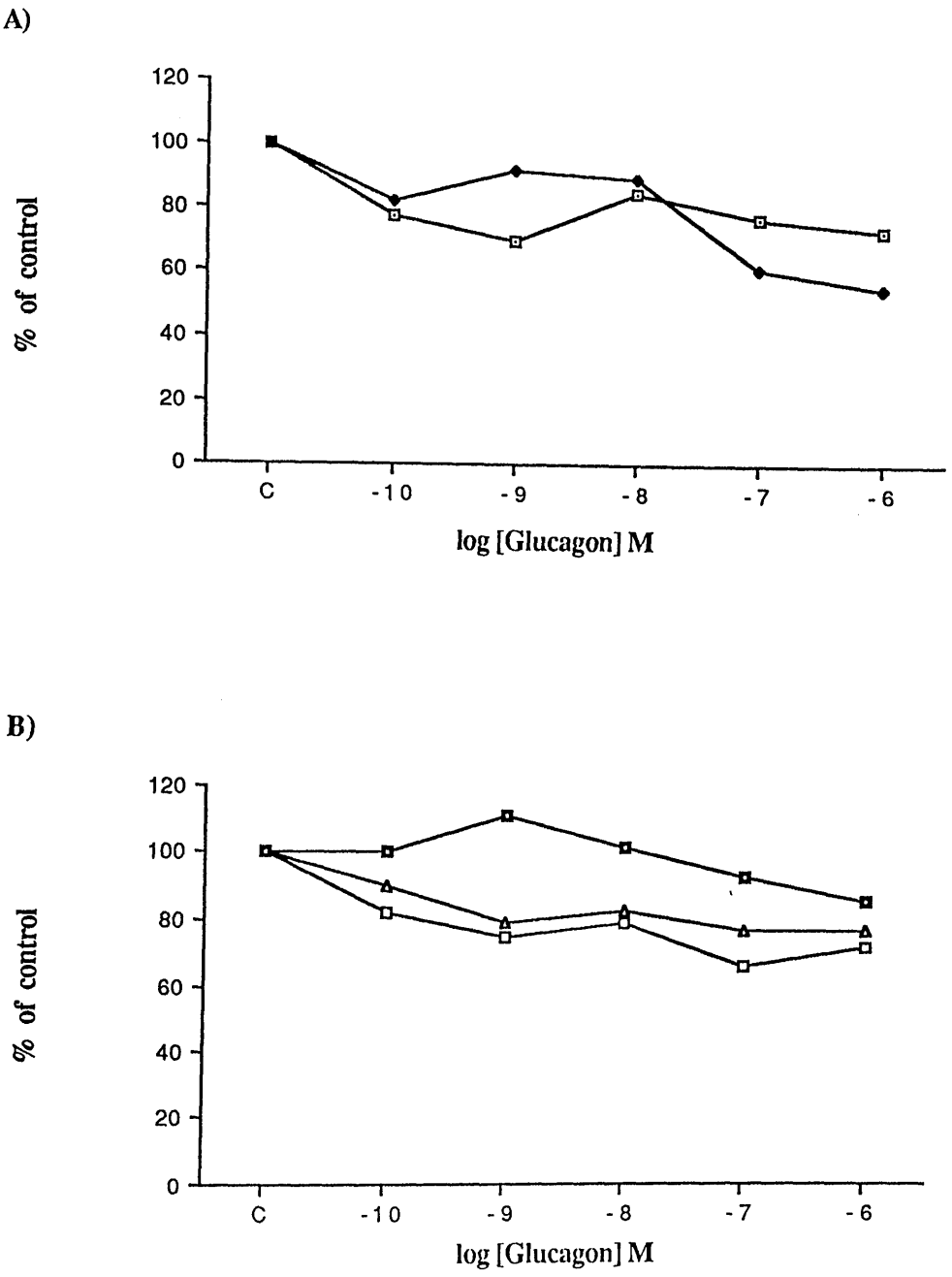


Figure 37 . Dose-response curves of (A) 7 α -hydroxylase [▫] and 5 α -reductase [•] and (B) 17-OHSD [▫], 6 β - [▲] and 16 α -hydroxylases [▫] activities to glucagon after 1/2 hour preincubation in hepatocytes obtained from 3 days STZ-treated diabetic male rat.
Results are expressed as percentage of the relevant control and mean ± S.D (N = 3). Absolute data is given in Table 33 .
C = control

maximum effect being seen at 10^{-9} M (about 70 to 80 % of control) (Figure 37). Glucagon had no effect on 17-OHSD activity. Female-specific 5α-reductase basal level was higher than the male-specific enzymes (6β- and 16α-hydroxylases) and the response to glucagon of the 5α-reductase activity had not reach its maximum even at 10^{-6} M.

Experiments involving preincubation with glucagon for more than 1/2 hour were not conducted.

5.1.3 PREINCUBATION OF MALE RAT HEPATOCYTES WITH GLUCAGON IN THE PRESENCE OF THE PROTEIN KINASE INHIBITOR K-252a

5.1.3.1 Preincubation with glucagon for 1/2 hour in the presence of K-252a (20 nM)

No significant changes in enzyme activities were observed after hepatocytes were preincubated for **1/2 hour** with glucagon in the presence of the non-selective protein kinase inhibitor, K-252a (Table 34). The U-shaped dose-response curves induced by glucagon (refer to Section **5.1.1.2**) were abolished in the presence of 20 nM K-252a (Figure 38). As shown in Table 34, K-252a alone produced no significant changes in androst-4-ene-3,17-dione metabolism.

5.1.3.2 Preincubation with glucagon for 24 hours in the presence of K-252a

In the presence of K-252a (20 nM), glucagon, at lower concentrations (10^{-10} to 10^{-8} M) elicited a dose-dependent increase in all the enzyme activities with

Drug concentration	Enzyme activities (pmoles / min / million cells)				
	7α - OHase	6β - OHase	16 α -OHase	17 - OHSD	5 α - reductase
Control (DMSO)	14 ± 4	27 ± 4	21 ± 2	28 ± 5	24 ± 2
K (20 nM) [a] alone	13 ± 2	30 ± 3	20 ± 3	27 ± 6	25 ± 1
K + G (0.1 nM)	13 ± 1	27 ± 3	19 ± 1	27 ± 6	23 ± 1
K + G (1 nM)	15 ± 2	30 ± 2	23 ± 1	29 ± 4	23 ± 3
K + G (10 nM)	16 ± 2	30 ± 2	23 ± 2	26 ± 5	26 ± 3
K + G (100 nM)	12 ± 2	30 ± 4	24 ± 4	33 ± 2	25 ± 2
K + G (1μ M)	17 ± 3	29 ± 6	24 ± 3	29 ± 2	24 ± 1

Table 34. Dose-response effects of 7 α -, 6 β -, 16 α -hydroxylases (OHases), 17-oxosteroid dehydrogenase (OHSD) and 5 α -reductase activities to glucagon (G) after 1/2 hour preincubation in the presence of 20 nM K-252a (K) in hepatocytes obtained from normal male rat. K-252a was dissolved in dimethyl-sulphoxide (DMSO). Results expressed as mean ± S.D (N = 3); * P < 0.05 with respect to [a] .

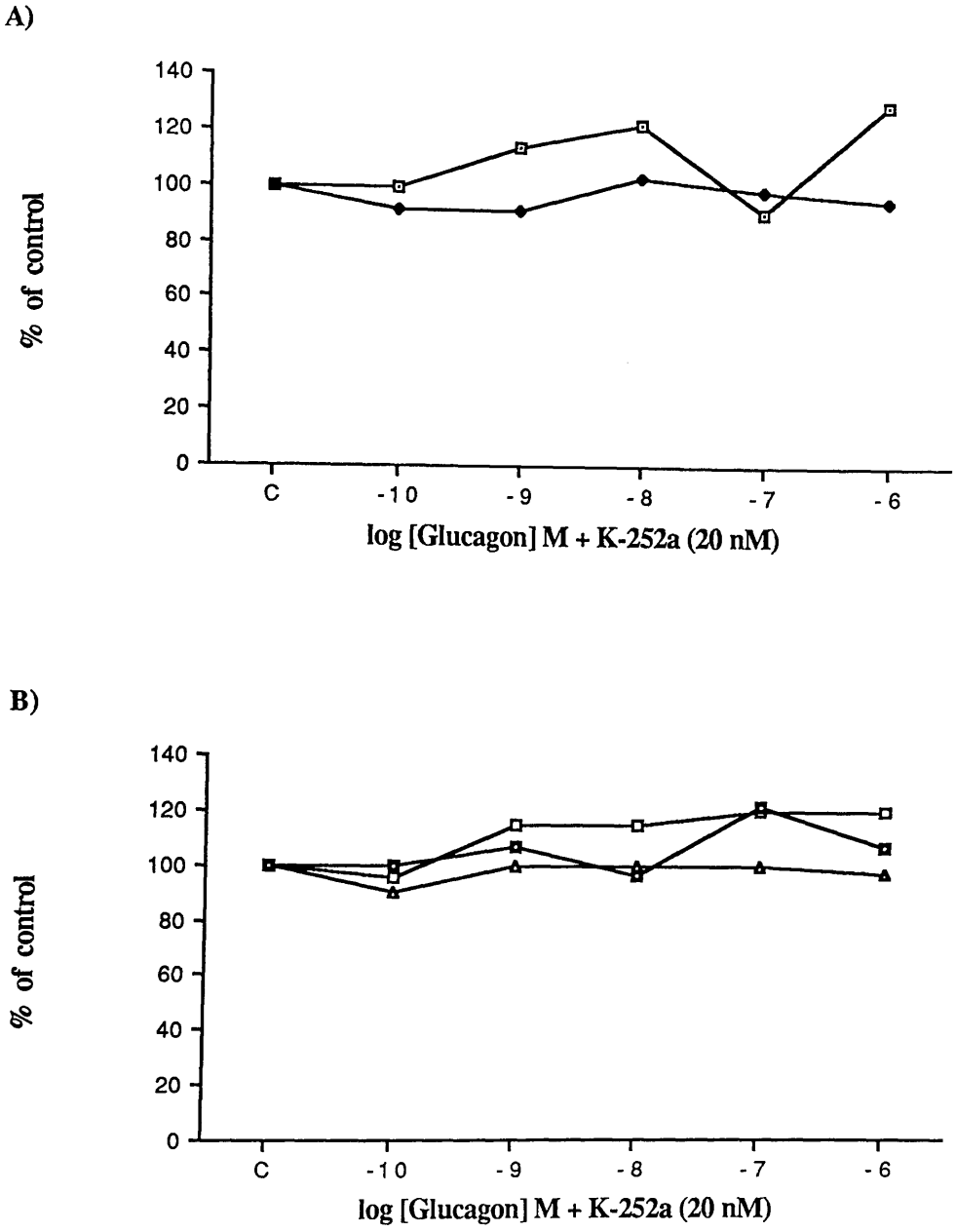
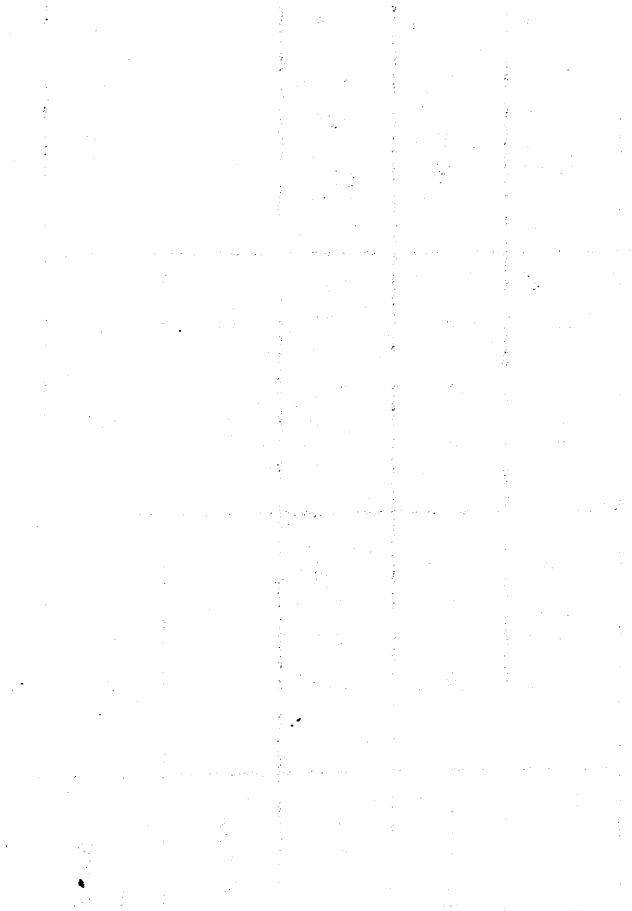


Figure 38 . Dose-response curves of (A) 7 α -hydroxylase [▪] and 5 α -reductase [•] and (B) 17-OHSD [▪], 6 β - [▲] and 16 α -hydroxylases [□] activities to glucagon after 1/2 hour preincubation in the presence of 20 nM of K-252a in hepatocytes obtained from normal male rat as described in the Methods section. Results are expressed as percentage of the relevant control and mean ± S.D (N = 3). Absolute data is given in Table 34 .
C = control (20 nM K-252a dissolved in the vehicle).

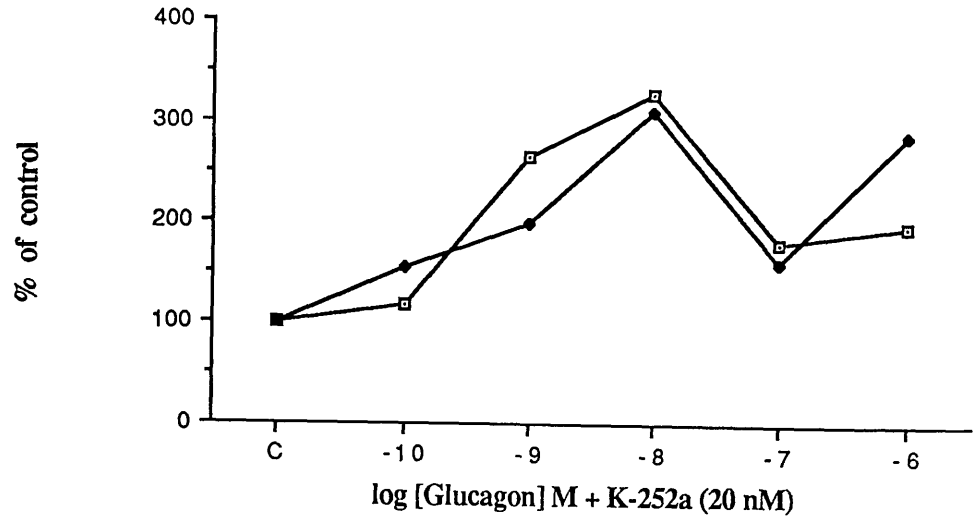
maximum effect of glucagon seen at 10^{-8} M (Table 35); at higher concentrations the effect of glucagon was markedly reduced despite still being significantly greater than their respective controls (Figure 39). At 10^{-8} M, glucagon increased 7α-hydroxylase and 5α-reductase activity to 333 % and 315 % of control respectively (Figure 39A), while 6β- and 16α-hydroxylases and 17-OHSD activities rose to 410 %, 500 % and 231 % of control respectively (Figure 39B).



Drug concentration	Enzyme activities (pmoles / min / million cells)				
	7α - OHase	6β - OHase	16 α-OHase	17 - OHSD	5 α-reductase
Control (DMSO)	5 ± 1	9 ± 1	6 ± 1	15 ± 1	14 ± 1
K (20 nM) [a] alone	6 ± 1	10 ± 1	6 ± 1	16 ± 6	13 ± 3
K + G (0.1 nM)	7 ± 2	13 ± 2	9 ± 1 *	21 ± 6	20 ± 1 *
K + G (1 nM)	16 ± 4 *	24 ± 8 *	25 ± 4 *	24 ± 5	26 ± 1 *
K + G (10 nM)	20 ± 2 *	41 ± 9 *	30 ± 8 *	37 ± 1 *	41 ± 9 *
K + G (100 nM)	11 ± 2 *	19 ± 2 *	16 ± 3 *	37 ± 8 *	21 ± 3 *
K + G (1μM)	12 ± 2 *	21 ± 1 *	22 ± 0 *	39 ± 1 *	38 ± 1 *

Table 35. Dose-response effects of 7 α -, 6 β -, 16 α -hydroxylases (OHases), 17-oxosteroid dehydrogenase (OHSD) and 5 α -reductase activities to glucagon (G) after 24 hour preincubation in the presence of 20 nM K-252a (K) in hepatocytes obtained from normal male rat. K-252a was dissolved in dimethyl-sulphoxide (DMSO). Results expressed as mean ± S.D (N = 3): * $P < 0.05$ with respect to [a] .

A)



B)

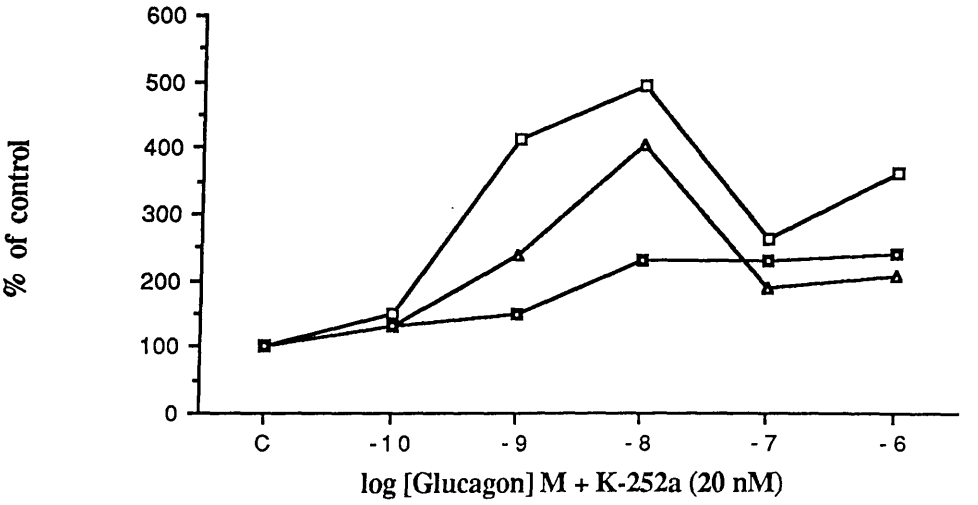


Figure 39 . Dose-response curves of (A) 7 α -hydroxylase [▪] and 5 α -reductase [•] and (B) 17-OHSD [▫], 6 β - [▲] and 16 α -hydroxylases [▫] activities to glucagon after 24 hour preincubation in the presence of 20 nM of K-252a in hepatocytes obtained from normal male rat as described in the Methods section. Results are expressed as percentage of the relevant control and mean ± S.D (N = 3). Absolute data is given in Table 35 .
C = control (20 nM K-252a dissolved in the vehicle).

6.0 INSULIN AND GLUCAGON

6.1 EFFECT OF COMBINATIONS OF INSULIN AND GLUCAGON ON ANDROST-4-ENE-3,17-DIONE METABOLISM

6.1.1 HEPATOCYTES FROM NORMAL RAT

6.1.1.1 Preincubation with insulin *and* glucagon for 1/2 hour

In this experiment only two concentrations (10^{-9} and 10^{-6} M) for insulin and glucagon were chosen and dose-response experiments were conducted based on these two concentrations. Various combinations of insulin and glucagon were added directly to the cultured hepatocytes and the cells assayed as described in Methods Section **2.6.**

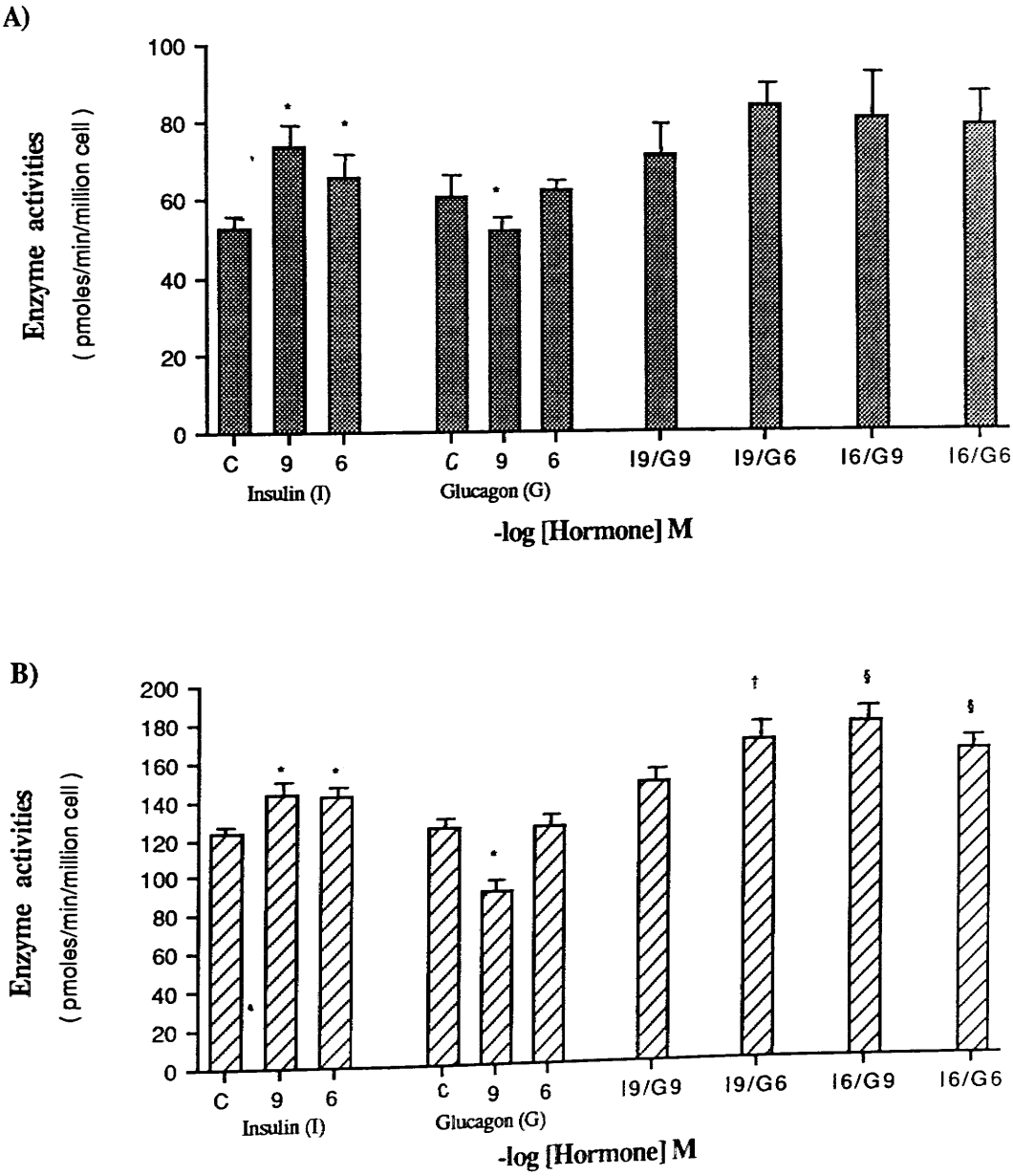
As shown in Table 36 and Figure 40, insulin caused a significant dose-dependent increase in all of the enzyme activities. Glucagon significantly decreased all of the enzyme activities at 10^{-9} M. At 10^{-6} M glucagon, however, all enzyme activities were at the control level. 10^{-9} M glucagon significantly decreased the effect of 10^{-9} M insulin on 16α-hydroxylase and 17-OHSD whereas the 7α- and 6β-hydroxylases and 5α-reductase activities were not affected. At supraphysiological concentration (10^{-6} M), glucagon's effect on 10^{-9} M insulin resulted in selective changes in enzyme activities, only seen with cytochrome P-450-independent enzymes. The effect of 10^{-9} M insulin on 17-OHSD activity was significantly blocked while the potentiation of insulin effect on 5α-reductase activity could be seen in the presence of 10^{-6} M glucagon.

It is evident that 10^{-9} M glucagon potentiated the effect of 10^{-6} M insulin on 6β-

Hormone concentration	Enzyme activities (pmoles / min / million cells)				
	7α - OHase	6β - OHase	16 α -OHase	17 - OHSD	5α - reductase
Control Insulin (I)	53 ± 2	120 ± 4	93 ± 5	.121 ± 10	123 ± 1
10^{-9} M (I) [a]	74 ± 5 *	154 ± 8 *	120 ± 4 *	143 ± 4 *	143 ± 6 *
10^{-6} M (I) [b]	66 ± 5 *	148 ± 3 *	144 ± 2 *	151 ± 2 *	142 ± 5 *
Control Glucagon (G)	.61 ± 4	109 ± 1	103 ± 8	116 ± 2	124 ± 3
10^{-9} M (G)	52 ± 2 *	89 ± 2 *	88 ± 5 *	84 ± 7 *	91 ± 2 *
10^{-6} M (G)	63 ± 2	109 ± 6	101 ± 3	119 ± 2	125 ± 5
10^{-9} (I) + 10^{-9} (G)	72 ± 6	167 ± 7	95 ± 4 †	120 ± 3 †	147 ± 4
10^{-9} (I) + 10^{-6} (G)	85 ± 5	168 ± 8	120 ± 4	132 ± 4 †	168 ± 9 †
10^{-6} (I) + 10^{-9} (G)	82 ± 12	192 ± 6 §	116 ± 6 §	155 ± 6	177 ± 8 §
10^{-6} (I) + 10^{-6} (G)	80 ± 6	177 ± 6 §	126 ± 2 §	133 ± 7 §	162 ± 2 §

Table 36 . Dose-response effects of 7 α -, 6 β -, 16 α -hydroxylases (OHases), 17-oxosteroid oxidoreductase (OHSD) and 5 α -reductase activities to insulin (I) or glucagon (G) or to various combinations of both hormones after 1/2 hour of preincubation in hepatocytes of normal male rat. Results expressed as mean ± S.D (N = 3). * $P < 0.05$ as compared to the respective controls; † $P < 0.05$ with respect to [a] ; § $P < 0.05$ with respect to [b] .

Figure 40. Dose-response effects of (A) 7 α -hydroxylase [▨], (B) 5 α -reductase [▨], (C) 6 β - [▤] and (D)16 α -hydroxylases [■] and (E) 17-OHSD [▨] activities to insulin (I) or glucagon (G) or to various combinations of both hormones after 1/2 hour of preincubation in hepatocytes obtained from normal male rat. Results are expressed as percentage of the relevant control and mean ± S.D (N = 3). Analysis of data : * $P < 0.05$ when compared to the respective hormone control; † $P < 0.05$ with respect to 10^{-9} M insulin; § $P < 0.05$ with respect to 10^{-6} M insulin.



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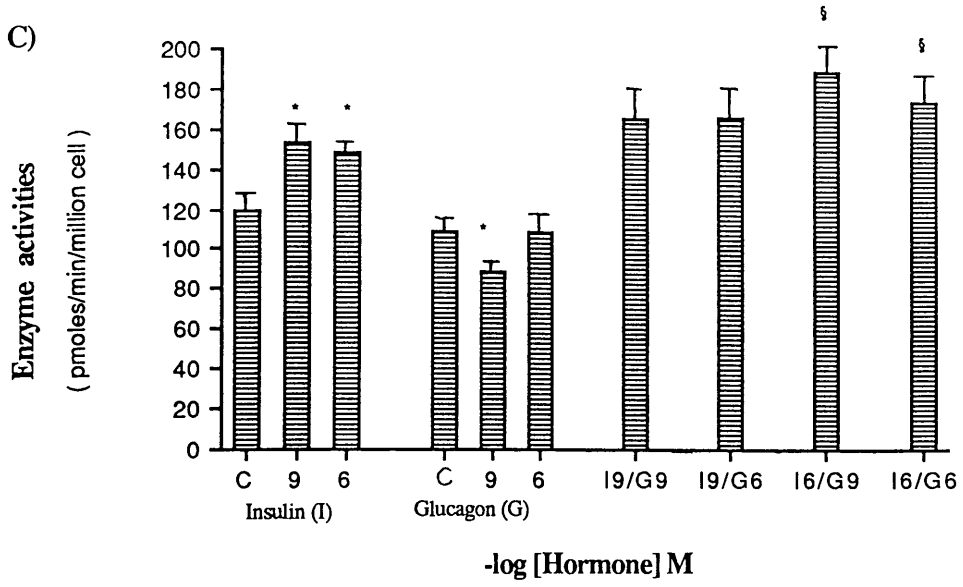
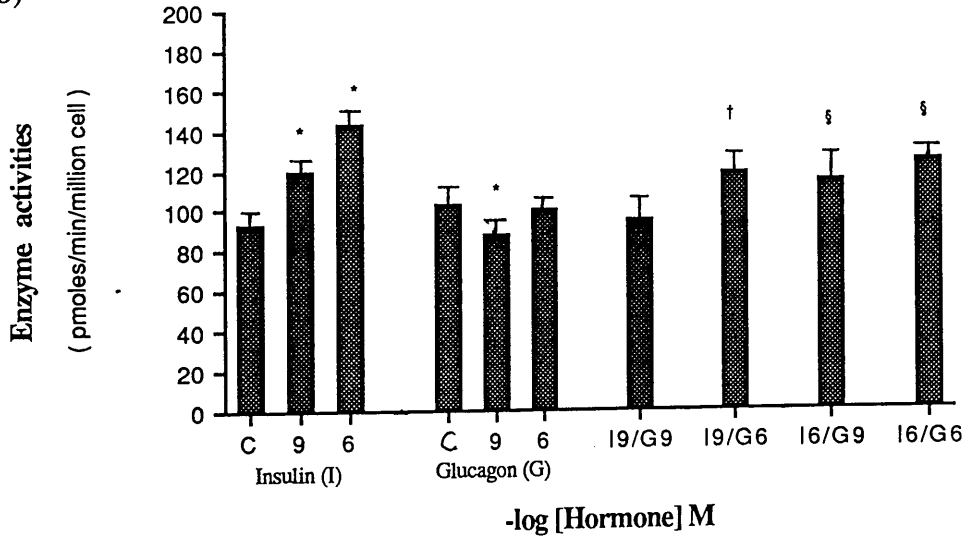
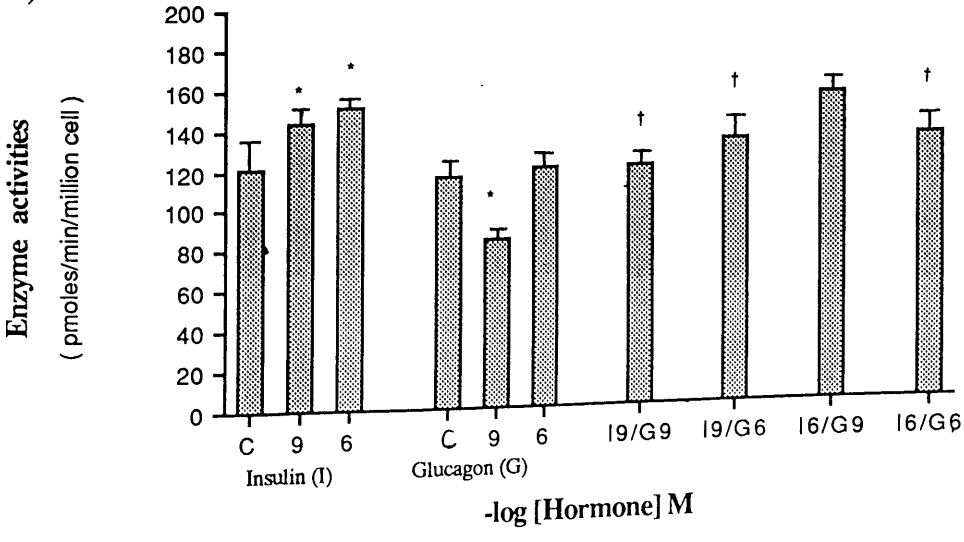

Enzyme activities
(pmoles/min/million cell)
200
180
160
140
120
100
80
60
40
20
0
C 9 6 C 9 6 I9/G9 I9/G6 I6/G9 I6/G6
Insulin (I)
Glucagon (G)
-log [Hormone] M

D)


Enzyme activities
(pmoles/min/million cell)
200
180
160
140
120
100
80
60
40
20
0
C 9 6 C 9 6 I9/G9 I9/G6 I6/G9 I6/G6
Insulin (I)
Glucagon (G)
-log [Hormone] M

E)


Enzyme activities
(pmoles/min/million cell)
200
180
160
140
120
100
80
60
40
20
0
C 9 6 C 9 6 I9/G9 I9/G6 I6/G9 I6/G6
Insulin (I)
Glucagon (G)
-log [Hormone] M

hydroxylase and 5α-reductase activity while an antagonistic effect was observed with 16α-hydroxylase. 10^{-9} M glucagon appears to block the action of 10^{-6} M insulin on 7α-hydroxylase and 17-OHSD activity. Similar effects could be seen with 10^{-6} M insulin in the presence of 10^{-6} M glucagon. Potentiation of insulin (10^{-6} M) effect by glucagon could be observed with 6β–hydroxylase and 5α-reductase while antagonism of glucagon on insulin effect could be seen with 16α-hydroxylase and 17-OHSD. 10^{-6} M glucagon appears to block the effect of 10^{-6} M insulin on 7α-hydroxylase activity.

6.1.2 HEPATOCYTES FROM 3-DAYS STZ-TREATED RAT

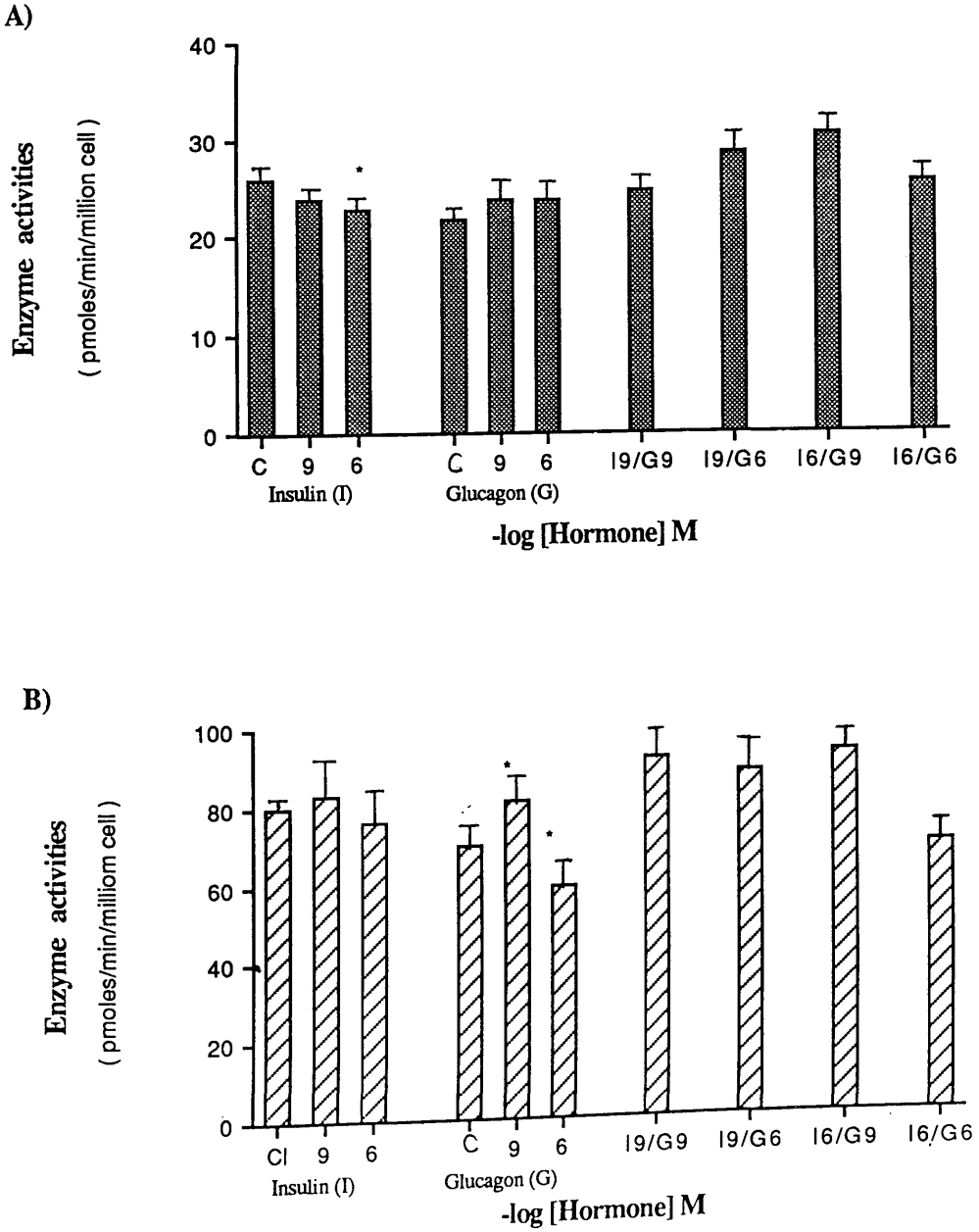
6.1.2.1 Preincubation with insulin *and* glucagon for 1/2 hour

As expected insulin at 10^{-9} and 10^{-6} M concentrations had little effect on the enzyme activities in hepatocytes from STZ-treated diabetic rats (Table 37 and Figure 41). Comparing the control levels, all of the enzyme activities in the diabetic rat hepatocytes were markedly lower (refer to Table 36 and 37) than in the hepatocytes from normal rat. Both 10^{-9} M and 10^{-6} M glucagon produced no significant changes on 10^{-9} M insulin effect on the female specific 7α-hydroxylase and 5α-reductase activities. On the other hand, 10^{-9} M glucagon significantly increased the effect of 10^{-9} M insulin on 16α-hydroxylase activity, whereas 10^{-6} M glucagon significantly increased the effect of 10^{-9} M insulin on 6β-hydroxylase and 17-OHSD. However at 10^{-6} M insulin, both physiological and supraphysiological concentrations did not significantly affect the effect of the former on all the enzyme activities.

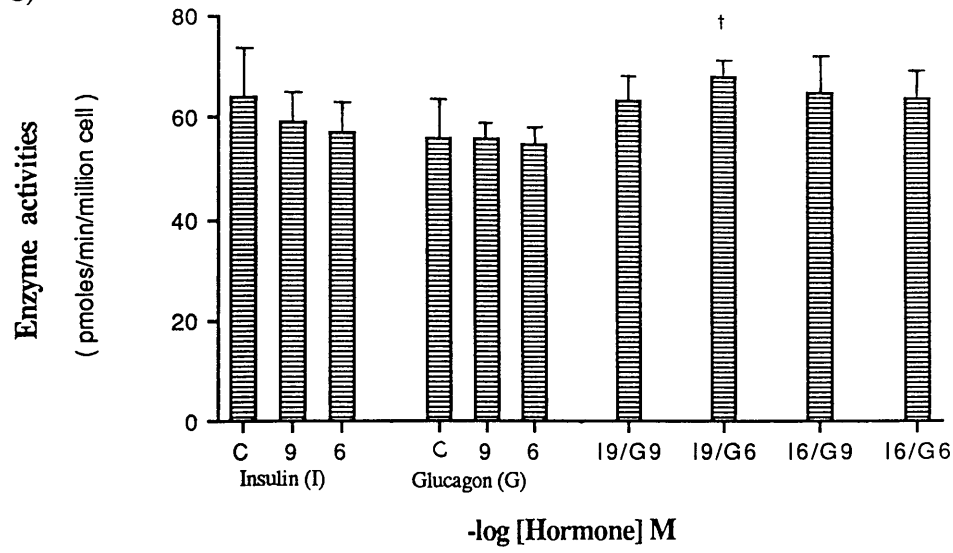
Hormone concentration	Enzyme activities (pmoles / min / million cells)				
	7α - OHase	6β - OHase	16 α -OHase	17 - OHSD	5α - reductase
Control Insulin (I)	26 ± 2	64 ± 10	54 ± 4	109 ± 3	80 ± 3
10^{-9} M (I) [a]	24 ± 1	59 ± 4	53 ± 3	104 ± 6	83 ± 9
10^{-6} M (I) [b]	23 ± 2	57 ± 3	53 ± 4	100 ± 4	76 ± 10
Control Glucagon (G)	22 ± 1	56 ± 7	49 ± 2	89 ± 4	70 ± 4
10^{-9} M (G)	24 ± 2	56 ± 3	48 ± 5	96 ± 3	81 ± 8
10^{-6} M (G)	24 ± 3	55 ± 3	48 ± 2	92 ± 4	59 ± 5
10^{-9} (I) + 10^{-9} (G)	25 ± 2	64 ± 4	61 ± 1 †	105 ± 1	92 ± 6
10^{-9} (I) + 10^{-6} (G)	29 ± 4	69 ± 3 †	53 ± 5	122 ± 4 †	88 ± 4
10^{-6} (I) + 10^{-9} (G)	31 ± 4	66 ± 6	56 ± 4	108 ± 7	93 ± 3
10^{-6} (I) + 10^{-6} (G)	26 ± 2	65 ± 4	53 ± 2	94 ± 3	69 ± 2

Table 37 . Dose-response effects of 7 α -, 6 β -, 16 α -hydroxylases (OHases), 17-oxosteroid oxidoreductase (OHSD) and 5 α -reductase activities to insulin (I) or glucagon (G) or to various combinations of both hormones after 1/2 hour of preincubation in hepatocytes obtained from 3-days STZ-treated diabetic male rat. Results expressed as mean ± S.D (N = 3). * $P < 0.05$ as compared to the respective controls; † $P < 0.05$ with respect to [a] : § $P < 0.05$ with respect to [b] .

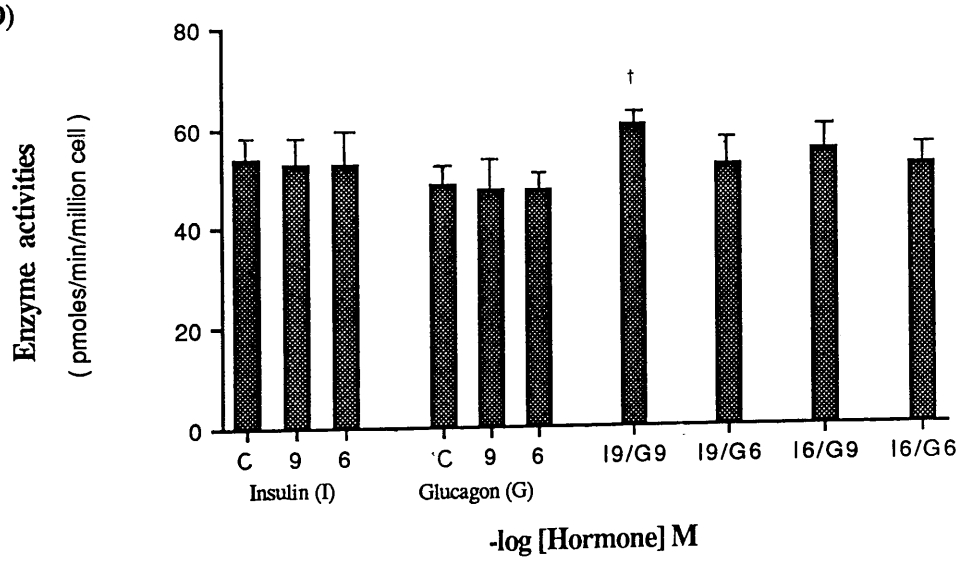
Figure 41 . Dose-response effects of (A) 7 α -hydroxylase [▨], (B) 5 α -reductase [▨], (C) 6 β - [▤] and (D) 16 α -hydroxylases [■] and (E)17-OHSD [▩] activities to insulin (I) or glucagon (G) or to various combinations of both hormones after 1/2 hour of preincubation in hepatocytes obtained from 3 days STZ-treated diabetic male rat.
Results are expressed as percentage of the relevant control and mean ± S.D (N = 3). Analysis of data : * $P < 0.05$ when compared to the respective hormone control; † $P < 0.05$ with respect to 10^{-9} M insulin; § $P < 0.05$ with respect to 10^{-6} M insulin.



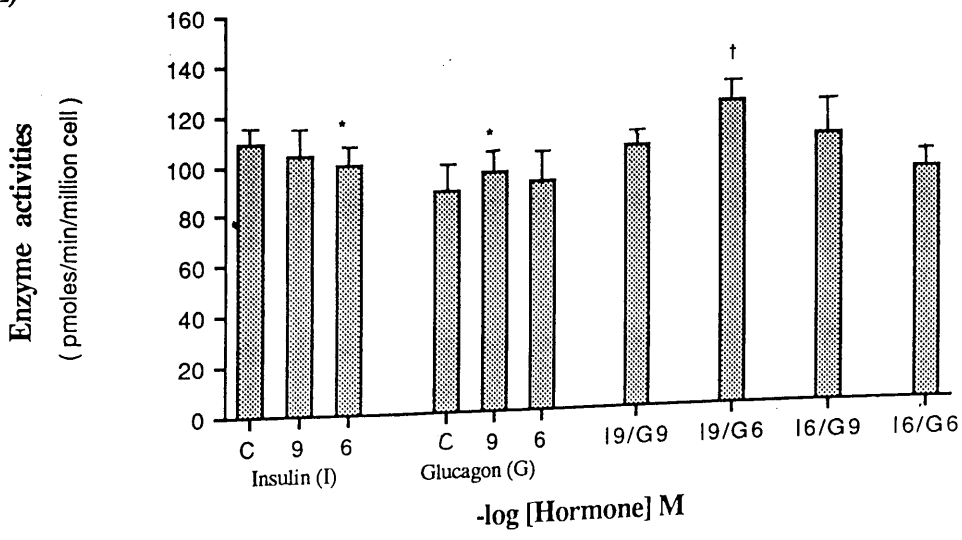
C)


Enzyme activities
(pmoles/min/million cell)
80
60
40
20
0
†
C 9 6 C 9 6 I9/G9 I9/G6 I6/G9 I6/G6
Insulin (I)
Glucagon (G)
-log [Hormone] M

D)


Enzyme activities
(pmoles/min/million cell)
80
60
40
20
0
†
C 9 6 C 9 6 I9/G9 I9/G6 I6/G9 I6/G6
Insulin (I)
Glucagon (G)
-log [Hormone] M

E)


Enzyme activities
(pmoles/min/million cell)
160
140
120
100
80
60
40
20
0
*
*
†
C 9 6 C 9 6 I9/G9 I9/G6 I6/G9 I6/G6
Insulin (I)
Glucagon (G)
-log [Hormone] M

7.0 [I-N$^{\alpha}$-trinitrophenylhistidine,12-homoarginine] glucagon (TH-GLUCAGON)

7.1 EFFECT OF TH-GLUCAGON ON THE METABOLISM OF ANDROST-4-ENE- 3,17-DIONE IN HEPATOCYTES ISOLATED FROM NORMAL MALE RATS

The effect of [I-N$^{\alpha}$ - trinitrophenylhistidine, 12-homoarginine] glucagon, (TH-glucagon), on androst-4-ene-3,17-dione metabolism was only tested on hepatocytes from normal rat.

7.1.1 Preincubation with TH-glucagon for 1/2, 1 and 2 hours

Preincubation of hepatocytes with TH-glucagon for **1/2 hour** resulted in a dose-dependent increase in 7α- , 6β- and 16α-hydroxylases , 17-OHSD and 5α-reductase activities (Table 38) with maximum effect observed at 10^{-8} M concentration. At higher concentrations the effect of TH-glucagon was markedly reduced despite being significantly greater than their respective controls even at 10^{-6} M concentration (Figure 42).

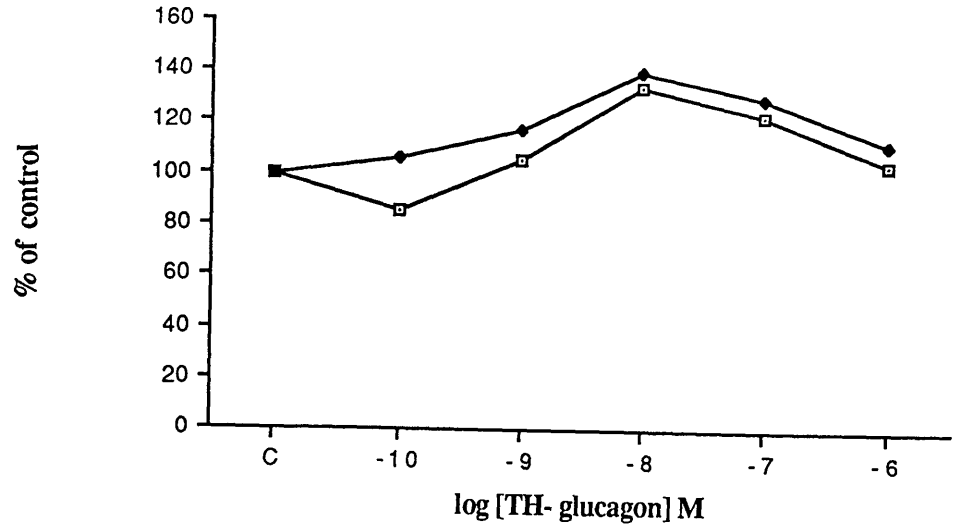
At **1 hour** preincubation, TH-glucagon gave little change in any of the enzyme activities studied (Table 39). Only at 10^{-8} M concentration did TH-glucagon significantly increased the cytochrome P-450 dependent enzymes, 7α- , 6β- and 16α-hydroxylases but had no effect on the non-cytochrome P-450 dependent enzymes, 17-OHSD and 5α-reductase though the significance in the differences seen is not known (Figure 43).

The effect of TH-glucagon on the enzyme activities after **2 hour** preincubation varies according to the enzyme studied. 7α-hydroxylase and 17-OHSD activities were

TH-glucagon concentration	Enzyme activities (pmoles / min / million cells)				
	7 α - OHase	6 β - OHase	16 α -OHase	17 - OHSD	5 α - reductase
Control	28 ± 1	69 ± 1	103 ± 5	72 ± 4	59 ± 4
10^{-10} M	24 ± 1 *	67 ± 1 *	102 ± 6	88 ± 2 *	63 ± 2
10^{-9} M	30 ± 2 *	80 ± 3 *	116 ± 2 *	88 ± 6 *	70 ± 7 *
10^{-8} M	38 ± 2 *	103 ± 2 *	178 ± 4 *	94 ± 4 *	84 ± 3 *
10^{-7} M	35 ± 1 *	83 ± 7 *	136 ± 5 *	96 ± 5 *	78 ± 2 *
10^{-6} M	30 ± 1 *	76 ± 3 *	115 ± 3 *	89 ± 2 *	68 ± 3 *

Table 38 . Dose-response effects of 7 α -, 6 β -, 16 α -hydroxylases (OHases), 17-oxosteroid oxidoreductase (OHSD) and 5 α -reductase activities to TH-glucagon after 1/2 hour preincubation in hepatocytes obtained from normal male rat. Results expressed as mean ± S.D (N = 3); * $P < 0.05$ as compared to respective controls.

A)



B)

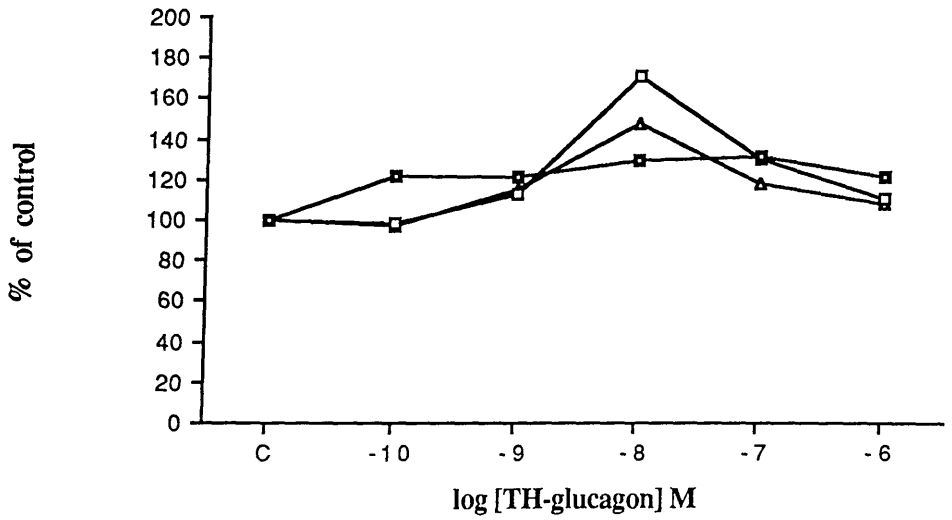
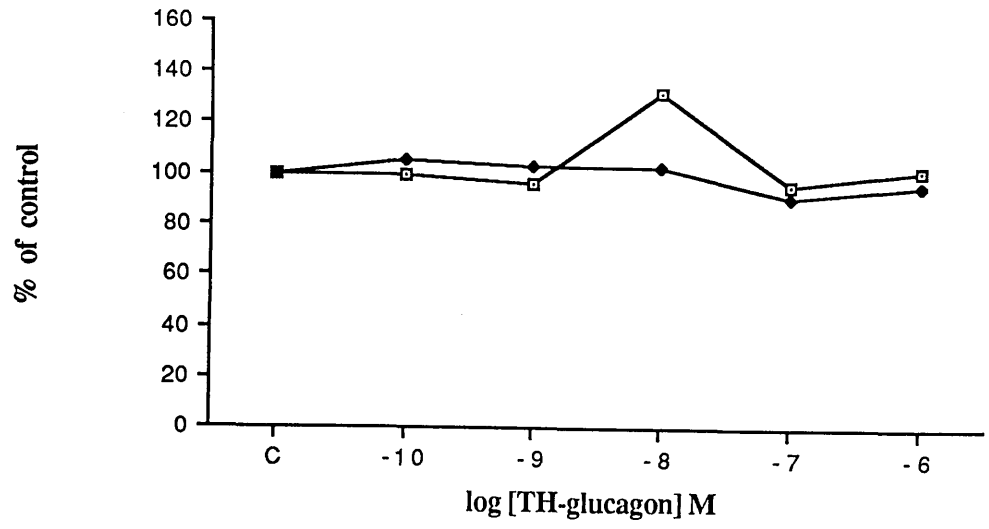


Figure 42 . Dose-response curves of (A) 7 α -hydroxylase [■] and 5 α -reductase [•] and (B) 17-OHSD [▫], 6 β - [▲] and 16 α -hydroxylases [▫] to TH-glucagon after 1/2 hour preincubation in hepatocytes obtained from normal male rat.
Results are expressed as percentage of the relevant control and mean ± S.D (N = 3). Absolute data is given in Table 38 .
C = control

TH-glucagon concentration	Enzyme activities (pmoles / min / million cells)				
	7α - OHase	6 β - OHase	16 α -OHase	17 - OHSD	5 α - reductase
Control	31 ± 3	78 ± 6	102 ± 4	120 ± 4	78 ± 7
10^{-10} M	31 ± 1	77 ± 5	107 ± 1	116 ± 3	83 ± 6
10^{-9} M	30 ± 3	73 ± 5	104 ± 4	115 ± 4	81 ± 6
10^{-8} M	40 ± 5 *	94 ± 6 *	137 ± 7 *	119 ± 9	81 ± 2
10^{-7} M	30 ± 3	75 ± 4	104 ± 6	116 ± 5	72 ± 5
10^{-6} M	32 ± 3	83 ± 7	111 ± 2 *	122 ± 4	76 ± 9

Table 39 . Dose-response effects of 7 α -, 6 β -, 16 α -hydroxylases (OHases), 17-oxosteroid oxidoreductase (OHSD) and 5 α -reductase activities to TH-glucagon after 1 hour preincubation in hepatocytes obtained from normal male rat. Results expressed as mean ± S.D (N = 3); * $P < 0.05$ as compared to respective controls.

A)



B)

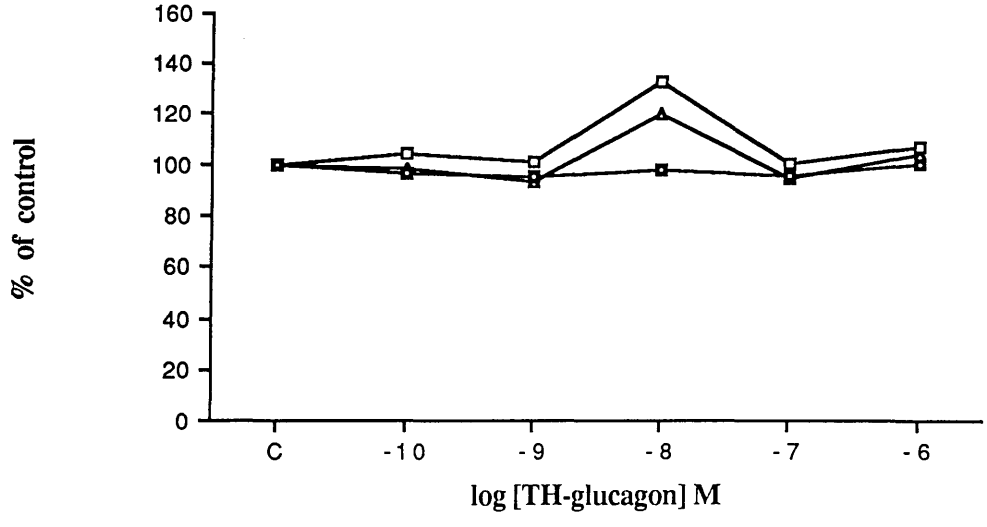


Figure 43 . Dose-response curves of (A) 7 α -hydroxylase [▪] and 5 α -reductase [•] and (B) 17-OHSD [▪], 6 β - [▲] and 16 α -hydroxylases [▫] to TH-glucagon after 1 hour preincubation in hepatocytes obtained from normal male rat.
Results are expressed as percentage of the relevant control and mean ± S.D (N = 3). Absolute data is given in Table 39 .
C = control

not altered. At low concentration (10^{-10} - 10^{-9} M) TH-glucagon increased 6β- and 16α-hydroxylases and 5α-reductase activity but at 10^{-6} M the enzyme activities were reduced significantly below control (Table 40 and Figure 44).

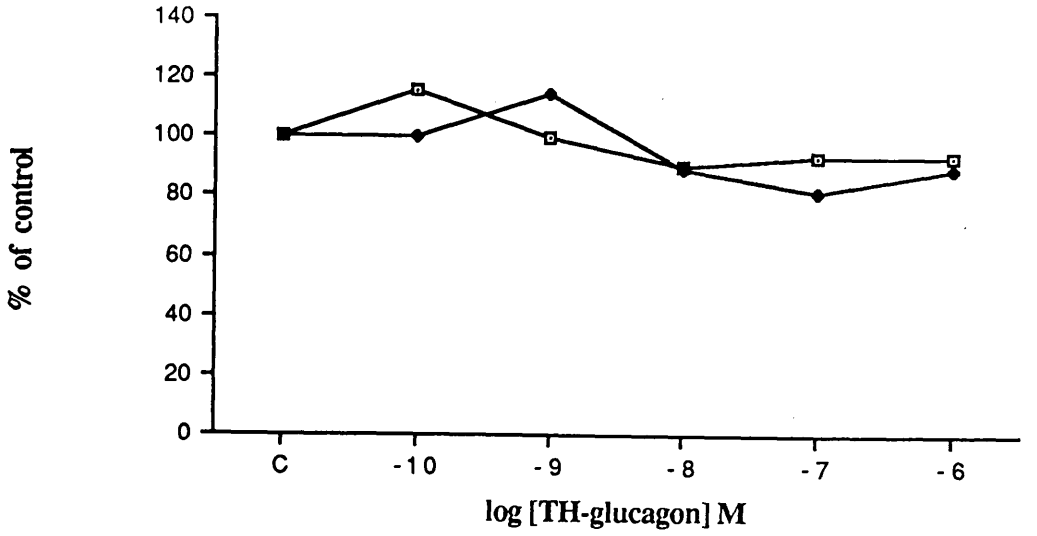
7.1.2 Preincubation with TH-glucagon for 24 hours

After **24 hour** of preincubation, TH-glucagon caused a marked, dose-dependent decrease in all of the enzyme activities (Table 41). Maximum response could be observed at 10^{-9} M with the activity of the 7α-hydroxylase and 5α-reductase reduced to about 40 % and 60 % of respective control and the 6β- and 16α-hydroxylase and 17-OHSD activities reduced to about 40 % , 25 % and 55 % of control respectively (Figure 45). Significant reduction in enzyme activity could be seen at concentrations as low as 10^{-10} M.

TH-glucagon concentration	Enzyme activities (pmoles / min / million cells)				
	7α - OHase	6β - OHase	16 α -OHase	17 - OHSD	5 α - reductase
Control	32 ± 6	64 ± 4	83 ± 5	120 ± 5	79 ± 6
10^{-10} M	37 ± 4	72 ± 5 *	99 ± 2 *	130 ± 5	79 ± 5
10^{-9} M	32 ± 4	68 ± 3	93 ± 2 *	123 ± 9	91 ± 3 *
10^{-8} M	29 ± 2	50 ± 2 *	59 ± 4 *	119 ± 4	71 ± 3
10^{-7} M	30 ± 1	60 ± 3	79 ± 4	115 ± 3	65 ± 6 *
10^{-6} M	30 ± 2	58 ± 1 *	71 ± 4 *	118 ± 1	70 ± 3 *

Table 40 . Dose-response effects of 7 α -, 6 β -, 16 α -hydroxylases (OHases), 17-oxosteroid oxidoreductase (OHSD) and 5 α -reductase activities to TH-glucagon after 2 hour preincubation in hepatocytes obtained from normal male rat. Results expressed as mean ± S.D (N = 3); * $P < 0.05$ as compared to respective controls.

A)



B)

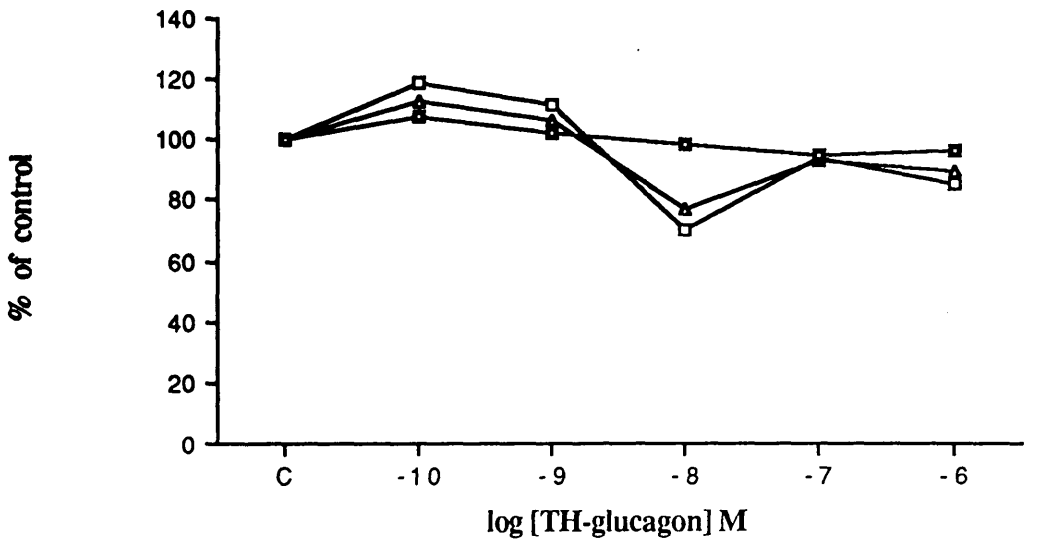


Figure 44 . Dose-response curves of (A) 7 α -hydroxylase [■] and 5 α -reductase [●] and (B) 17-OHSD [■], 6 β - [▲] and 16 α -hydroxylases [□] to TH-glucagon after 2 hour preincubation in hepatocytes obtained from normal male rat.
Results are expressed as percentage of the relevant control and mean ± S.D (N = 3). Absolute data is given in Table 40 .
C = control

TH-glucagon concentration	Enzyme activities (pmoles / min / million cells)				
	7 α - OHase	6 β - OHase	16 α -OHase	17 - OHSD	5 α - reductase
Control	69 ± 6	86 ± 3	97 ± 3	82 ± 7	62 ± 2
10^{-10} M	51 ± 4 *	56 ± 5 *	54 ± 8 *	63 ± 3 *	41 ± 1 *
10^{-9} M	29 ± 1 *	37 ± 3 *	25 ± 2 *	45 ± 2 *	37 ± 2 *
10^{-8} M	27 ± 3 *	33 ± 2 *	26 ± 1 *	45 ± 5 *	34 ± 3 *
10^{-7} M	26 ± 2 *	34 ± 3 *	24 ± 2 *	46 ± 5 *	33 ± 2 *
10^{-6} M	24 ± 2 *	33 ± 2 *	27 ± 2 *	45 ± 6 *	34 ± 2 *

Table 41 . Dose-response effects of 7 α -, 6 β -, 16 α -hydroxylases (OHases), 17-oxosteroid oxidoreductase (OHSD) and 5 α -reductase activities to TH-glucagon after 24 hour preincubation in hepatocytes obtained from normal male rat. Results expressed as mean ± S.D (N = 3); * $P < 0.05$ as compared to respective controls.

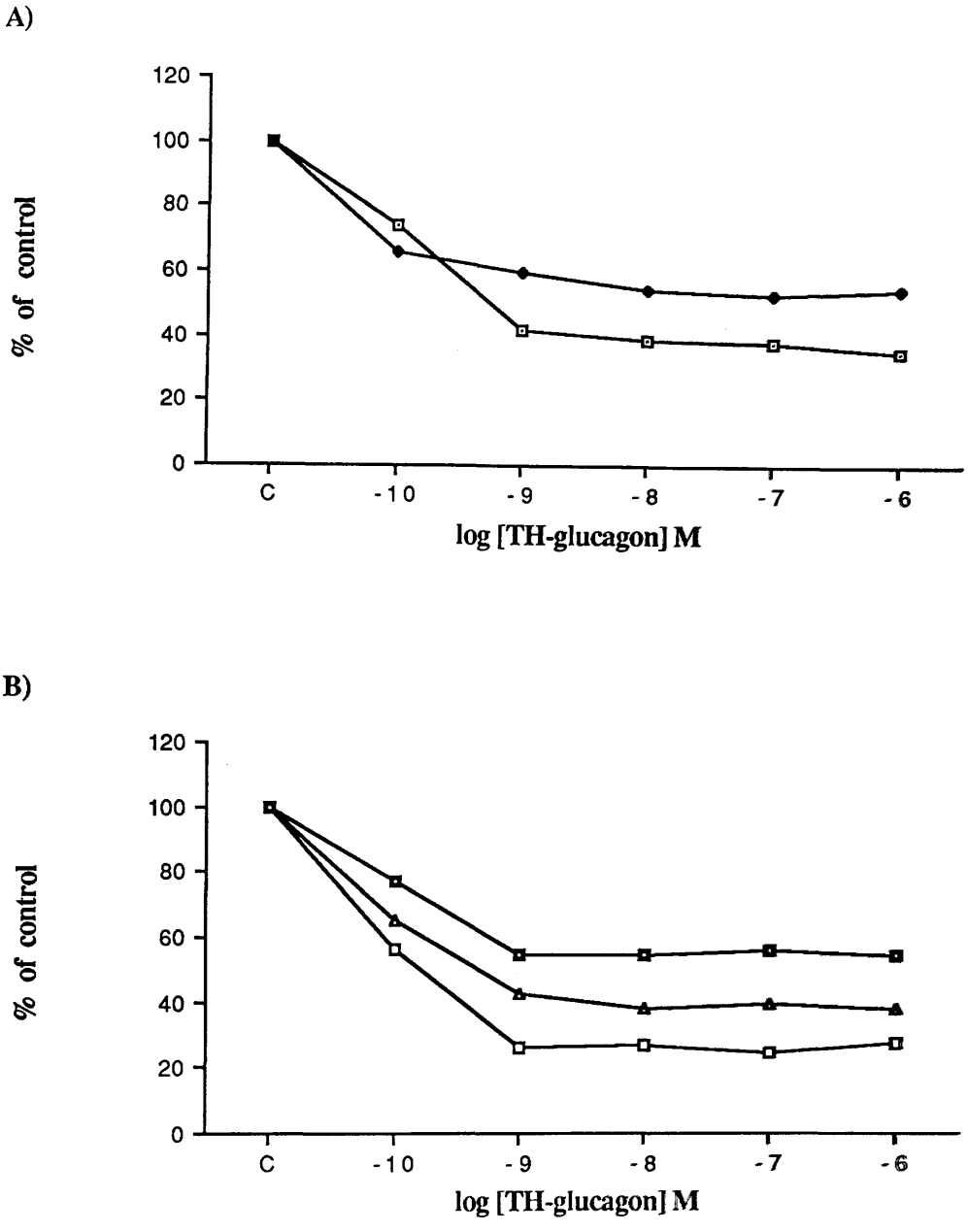


Figure 45 . Dose-response curves of (A) 7 α -hydroxylase [▪] and 5 α -reductase [•] and (B) 17-OHSD [▫], 6 β - [▲] and 16 α -hydroxylases [▫] to TH-glucagon after 24 hour preincubation in hepatocytes obtained from normal male rat.
Results are expressed as percentage of the relevant control and mean ± S.D (N = 3). Absolute data is given in Table 41 .
C = control

8.0 ORAL HYPOGLYCAEMIC AGENTS

8.1 THE EFFECTS OF PHENFORMIN AND TOLBUTAMIDE ON THE METABOLISM OF ANDROST-4-ENE-3,17-DIONE

8.1.1 HEPATOCYTES FROM NORMAL RAT

8.1.1.1 Preincubation with phenformin or tolbutamide alone

The hepatocytes were treated with the oral hypoglycaemic agents as described in the Methods Section **2.5.6.** Phenformin and tolbutamide dose/response curves were examined using concentrations of the drugs from 10^{-6} to 10^{-3} M.

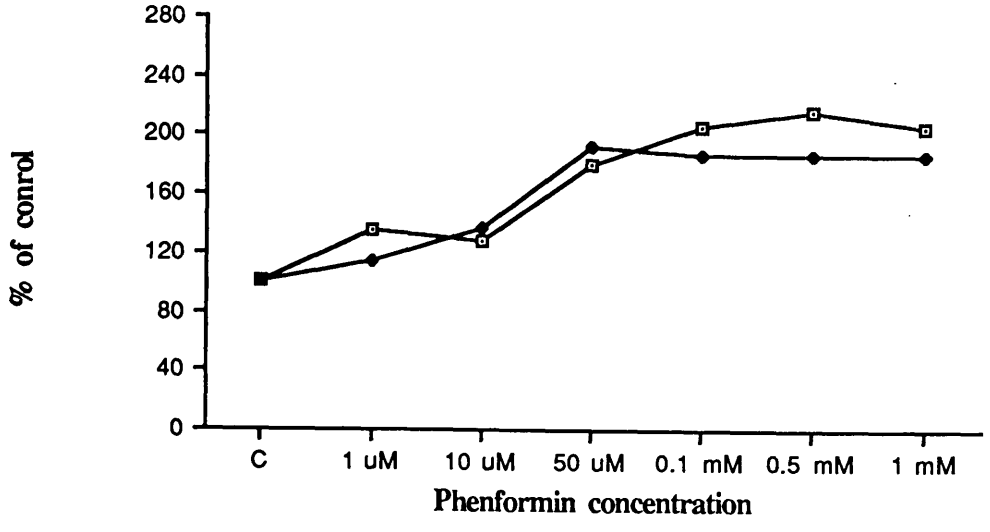
Phenformin significantly increased steroid metabolism at concentrations as low as 10^{-6} M (Table 42; Figure 46) and maximum response observed was at 5×10^{-5} M (182 % of control for 7α-hydroxylase; 194 % of control for 5α-reductase; 214 % of control for 6β-hydroxylase; 140 % of control for 16α-hydroxylase; 186 % of control for 17-OHSD). No selective effect of phenformin on the enzymes tested is evident.

Similarly, **tolbutamide** induced a dose-dependent effect on all of the enzyme activities. Again, a significant increase in activity could be seen at concentrations as low as 10^{-6} M but maximum response was achieved at a slightly higher concentration (10^{-4} M). However, at the highest concentration used i.e. 10^{-3} M, enzymes activity decreased below the maximum seen despite being significantly above the control level (Figure 47). Maximum responses achieved were lower than attained by phenformin ; At 10^{-4} M tolbutamide increased 7α- and 6β-hydroxylases activity 52 % above control, 75 % above control for 16α-hydroxylase, 102 % and 63 % above control for 17-

Phenformin concentration	Enzyme activities (pmoles / min / million cells)				
	7α - OHase	6β - OHase	16 α -OHase	17 - OHSD	5 α - reductase
Control	49 ± 3	70 ± 1	82 ± 6	111 ± 4	107 ± 7
1 uM	66 ± 3 *	101 ± 8 *	89 ± 3	154 ± 1 *	122 ± 6 *
10 uM	63 ± 1 *	102 ± 5 *	88 ± 1	175 ± 2 *	148 ± 7 *
50 uM	89 ± 4 *	150 ± 23 *	115 ± 5 *	206 ± 4 *	208 ± 10 *
0.1 mM	102 ± 10 *	153 ± 8 *	122 ± 2 *	200 ± 5 *	202 ± 4 *
0.5 mM	107 ± 6 *	145 ± 6 *	131 ± 6 *	200 ± 9 *	201 ± 5 *
1 mM	102 ± 4 *	148 ± 10 *	134 ± 10 *	198 ± 4 *	202 ± 6 *

Table 42 . Dose-response effects of 7 α -, 6 β -, 16 α -hydroxylases (OHases), 17-oxosteroid oxidoreductase (OHSD) and 5 α -reductase activities to phenformin after 24 hour preincubation in hepatocytes obtained from normal male rat. Results expressed as mean ± S.D (N = 3); * P < 0.05 as compared to respective controls.

A)



B)

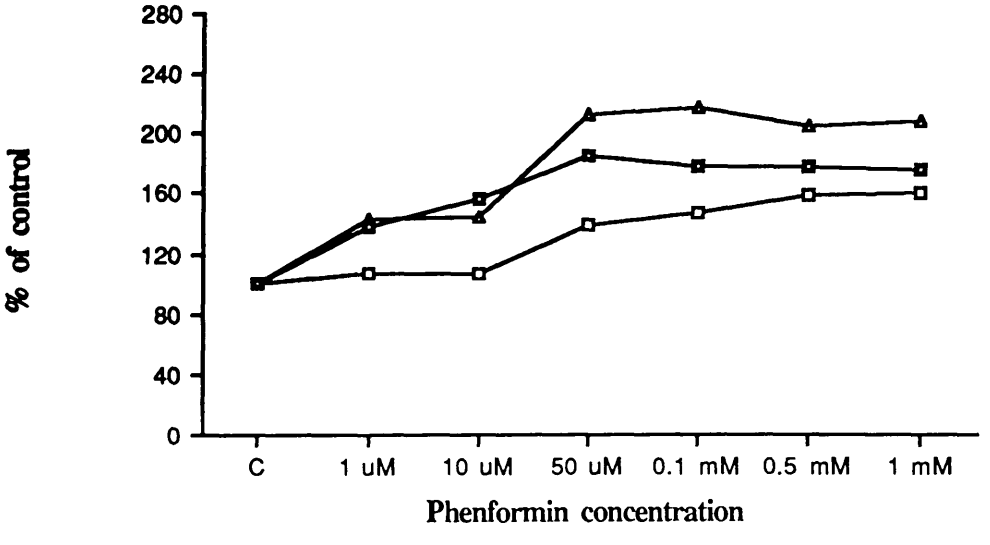
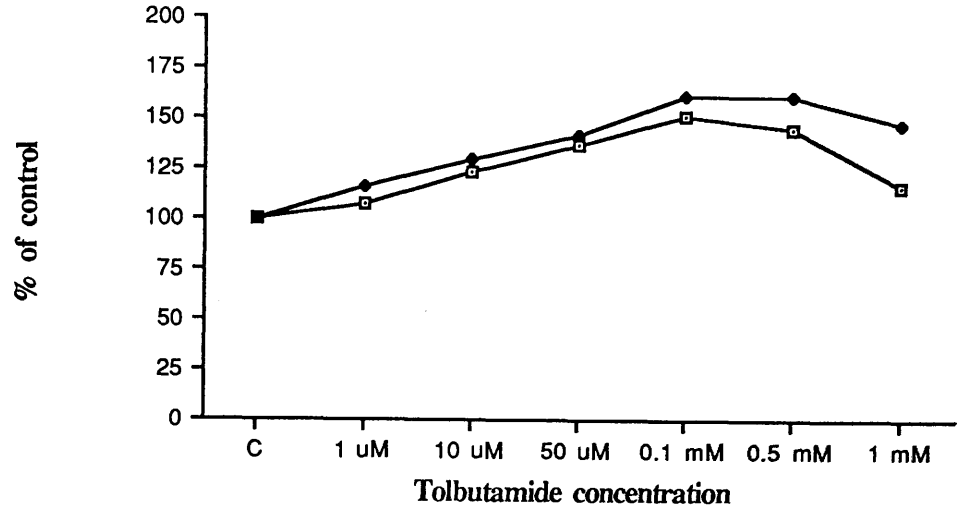


Figure 46 . Dose-response curves of (A) 7 α -hydroxylase [▪] and 5 α -reductase [•] and (B) 17-OHSD [▪], 6 β -[▲] and 16 α -hydroxylases [▫] activities to phenformin after 24 hour preincubation in hepatocytes obtained from normal male rat.
Results are expressed as percentage of the relevant control and mean ± S.D (N = 3). Absolute data is given in Table 42 .
C = control

Tolbutamide concentration	Enzyme activities (pmoles / min / million cells)				
	7 α - OHase	6 β - OHase	16 α -OHase	17 - OHSD	5 α - reductase
Control	63 ± 4	95 ± 5	81 ± 5	115 ± 4	115 ± 4
1 uM	68 ± 10	104 ± 3 *	92 ± 6 *	146 ± 2 *	133 ± 2 *
10 uM	78 ± 8 *	112 ± 4 *	128 ± 3 *	151 ± 8 *	150 ± 5 *
50 uM	86 ± 5 *	116 ± 5 *	140 ± 6 *	197 ± 6 *	165 ± 4 *
0.1 mM	96 ± 5 *	144 ± 4 *	142 ± 9 *	232 ± 8 *	187 ± 7 *
0.5 mM	92 ± 5 *	137 ± 9 *	146 ± 6 *	237 ± 7 *	187 ± 10 *
1 mM	74 ± 4 *	112 ± 6 *	103 ± 6 *	195 ± 4 *	171 ± 13 *

Table 43 , Dose-response effects of 7 α -, 6 β -, 16 α -hydroxylases (OHases), 17-oxosteroid oxidoreductase (OHSD) and 5 α -reductase activities to tolbutamide after 24 hour preincubation in hepatocytes obtained from normal male rat. Results expressed as mean ± S.D (N = 3); * $P < 0.05$ as compared to respective controls.

A)



B)

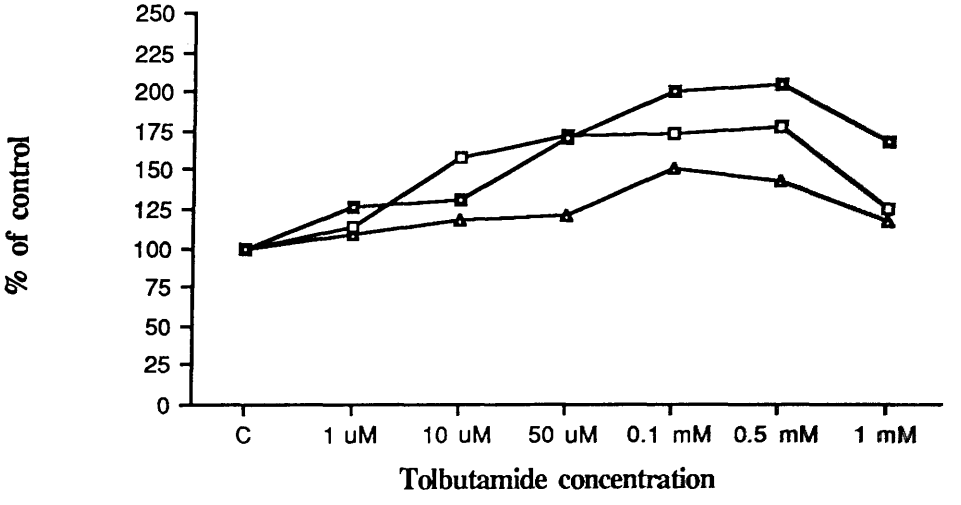


Figure 47 . Dose-response curves of (A) 7 α -hydroxylase [▪] and 5 α -reductase [•] and (B) 17-OHSD [▪], 6 β - [▲] and 16 α -hydroxylases [▫] activities to tolbutamide after 24 hour preincubation in hepatocytes obtained from normal male rat. Results are expressed as percentage of the relevant control and mean ± S.D (N = 3). Absolute data is given in Table 43 .
C = control

OHSD and 5α-reductase respectively (Table 43 and Figure 47)

8.1.1.2 Dose-response curve for insulin in the presence of phenformin or tolbutamide (10^{-3} M)

In this experiment the hepatocytes were first preincubated with phenformin or tolbutamide (10^{-3} M) in serum-free medium for 24 hours. Subsequently, insulin (from 10^{-10} to 10^{-6} M) was added and then left in the incubator for an additional 30 minutes after which the steroid assay was carried out.

Insulin at 10^{-9} M alone significantly increased all the enzyme activities (between 120 % to 170 % of control). **Phenformin** (10^{-3} M) alone increased all the enzyme activities to the same extent as insulin (Table 44). When compared to 1 mM phenformin alone as control, the effect of increasing insulin concentrations in the presence of 10^{-3} M phenformin was enzyme dependent. No further increase in activity was observed for 7α- and 16α-hydroxylases except at 1 μM insulin concentration for the latter. Potentiation of phenformin effect by insulin could be observed with 6β-hydroxylase, 17-OHSD and 5α-reductase and was maximum at 0.1 nM insulin (Figure 48).

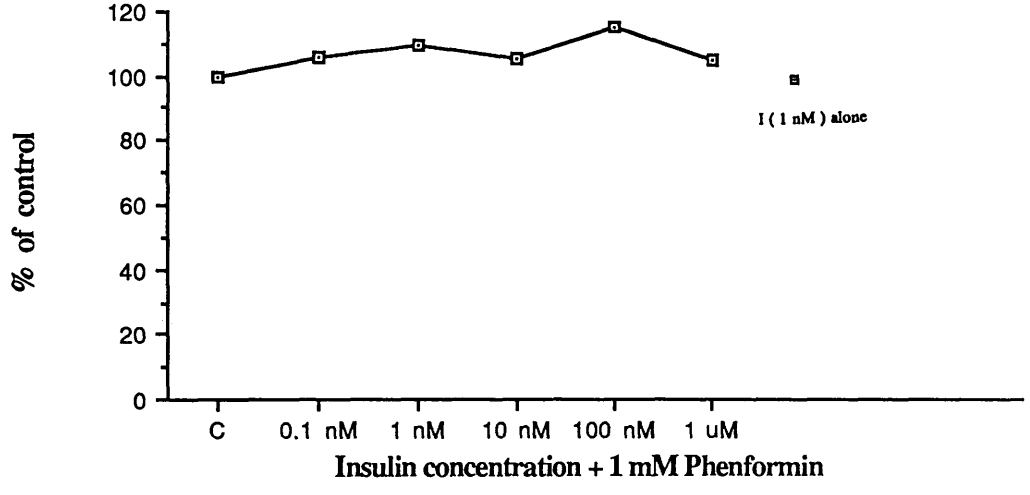
Similarly, **tolbutamide** (10^{-3} M) alone significantly increased all the enzyme activities (between 120 % and 160 % of control)(Table 45). Insulin in the presence of 10^{-3} M tolbutamide increased the 5α-reductase activity to the same extent as tolbutamide alone (Figure 49). In the presence of 1 mM tolbutamide, increasing the concentration of insulin resulted in a further increase in 7α-, 6β- and 16α-hydroxylases and 17-OHSD activities.

Drug concentration	Enzyme activities (pmoles / min / million cells)				
	7α - OHase	6β - OHase	16 α -OHase	17 - OHSD	5 α - reductase
Control	17 ± 2	43 ± 3	27 ± 3	79 ± 2	42 ± 2
1 nM I alone	29 ± 2	52 ± 4	34 ± 2	95 ± 3	59 ± 2
1 mM P alone §	31 ± 2	53 ± 3	34 ± 3	99 ± 3	60 ± 1
0.1 nM I + P	33 ± 2	65 ± 3 *	39 ± 3	104 ± 2	71 ± 1 *
1 nM I + P	34 ± 4	67 ± 3 *	38 ± 4	107 ± 1 *	65 ± 3
10 nM I + P	33 ± 4	63 ± 5 *	39 ± 2	107 ± 2 *	64 ± 2
100 nM I + P	36 ± 2	65 ± 2 *	41 ± 4	109 ± 1 *	71 ± 3 *
1 uM I + P	33 ± 5	67 ± 1 *	47 ± 3 *	113 ± 2 *	72 ± 2 *

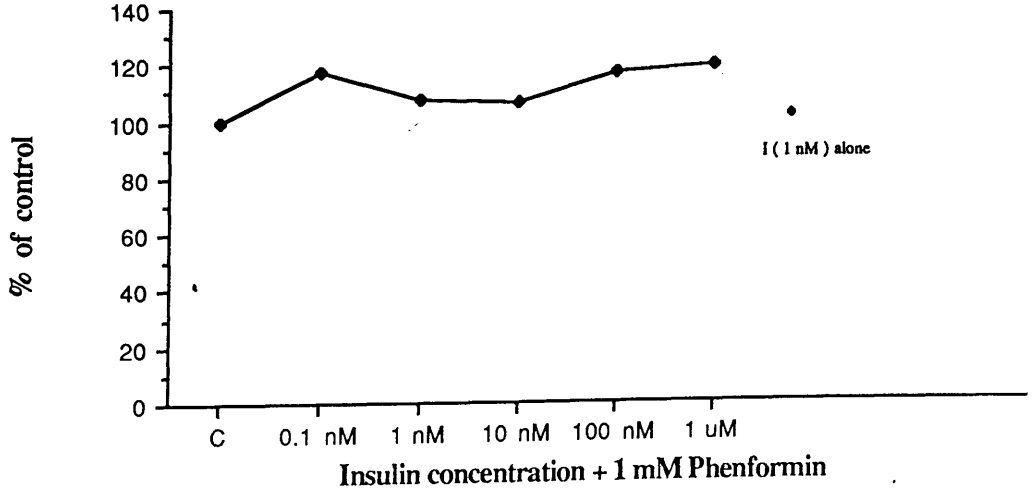
Table 44 . Dose-response effects of 7 α -, 6 β -, 16 α -hydroxylases (OHases), 17-oxosteroid oxidoreductase (OHSD) and 5 α -reductase activities to insulin (I) 1/2 hour preincubation after 24 hour exposure to 1 mM phenformin (P) in hepatocytes obtained from normal male rat. Results expressed as mean ± S.D (N = 3); $P < 0.05$ with respect to § .

Figure 48 . Dose-response effects of (A)7 α -hydroxylase [▪], (B) 5 α -reductase [◆], (C) 6 β - [▲] and (D) 16 α -hydroxylases [▫] and (E) 17-OHSD [▪] activities to insulin (I) 1/2 hour preincubation after 24 hour exposure to 1 mM phenformin (P) in hepatocytes obtained from normal male rat.
Results are expressed as percentage of the relevant control and mean ± S.D (N = 3). Absolute data is given in Table 44 .
C = control (1 mM Phenformin alone)

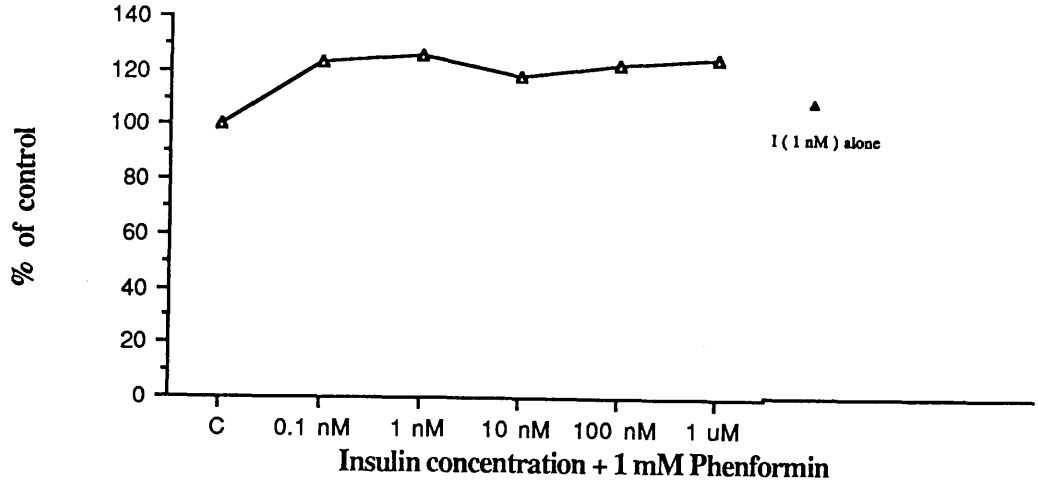
A)



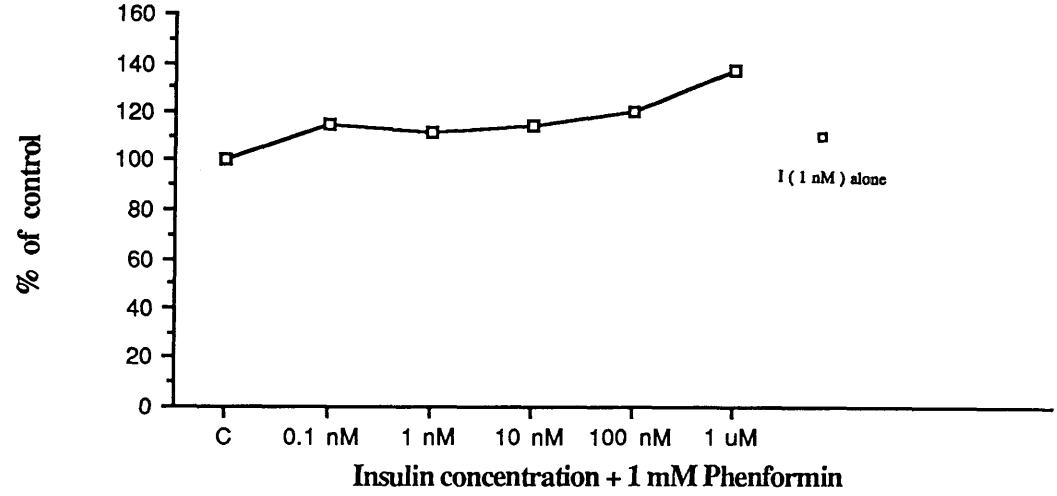
B)



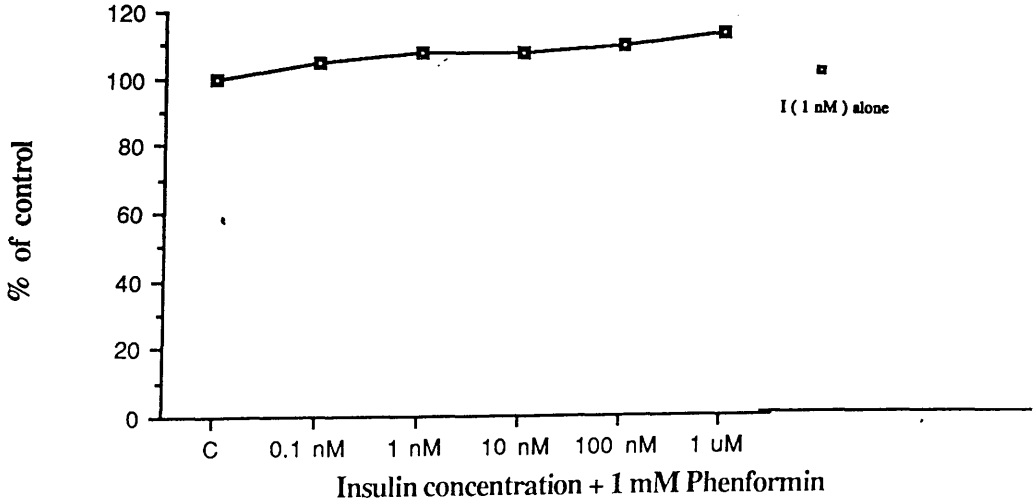
C)


140
120
100
80
60
40
20
0
% of control
I (1 nM) alone
C
0.1 nM
1 nM
10 nM
100 nM
1 uM
Insulin concentration + 1 mM Phenformin

D)


160
140
120
100
80
60
40
20
0
% of control
I (1 nM) alone
C
0.1 nM
1 nM
10 nM
100 nM
1 uM
Insulin concentration + 1 mM Phenformin

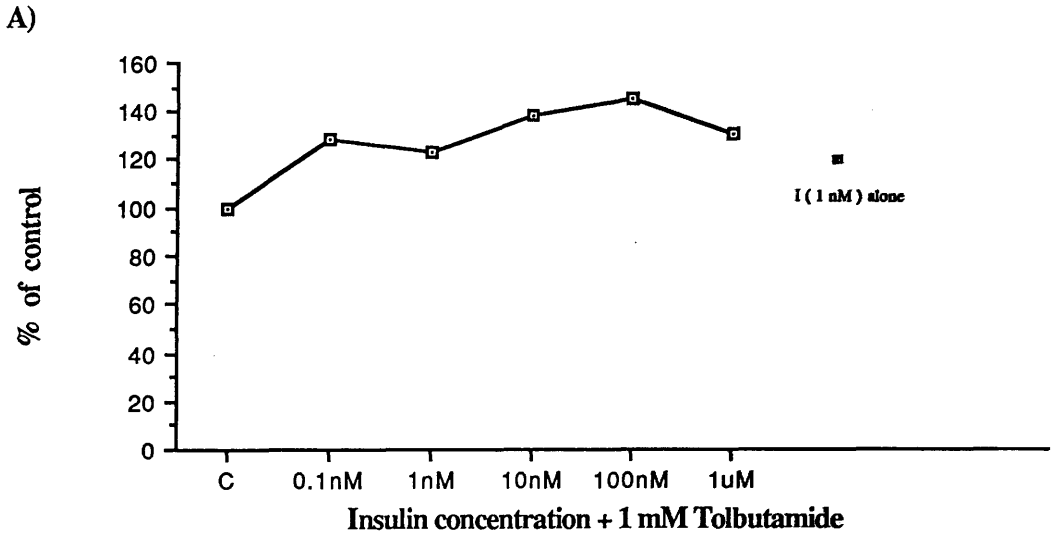
E)

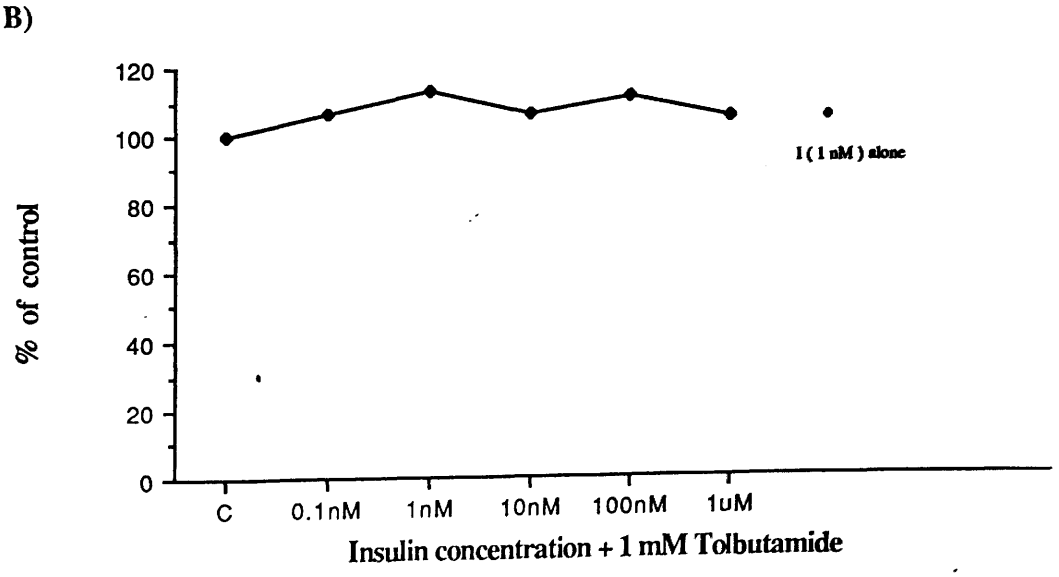

120
100
80
60
40
20
0
% of control
I (1 nM) alone
C
0.1 nM
1 nM
10 nM
100 nM
1 uM
Insulin concentration + 1 mM Phenformin

Drug concentration	Enzyme activities (pmoles / min / million cells)				
	7α - OHase	6 β - OHase	16 α -OHase	17 - OHSD	5 α - reductase
Control	28 ± 1	43 ± 3	29 ± 3	76 ± 2	42 ± 5
1 nM I alone	43 ± 3	57 ± 3	41 ± 3	94 ± 5	66 ± 5
1 mM T alone §	38 ± 3	58 ± 1	39 ± 2	98 ± 1	68 ± 6
0.1 nM I + T	49 ± 4 *	59 ± 4	38 ± 4	108 ± 6	73 ± 5
1 nM I + T	47 ± 2 *	61 ± 4	47 ± 2 *	92 ± 4	77 ± 6
10 nM I + T	53 ± 4 *	62 ± 4	49 ± 4 *	104 ± 2 *	73 ± 1
100 nM I + T	56 ± 4 *	68 ± 2 *	48 ± 2 *	108 ± 2 *	76 ± 3
1 uM I + T	50 ± 1 *	71 ± 4 *	47 ± 3 *	106 ± 3 *	72 ± 3

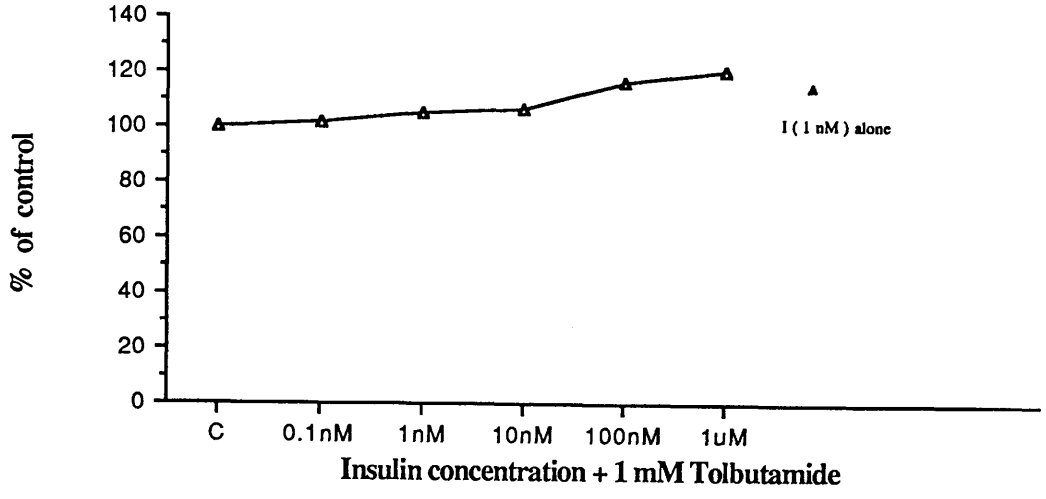
Table 45 . Dose-response effects of 7 α -, 6 β -, 16 α -hydroxylases (OHases), 17-oxosteroid oxidoreductase (OHSD) and 5 α -reductase activities to insulin (I) 1/2 hour preincubation after 24 hour exposure to 1 mM tolbutamide (T) in hepatocytes obtained from normal male rat. Results expressed as mean ± S.D (N = 3); $P < 0.05$ with respect to § .

Figure 49 . Dose-response effects of (A) 7 α -hydroxylase [▪], (B) 5 α -reductase [◆], (C) 6 β - [▲] and (D) 16 α -hydroxylases [▫] and (E) 17-OHSD [▪] activities to insulin (I) 1/2 hour preincubation after 24 hour exposure to 1 mM tolbutamide (T) in hepatocytes obtained from normal male rat.
Results are expressed as percentage of the relevant control and mean ± S.D (N = 3). Absolute data is given in Table 45 .
C = control (1 mM Tolbutamide alone)

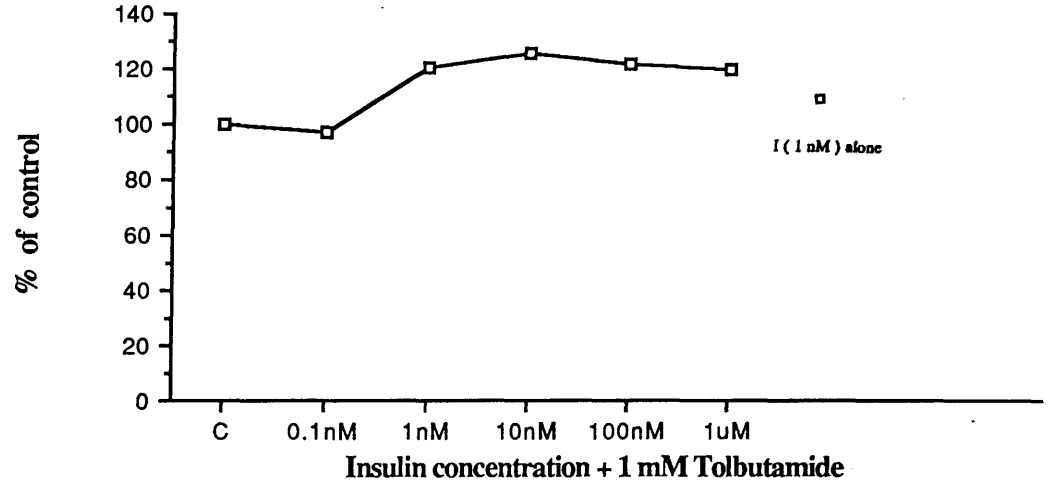




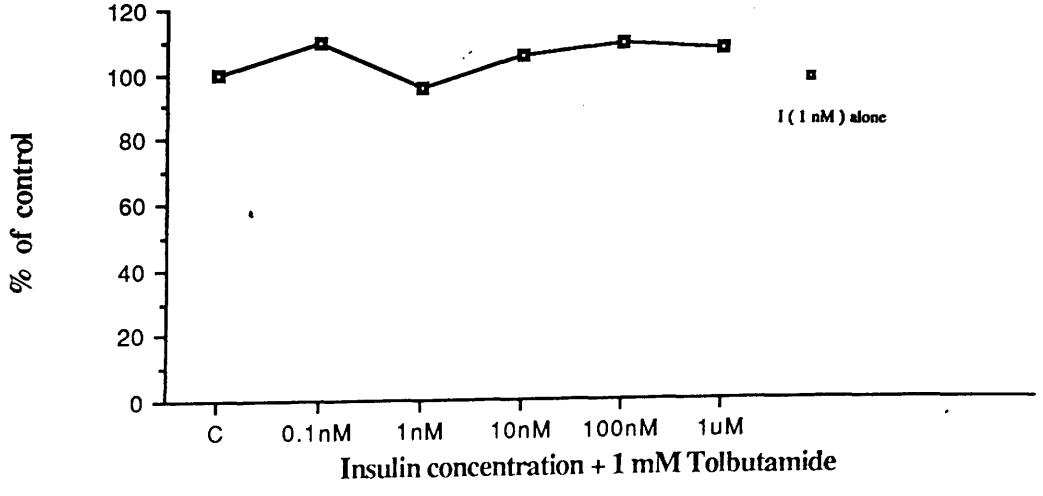
C)


140
120
100
80
60
40
20
0
% of control
I (1 nM) alone
C
0.1nM
1nM
10nM
100nM
1uM
Insulin concentration + 1 mM Tolbutamide

D)


140
120
100
80
60
40
20
0
% of control
I (1 nM) alone
C
0.1nM
1nM
10nM
100nM
1uM
Insulin concentration + 1 mM Tolbutamide

E)


120
100
80
60
40
20
0
% of control
I (1 nM) alone
C
0.1nM
1nM
10nM
100nM
1uM
Insulin concentration + 1 mM Tolbutamide

8.1.1.3 Dose-response curves for phenformin or tolbutamide in the presence of 10^{-9} M insulin

Both insulin (10^{-9} M) and phenformin (10^{-3} M) caused significant increases in all the enzyme activities (Table 46). In normal rat hepatocytes, **phenformin** gave a dose-related increase in all the enzyme activities (Figure 46). This effect of phenformin could also be seen in the presence of 10^{-9} M insulin (Figure 50).When compared to 1 nM insulin alone as control, phenformin significantly potentiated the effect of insulin (1 nM) on all the enzyme activities measured. This potentiating effect of phenformin could be observed at concentration as low as 1 μM for all the enzymes except 16α-hydroxylase (at 10 μM) (Table 46).

Similarly, tolbutamide (10^{-3} M) and insulin (10^{-9} M) cause significant increases in all the enzyme activities (Table 47). Tolbutamide in the presence of 10^{-9} M insulin increased 7α- and 6β-hydroxylase activities to the same extent as 10^{-9} M insulin (except at 1 μM tolbutamide). However, tolbutamide in the presence of 10^{-9} M insulin caused a dose-dependent increase in the activity of the cytochrome P-450 independent enzymes, 17-OHSD and 5α-reductase (Figure 51). Only at higher tolbutamide concentrations (> 0.1 mM) was potentiation of insulin's effect observed with the 16α-hydroxylase.

8.1.2 HEPATOCYTES FROM 3-DAYS STZ-TREATED DIABETIC RAT

8.1.2.1 Preincubation with phenformin or tolbutamide alone

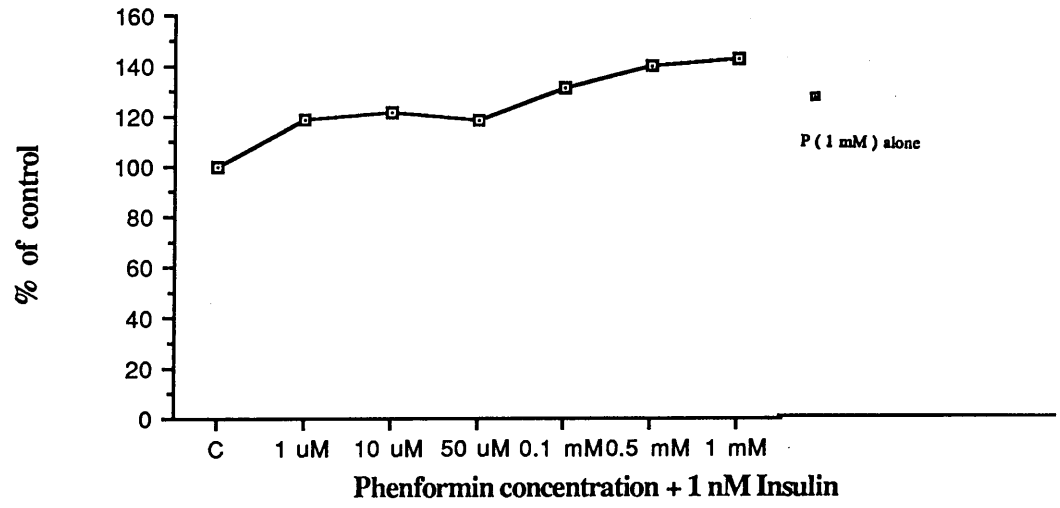
No selective effect of phenformin on the enzymes tested is evident (Table 48). In the diabetic rat hepatocytes, **phenformin** still exhibited a dose-dependent effect on all

Drug concentration	Enzyme activities (pmoles / min / million cells)				
	7α - OHase	6β - OHase	16 α -OHase	17 - OHSD	5 α - reductase
Control	56 ± 3	79 ± 7	53 ± 7	77 ± 4	81 ± 3
1 nM I alone §	85 ± 6	104 ± 7	72 ± 3	94 ± 2	94 ± 4
1 mM P alone	91 ± 7	113 ± 5	73 ± 3	103 ± 5	112 ± 5
1 uM P + I	101 ± 6 *	124 ± 6 *	71 ± 2	106 ± 3 *	119 ± 2 *
10 uM P + I	104 ± 5 *	169 ± 4 *	95 ± 5 *	116 ± 5 *	130 ± 2 *
50 uM P + I	101 ± 3 *	183 ± 4 *	93 ± 4 *	112 ± 6 *	117 ± 7 *
0.1 mM P + I	112 ± 4 *	174 ± 3 *	81 ± 6	116 ± 5 *	111 ± 6 *
0.5 mM P + I	120 ± 7 *	161 ± 9 *	71 ± 6	116 ± 5 *	126 ± 5 *
1 mM P + I	122 ± 7 *	180 ± 6 *	86 ± 4 *	120 ± 2 *	121 ± 4 *

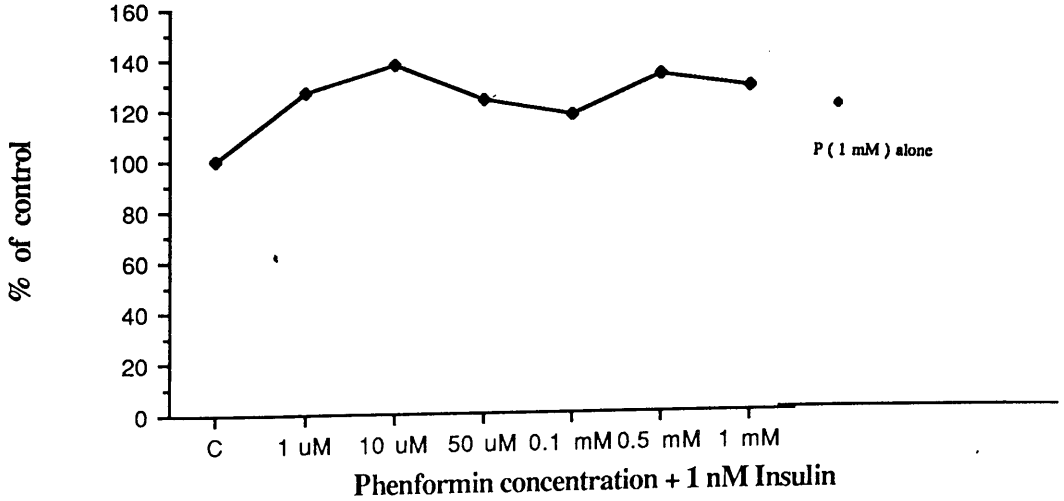
Table 46 . Dose-response effects of 7 α -, 6 β -, 16 α -hydroxylases (OHases), 17-oxosteroid oxidoreductase (OHSD) and 5 α -reductase activites to phenformin (P) after 24 hour preincubation followed by another 1/2 hour exposure to 1 nM insulin (I) in hepatocytes obtained from normal male rat. Results expressed as mean ± S.D (N = 3); * $P < 0.05$ with respect to § .

Figure 50 . Dose-response effects of (A) 7 α -hydroxylase [▫], (B) 5 α -reductase [•], (C) 6 β - [▲] and (D) 16 α -hydroxylases [▫] and (E) 17-OHSD [▫] activities to phenformin (P) after 24 hour preincubation followed by another 1/2 hour exposure to 1 nM insulin (I) in hepatocytes obtained from normal male rat. Results are expressed as percentage of the relevant control and mean ± S.D (N = 3). Absolute data is given in Table 46 .
C = control (1 nM Insulin alone)

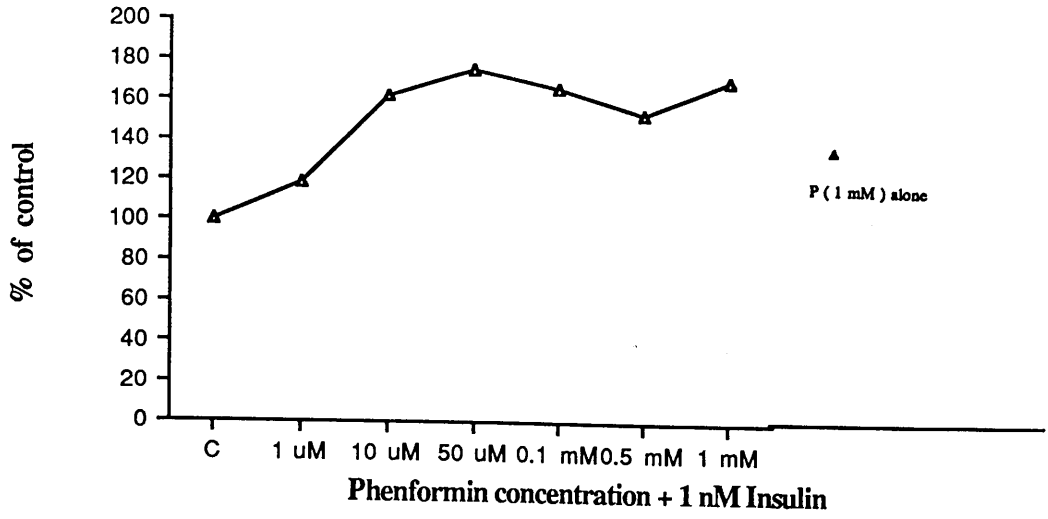
A)



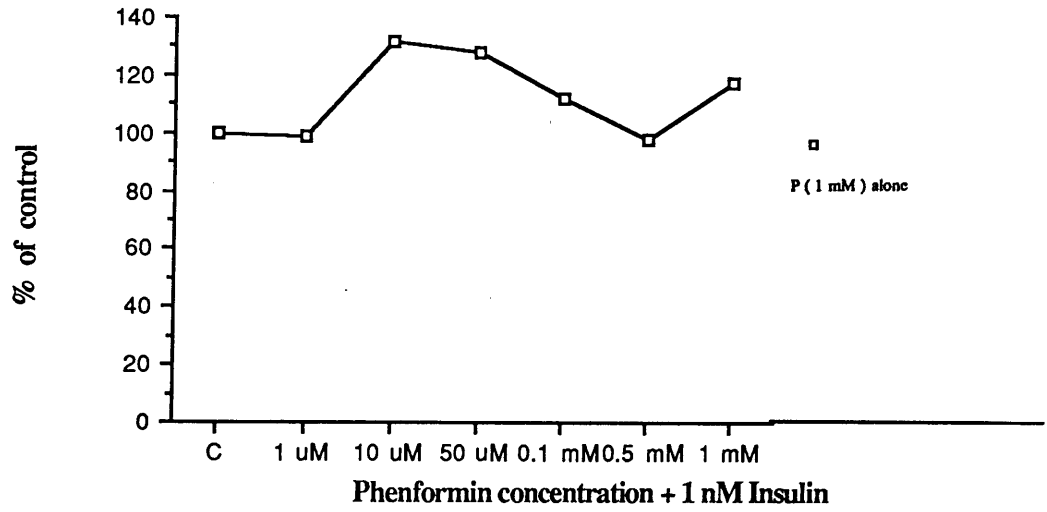
B)



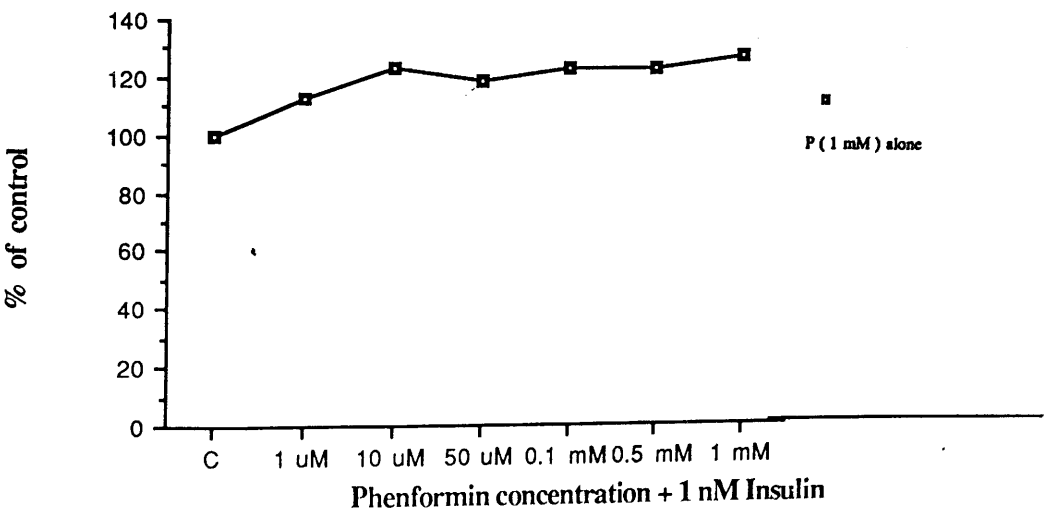
C)


200
180
160
140
120
100
80
60
40
20
0
% of control
P (1 mM) alone
C
1 uM
10 uM
50 uM
0.1 mM
0.5 mM
1 mM
Phenformin concentration + 1 nM Insulin

D)


140
120
100
80
60
40
20
0
% of control
P (1 mM) alone
C
1 uM
10 uM
50 uM
0.1 mM
0.5 mM
1 mM
Phenformin concentration + 1 nM Insulin

E)


140
120
100
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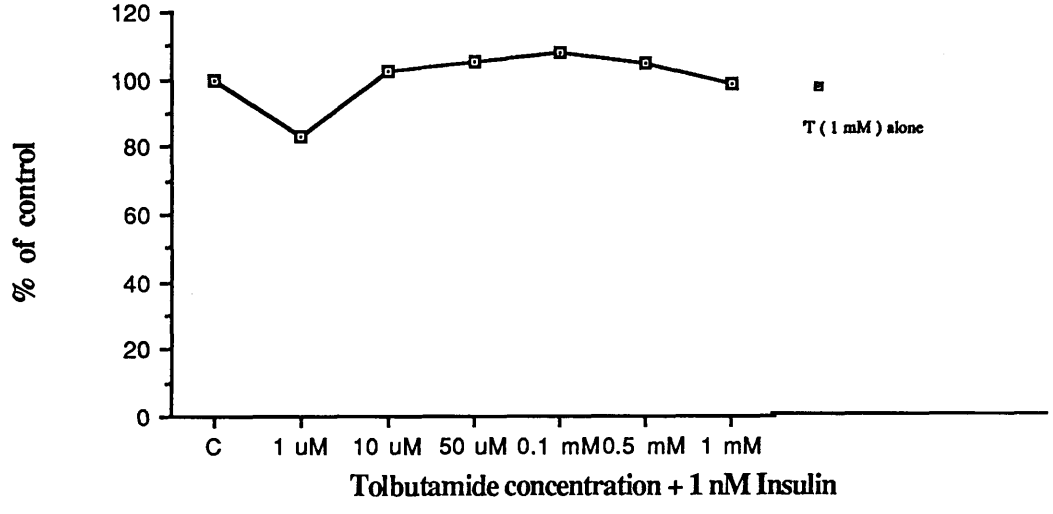
60
40
20
0
% of control
P (1 mM) alone
C
1 uM
10 uM
50 uM
0.1 mM
0.5 mM
1 mM
Phenformin concentration + 1 nM Insulin

Drug concentration	Enzyme activities (pmoles / min / million cells)				
	7α - OHase	6β - OHase	16 α -OHase	17 - OHSD	5 α - reductase
Control	63 ± 4	91 ± 2	72 ± 8	188 ± 9	94 ± 9
1 nM I§ alone	88 ± 5	129 ± 6	126 ± 5	238 ± 5	104 ± 5
1 mM T alone	83 ± 1	119 ± 1	108 ± 3	239 ± 8	108 ± 4
1 uM T + I	73 ± 1 *	113 ± 2 *	126 ± 7	254 ± 4 *	114 ± 2 *
10 uM T + I	91 ± 5	132 ± 2	119 ± 4	244 ± 11	129 ± 6 *
50 uM T + I	93 ± 9	134 ± 4	137 ± 4	297 ± 8 *	163 ± 7 *
0.1 mM T + I	96 ± 3	130 ± 6	141 ± 5 *	282 ± 6 *	157 ± 2 *
0.5 mM T + I	93 ± 4	136 ± 5	135 ± 8	286 ± 8 *	151 ± 8 *
1 mM T + I	88 ± 4	129 ± 6	137 ± 3 *	293 ± 8 *	154 ± 7 *

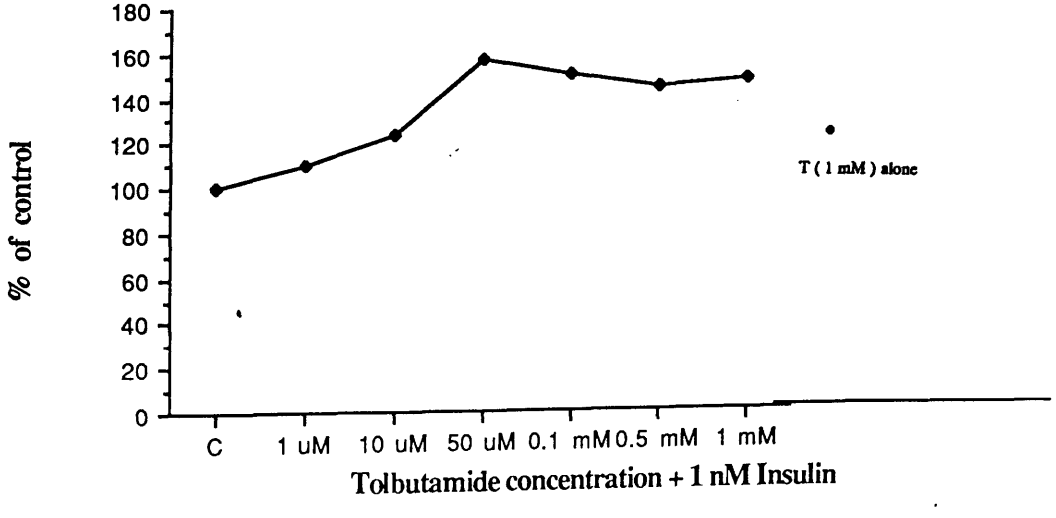
Table 47 . Dose-response effects of 7 α -, 6 β -, 16 α -hydroxylases (OHases), 17-oxosteroid oxidoreductase (OHSD) and 5 α -reductase activities to tolbutamide (T) after 24 hour preincubation followed by another 1/2 hour exposure to 1 nM insulin (I) in hepatocytes obtained from normal male rat. Results expressed as mean ± S.D (N = 3); * $P < 0.05$ with respect to § .

Figure 51 . Dose-response effects of (A) 7 α -hydroxylase [▫], (B) 5 α -reductase [•], (C) 6 β - [▲] and (D) 16 α -hydroxylases [▫] and (E) 17-OHSD [▫] activities to tolbutamide (T) after 24 hour preincubation followed by another 1/2 hour exposure to 1 nM insulin (I) in hepatocytes obtained from normal male rat. Results are expressed as percentage of the relevant control and mean ± S.D (N = 3). Absolute data is given in Table 47 .
C = control (1 nM Insulin alone)

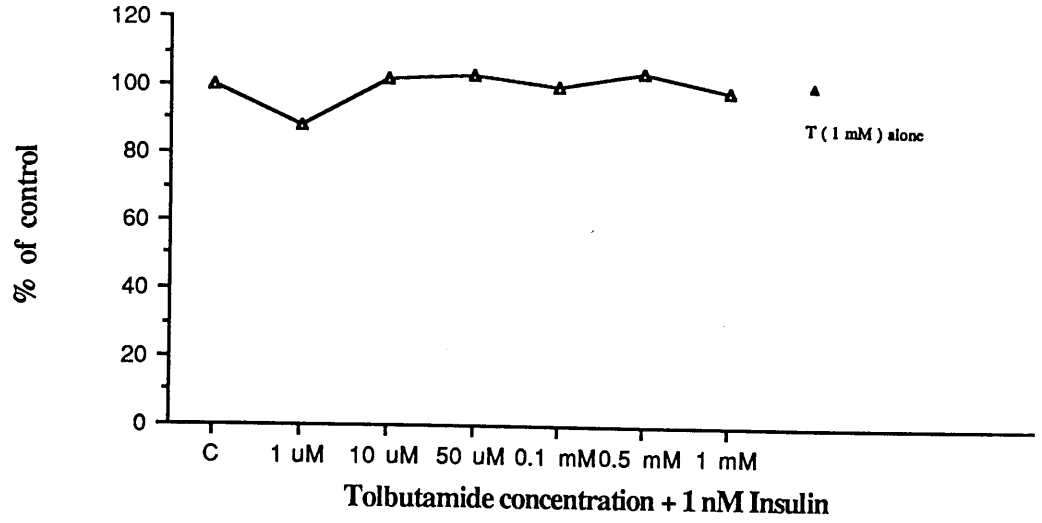
A)



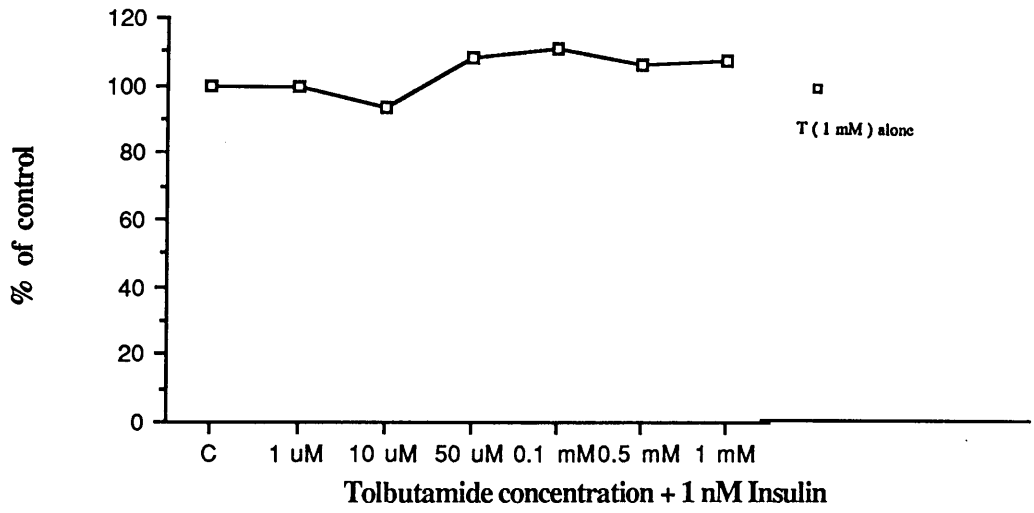
B)



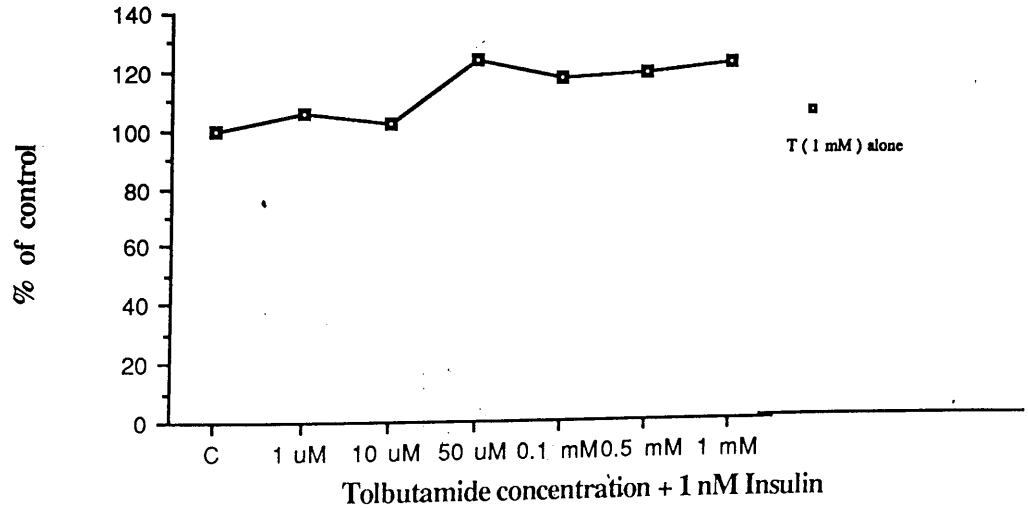
C)


120
100
80
60
40
20
0
% of control
T (1 mM) alone
C
1 uM
10 uM
50 uM
0.1 mM
0.5 mM
1 mM
Tolbutamide concentration + 1 nM Insulin

D)


120
100
80
60
40
20
0
% of control
T (1 mM) alone
C
1 uM
10 uM
50 uM
0.1 mM
0.5 mM
1 mM
Tolbutamide concentration + 1 nM Insulin

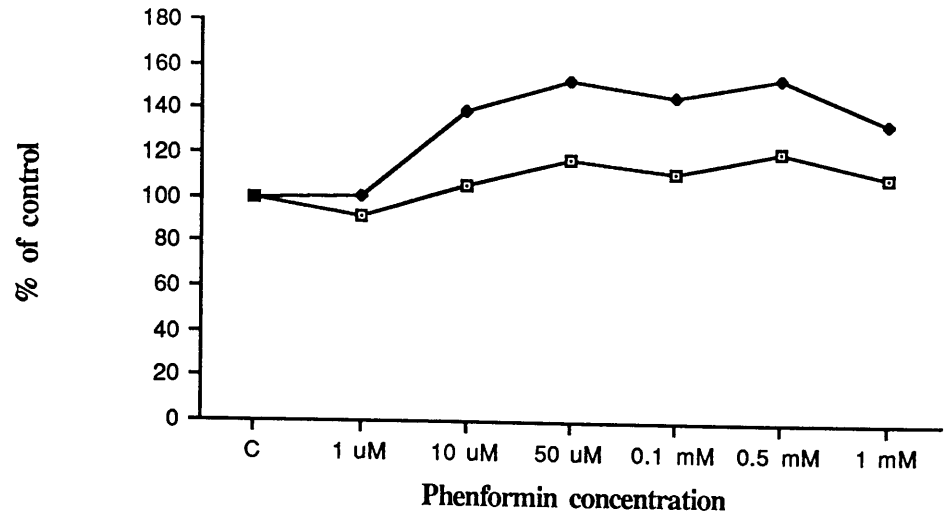
E)


140
120
100
80
60
40
20
0
% of control
T (1 mM) alone
C
1 uM
10 uM
50 uM
0.1 mM
0.5 mM
1 mM
Tolbutamide concentration + 1 nM Insulin

Phenformin concentration	Enzyme activities (pmoles / min / million cells)				
	7α - OHase	6β - OHase	16 α -OHase	17 - OHSD	5 α - reductase
Control	52 ± 4	56 ± 5	67 ± 2	59 ± 2	67 ± 1
1 uM	48 ± 8	55 ± 3	63 ± 4	58 ± 2	68 ± 3
10 uM	55 ± 2 *	74 ± 7 *	77 ± 4 *	65 ± 3 *	94 ± 5 *
50 uM	62 ± 2 *	79 ± 4 *	91 ± 6 *	86 ± 9 *	104 ± 5 *
0.1 mM	69 ± 2 *	74 ± 1 *	78 ± 2 *	94 ± 4 *	99 ± 6 *
0.5 mM	64 ± 3 *	66 ± 4 *	80 ± 3 *	84 ± 2 *	105 ± 8 *
1 mM	58 ± 7	65 ± 3 *	74 ± 4 *	74 ± 3 *	92 ± 3 *

Table 48. Dose-response effects of 7 α -, 6 β -, 16 α -hydroxylases (OHases), 17-oxosteroid oxidoreductase (OHSD) and 5 α -reductase activities to phenformin after 24 hour preincubation in hepatocytes obtained from 3-days STZ-treated diabetic male rat. Results expressed as mean ± S.D (N= 3); * $P < 0.05$ as compared to respective

A)



B)

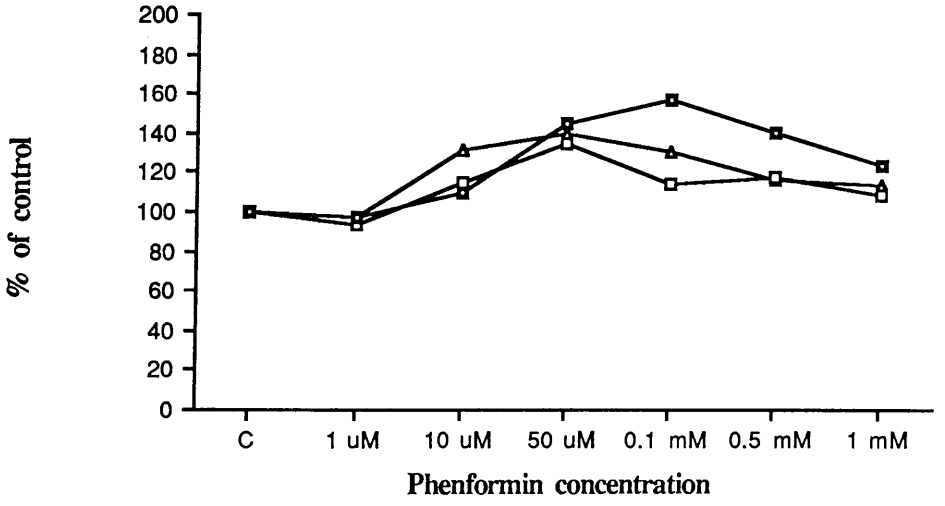


Figure 52 . Dose-response curves of (A) 7 α -hydroxylase [▪] and 5 α -reductase [•] and (B)17-OHSD [▪], 6 β - [▲] and 16 α -hydroxylases [▫] activities to phenformin after 24 hour preincubation in hepatocytes obtained from 3-days STZ-treated diabetic male rat.

Results are expressed as percentage of the relevant control and mean ± S.D (N = 3). Absolute data is given in Table 48 .

C = control

of the enzyme activities. However, the maximum response was much depressed when compared to that in the normal rat hepatocytes (Table 42 and 48). Phenformin significantly increased androst-4-ene-3,17-dione metabolism at concentrations as low as 10^{-5} M and maximum response was observed at 5 x 10^{-5} M (Figure 52). The basal level of all the enzyme activities (except 7α-hydroxylase) were also markedly reduced in the diabetic rat hepatocytes.

With the exception of the 16α-hydroxylase and 17-OHSD, all of the enzymes responded to **tolbutamide** dose-dependently (Table 49). Significant increases in activity could be seen at concentrations as low as 10^{-6} M for 6β-hydroxylase and 5α-reductase and 10^{-5} M for 7α-hydroxylase. Higher concentrations were needed for significant increases in 16α-hydroxylase and 17-OHSD activity (10^{-4} M or 5 x 10^{-4} M respectively). The maximum effect could be seen at 5 x 10^{-5} M tolbutamide (Figure 53).

8.1.2.2 Dose-response curve for insulin in the presence of phenformin or tolbutamide (10^{-3} M)

As shown in Table 50, **phenformin** (10^{-3} M) alone significantly increased all of the enzyme activities and to the same extent as insulin (10^{-9} M) . In the presence of 10^{-3} M phenformin , increasing concentrations of insulin did not increase the 16α-hydroxylase, 17-OHSD or 5α-reductase activities any further except at 10^{-8} M insulin for both 16α-hydroxylase and 17-OHSD (Figure 54). When compared to 1 mM phenformin alone as control, physiological insulin concentrations (10^{-10} and 10^{-9} M) potentiated the effect of 10^{-3} M phenformin on 7α- and 6β-hydroxylase. No potentiation or additive effect was observed at higher insulin concentration.

A similar observation was noted when 10^{-3} M **tolbutamide** was used with

Tolbutamide concentration	Enzyme activities (pmoles / min / million cells)				
	7α - OHase	6β - OHase	16 α -OHase	17 - OHSD	5 α - reductase
Control	37 ± 3	47 ± 4	68 ± 7	83 ± 4	69 ± 2
1 uM	40 ± 3	58 ± 2 *	72 ± 6	82 ± 6	89 ± 3 *
10 uM	54 ± 5 *	68 ± 8 *	71 ± 3	88 ± 3	89 ± 4 *
50 uM	64 ± 2 *	73 ± 4 *	71 ± 4	91 ± 5	96 ± 5 *
0.1 mM	58 ± 6 *	70 ± 2 *	93 ± 5 *	81 ± 5	90 ± 2 *
0.5 mM	62 ± 6 *	72 ± 5 *	77 ± 1 *	94 ± 4 *	93 ± 5 *
1 mM	49 ± 3 *	77 ± 4 *	67 ± 2	94 ± 3 *	83 ± 5 *

Table 49. Dose-response effects of 7 α -, 6 β -, 16 α -hydroxylases (OHases), 17-oxosteroid oxidoreductase (OHSD) and 5 α -reductase activities to tolbutamide after 24 hour preincubation in hepatocytes obtained from 3-days STZ-treated diabetic male rat. Results expressed as mean ± S.D (N = 3); * $P < 0.05$ as compared to respective controls.

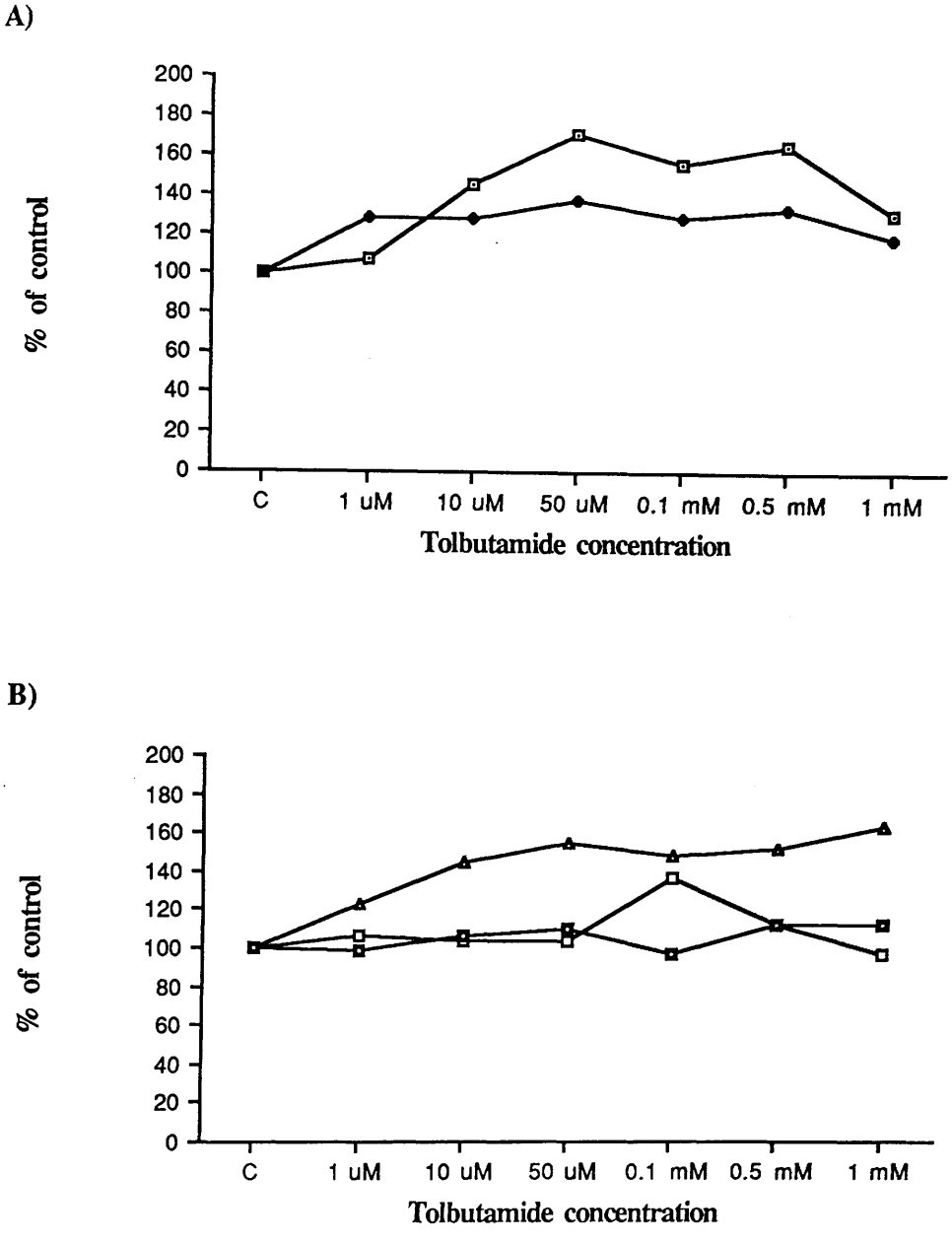


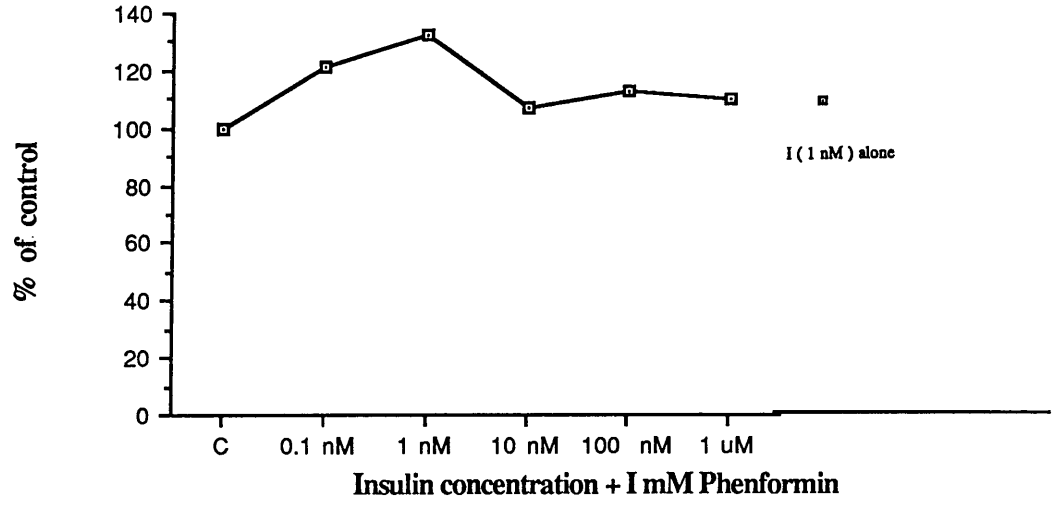
Figure 53 . Dose-response curves of (A) 7 α -hydroxylase [▪] and 5 α -reductase [•] and (B) 17-OHSD [▪], 6 β - [▲] and 16 α -hydroxylases [▫] activities to tolbutamide after 24 hour preincubation in hepatocytes obtained from 3-days STZ-treated diabetic male rat.
Results are expressed as percentage of the relevant control and mean ± S.D (N = 3). Absolute data is given in Table 49 .
C = control

Drug concentration	Enzyme activities (pmoles / min / million cells)				
	7 α - OHase	6 β - OHase	16 α -OHase	17 - OHSD	5 α - reductase
Control	29 ± 2	46 ± 3	40 ± 1	51 ± 2	54 ± 3
1 nM I alone	38 ± 6	57 ± 3	50 ± 4	66 ± 2	67 ± 2
1 mM P alone §	36 ± 2	53 ± 3	46 ± 2	64 ± 1	69 ± 4
0.1 nM I + P	44 ± 2 *	61 ± 2 *	51 ± 2	70 ± 4	71 ± 1
1 nM I + P	48 ± 2 *	61 ± 2 *	52 ± 3	71 ± 5	78 ± 6
10 nM I + P	39 ± 3	62 ± 4	56 ± 4 *	71 ± 2 *	74 ± 2
100 nM I + P	41 ± 2	62 ± 1 *	50 ± 3	64 ± 1	71 ± 3
1 uM I + P	40 ± 2	56 ± 2	48 ± 2	67 ± 2	72 ± 6

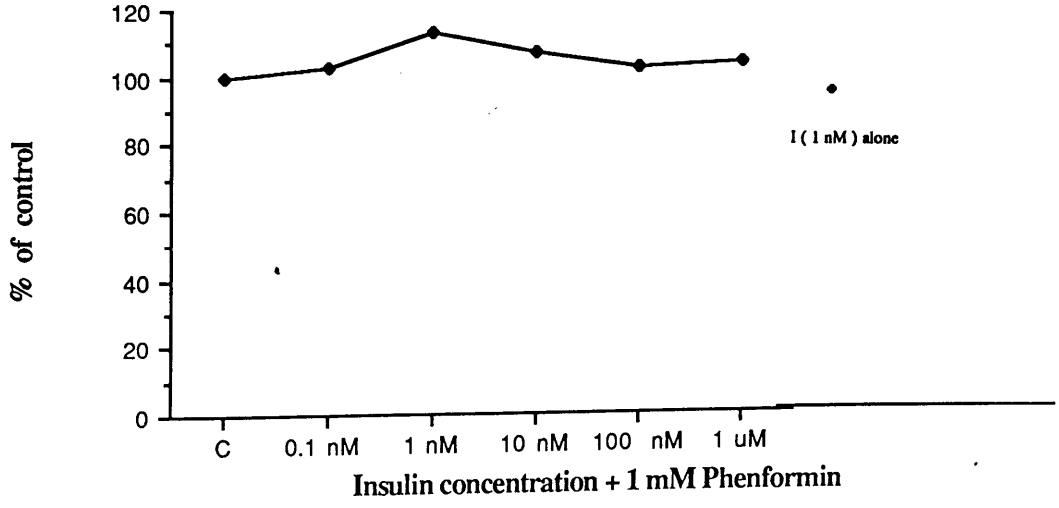
Table 50 . Dose-response effects of 7 α -, 6 β -, 16 α -hydroxylases (OHases), 17-oxosteroid oxidoreductase (OHSD) and 5 α -reductase activities to insulin (I) 1/2 hour preincubation after 24 hour exposure to 1 mM phenformin (P) in hepatocytes obtained from 3-days STZ-treated diabetic male rat. Results expressed as mean ± S.D (N = 3); * $P < 0.05$ with respect to § .

Figure 54 . Dose-response effects of (A) 7 α -hydroxylase [▫], (B) 5 α -reductase [◆], (C) 6 β - [▲] and (D) 16 α -hydroxylases [▫] and (E) 17-OHSD [▪] activities to insulin (I) 1/2 hour preincubation after 24 hour exposure to 1 mM phenformin (P) in hepatocytes obtained from 3-days STZ-treated diabetic male rat.
Results are expressed as percentage of the relevant control and mean ± S.D (N = 3). Absolute data is given in Table 50 .
C = control (1 mM Phenformin alone)

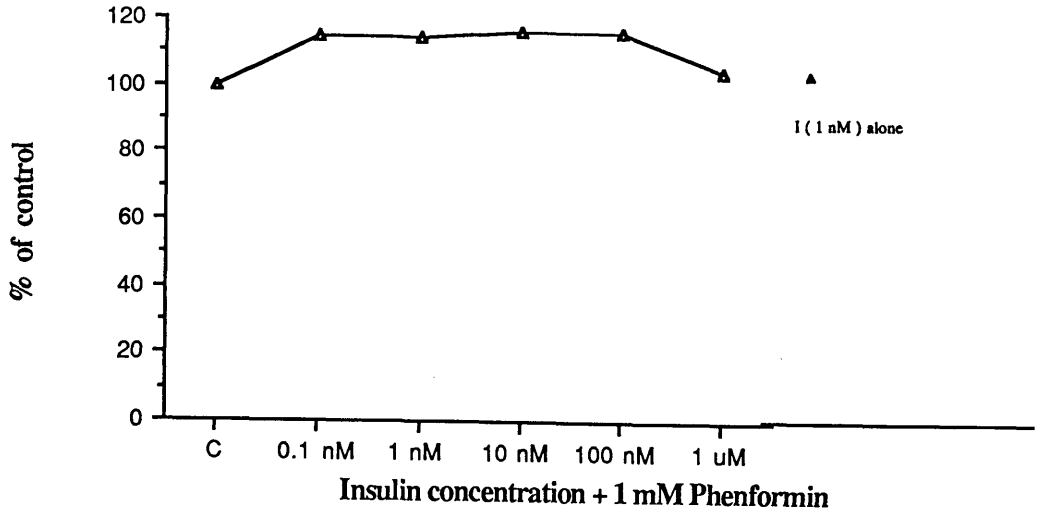
A)



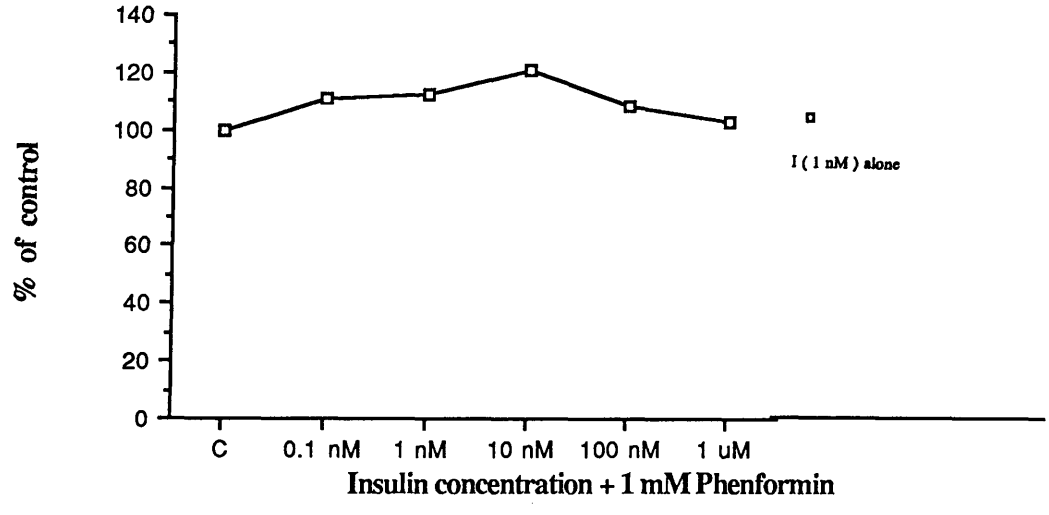
B)



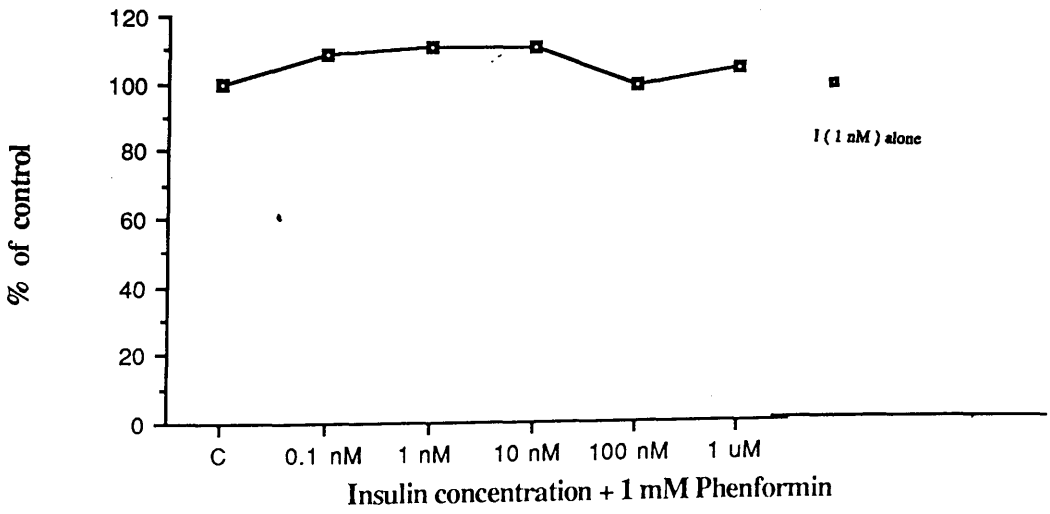
C)


120
100
80
60
40
20
0
% of control
I (1 nM) alone
C
0.1 nM
1 nM
10 nM
100 nM
1 uM
Insulin concentration + 1 mM Phenformin

D)


140
120
100
80
60
40
20
0
% of control
I (1 nM) alone
C
0.1 nM
1 nM
10 nM
100 nM
1 uM
Insulin concentration + 1 mM Phenformin

E)


120
100
80
60
40
20
0
% of control
I (1 nM) alone
C
0.1 nM
1 nM
10 nM
100 nM
1 uM
Insulin concentration + 1 mM Phenformin

increasing insulin concentrations (Table 51). With the exception of 16α-hydroxylase at 10^{-8} and 10^{-7} M insulin addition, no further increases in activity were seen with insulin in the presence of tolbutamide than would be seen with insulin (10^{-9} M) or tolbutamide (10^{-3} M) alone (Figure 55).

8.1.2.3 Dose-response curves for phenformin or tolbutamide in the presence of 10^{-9} M insulin

Insulin (10^{-9} M) or **phenformin** (10^{-3} M) alone significantly increased all of the enzyme activities above control (Table 52). With the exception of 7α-hydroxylase,the effect of 10^{-9} M insulin was significantly enhanced by increasing the concentrations of phenformin (at concentrations as low as 10^{-6} M) when compared to the effect of 10^{-9} M insulin alone. Phenformin is known to elicit lower responses in hepatocytes from diabetic rat than in normal rat (see Section **8.1.2.1**). In the presence of a physiological insulin concentration (10^{-9} M), the effect of phenformin on the enzyme activities was partially or fully restored. Thus, a combination of phenformin and insulin was able to restore some or all of the activity exhibited by phenformin in normal rat hepatocytes (compare between Figures 46, 52 and 56).

A similar observation was noted with **tolbutamide** in the presence of insulin (10^{-9} M). Tolbutamide (10^{-3} M) and insulin (10^{-9} M) both increased the enzyme activities to about the same extent (Table 53). No further increase in 7α- and 16α-hydroxylase or 5α-reductase activities were visible when increasing concentrations of tolbutamide were added to the medium in the presence of 10^{-9} M insulin (Figure 57). Increasing the concentration of tolbutamide (as low as 1 μM for 6β-hydroxylase and 0.1 mM for 17-OHSD) potentiated the effect of insulin when compared to 1 nM insulin alone.

Drug concentration	Enzyme activities (pmoles / min / million cells)				
	7α - OHase	6β - OHase	16 α -OHase	17 - OHSD	5 α - reductase
Control	38 ± 4	72 ± 5	77 ± 5	66 ± 3	89 ± 2
1 nM I alone	51 ± 4	87 ± 4	89 ± 4	92 ± 5	115 ± 4
1 mM T alone §	53 ± 4	96 ± 5	100 ± 2	91 ± 3	113 ± 4
0.1 nM I + T	54 ± 3	93 ± 7	105 ± 3	91 ± 7	113 ± 6
1 nM I + T	59 ± 2	93 ± 5	99 ± 5	84 ± 6	110 ± 3
10 nM I + T	54 ± 6	92 ± 6	90 ± 2 *	83 ± 4	116 ± 3
100 nM I + T	54 ± 3	90 ± 4	86 ± 3 *	86 ± 3	116 ± 2
1 uM I + T	59 ± 3	92 ± 4	95 ± 2	86 ± 4	116 ± 7

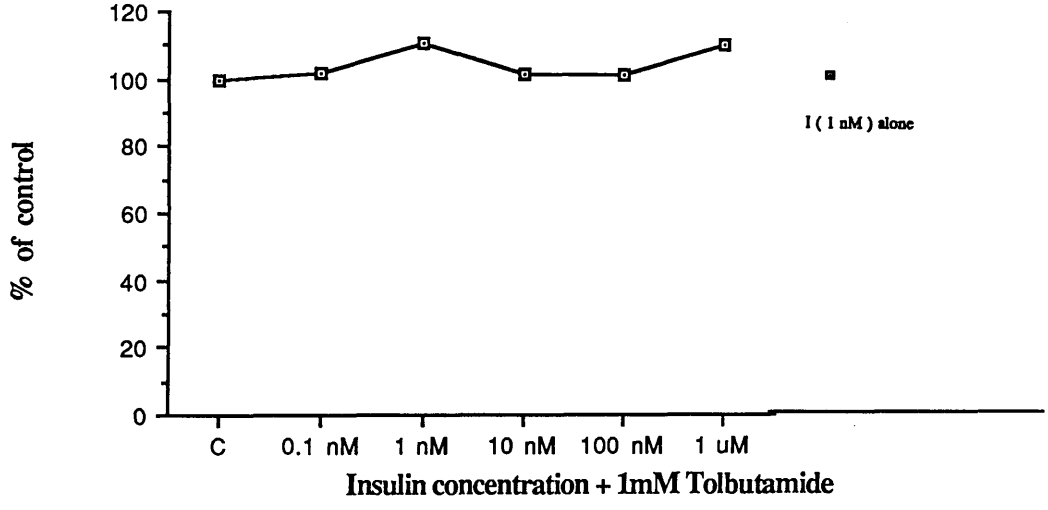
Table 51. Dose-response effects of 7 α -, 6 β -, 16 α -hydroxylases (OHases), 17-oxosteroid oxidoreductase (OHSD) and 5 α -reductase activities to insulin (I) 1/2 hour preincubation after 24 hour exposure to 1 mM tolbutamide (T) in hepatocytes obtained from 3-days STZ-treated diabetic male rat. Results expressed as mean ± S.D (N = 3); * $P < 0.05$ with respect to § .

Figure 55 . Dose-response effects of (A) 7 α -hydroxylase [▫], (B) 5 α -reductase [◆], (C) 6 β - [▲] and (D) 16 α -hydroxylases [▫] and (E) 17-OHSD [▪] activities to insulin (I) 1/2 hour preincubation after 24 hour exposure to 1 mM tolbutamide (T) in hepatocytes obtained from 3-days STZ-treated diabetic male rat.

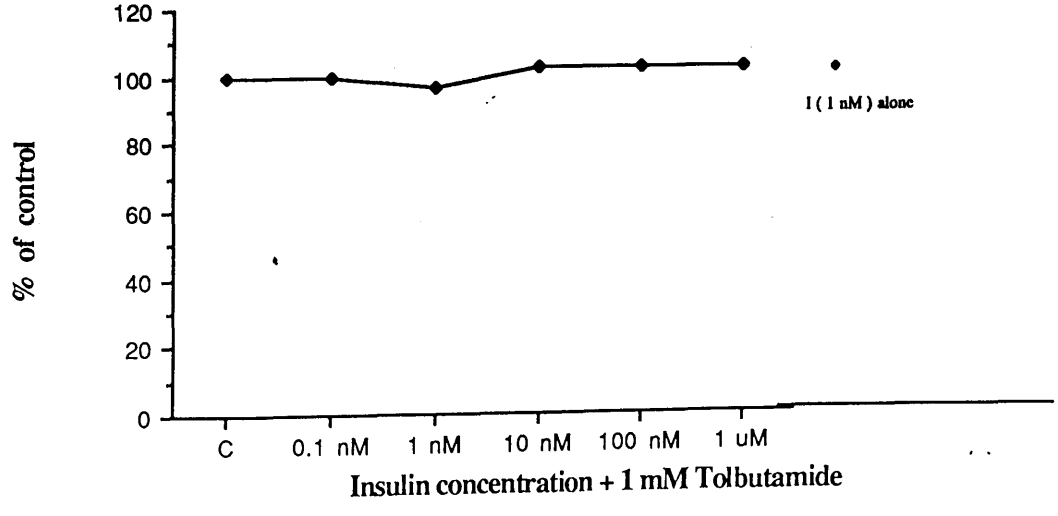
Results are expressed as percentage of the relevant control and mean ± S.D (N = 3). Absolute data is given in Table 51 .

C = control (1 mM Tolbutamide alone)

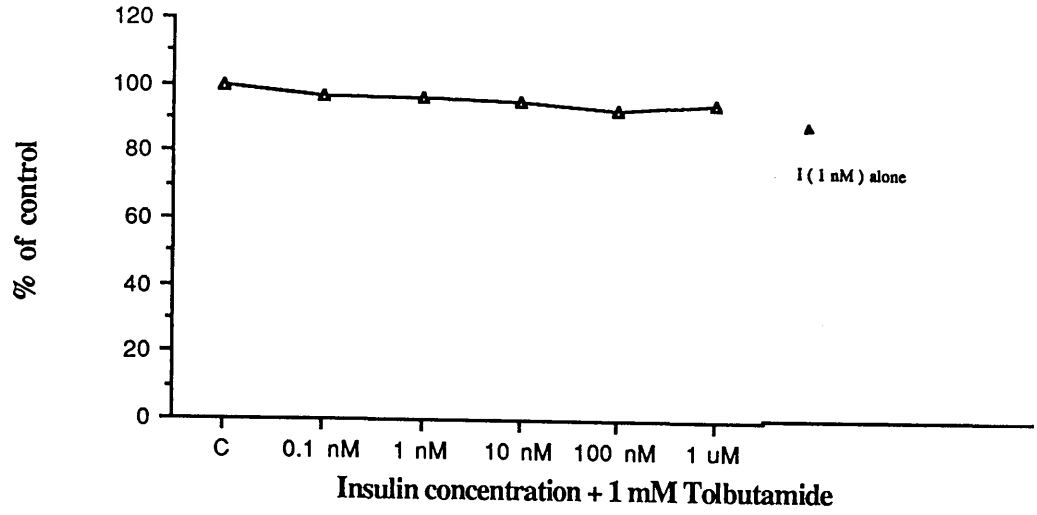
A)



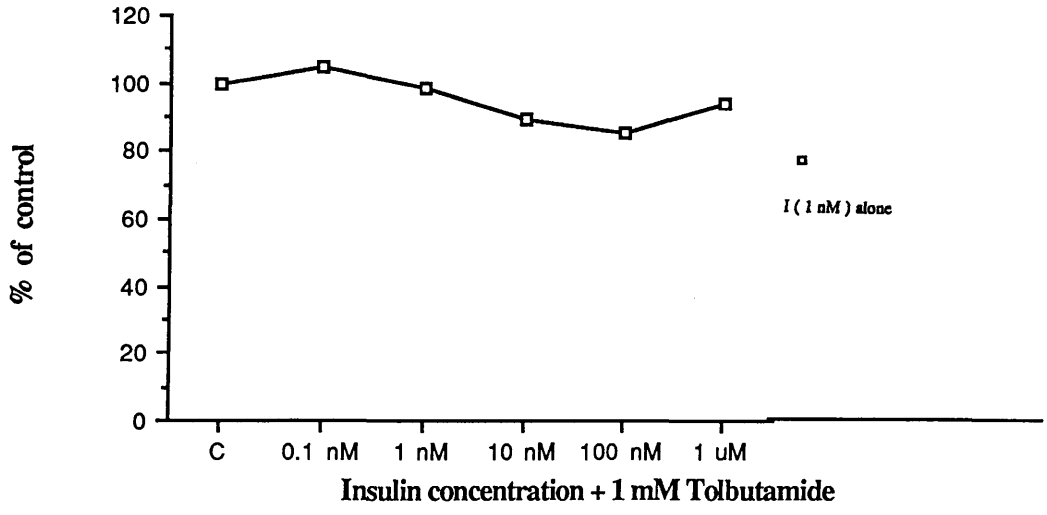
B)



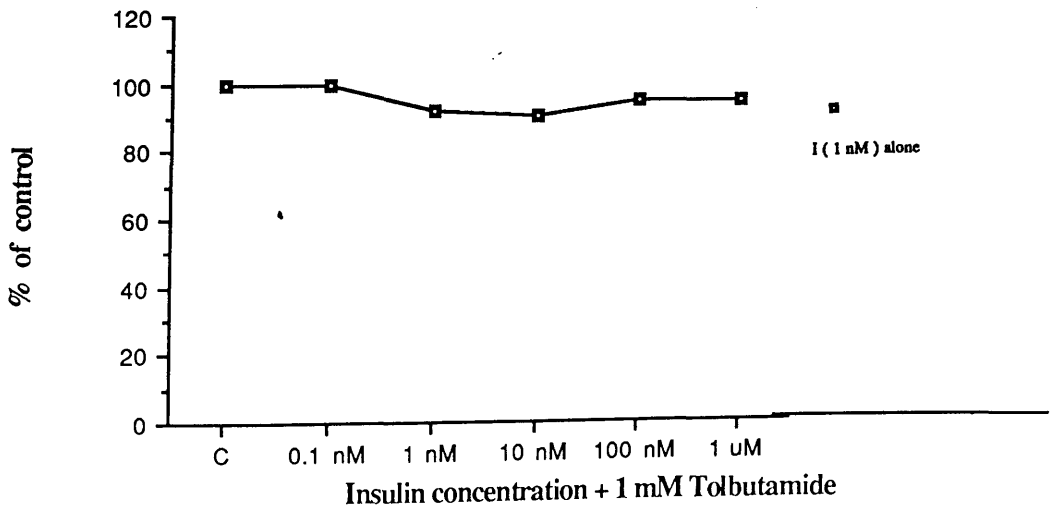
C)


120
100
80
60
40
20
0
% of control
I (1 nM) alone
C
0.1 nM
1 nM
10 nM
100 nM
1 uM
Insulin concentration + 1 mM Tolbutamide

D)


120
100
80
60
40
20
0
% of control
I (1 nM) alone
C
0.1 nM
1 nM
10 nM
100 nM
1 uM
Insulin concentration + 1 mM Tolbutamide

E)

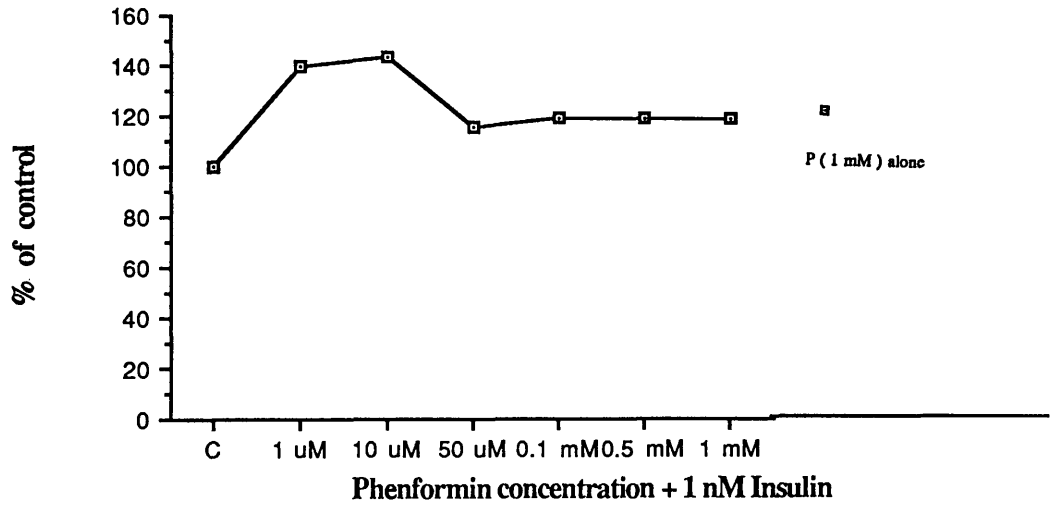

120
100
80
60
40
20
0
% of control
I (1 nM) alone
C
0.1 nM
1 nM
10 nM
100 nM
1 uM
Insulin concentration + 1 mM Tolbutamide

Drug concentration	Enzyme activities (pmoles / min / million cells)				
	7 α - OHase	6 β - OHase	16 α -OHase	17 - OHSD	5 α - reductase
Control	21 ± 3	28 ± 1	45 ± 4	45 ± 2	49 ± 6
1 nM I alone §	25 ± 3	33 ± 1	53 ± 3	60 ± 5	62 ± 6
1 mM P alone	30 ± 4	44 ± 4	70 ± 5	79 ± 4	73 ± 3
1 uM P + I	35 ± 2 *	49 ± 3 *	75 ± 2 *	79 ± 3 *	77 ± 2 *
10 uM P + I	36 ± 6	45 ± 3 *	76 ± 3 *	78 ± 3 *	76 ± 4 *
50 uM P + I	29 ± 2	40 ± 3 *	76 ± 4 *	70 ± 6	79 ± 1 *
0.1 mM P + I	30 ± 1	40 ± 1 *	75 ± 1 *	77 ± 2 *	82 ± 5 *
0.5 mM P + I	30 ± 2	41 ± 5	79 ± 5 *	84 ± 3 *	81 ± 7 *
1 mM P + I	30 ± 2	40 ± 4	76 ± 6 *	71 ± 5	83 ± 5 *

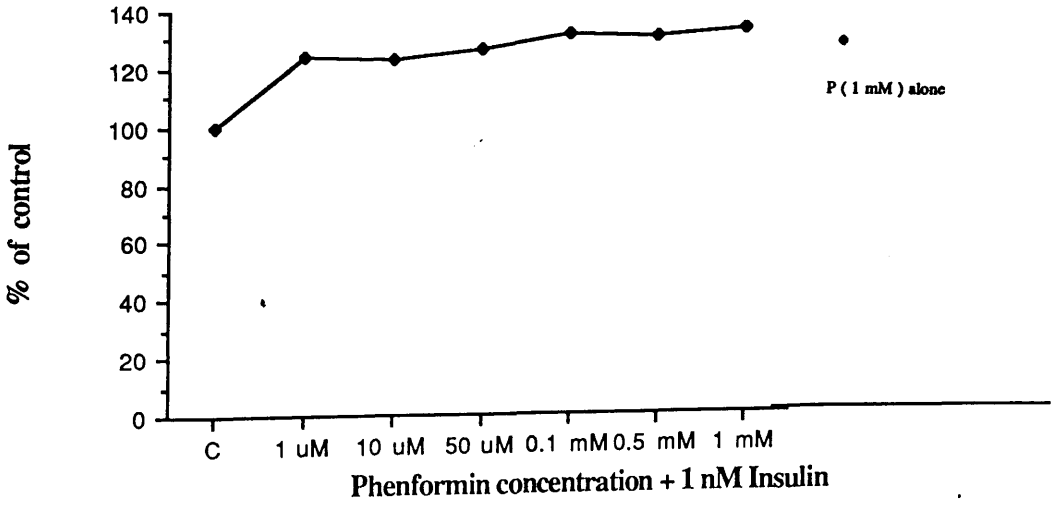
Table 52 . Dose-response effects of 7 α -, 6 β -, 16 α -hydroxylases (OHases), 17-oxosteroid oxidoreductase (OHSD) and 5 α -reductase activities to phenformin (P) after 24 hour preincubation followed by another 1/2 hour exposure to 1 nM insulin (I) in hepatocytes obtained from 3-days STZ-treated diabetic male rat. Results expressed as mean ± S.D (N = 3); * $P < 0.05$ with respect to § .

Figure 56 . Dose-response effects of (A) 7 α -hydroxylase [▫], (B) 5 α -reductase [•], (C) 6 β - [▲] and (D) 16 α -hydroxylases [▫] and (E) 17-OHSD [▪] activities to phenformin (P) after 24 hour preincubation followed by another 1/2 hour exposure to 1 nM insulin (I) in hepatocytes obtained from 3-days STZ-treated diabetic male rat.
Results are expressed as percentage of the relevant control and mean ± S.D (N = 3). Absolute data is given in Table 52 .
C = control (1 nM Insulin alone)

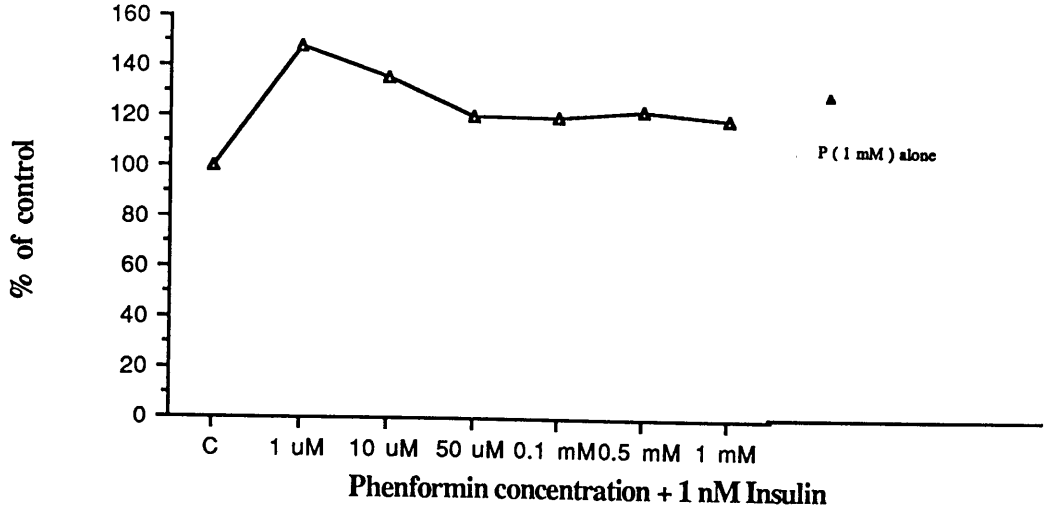
A)



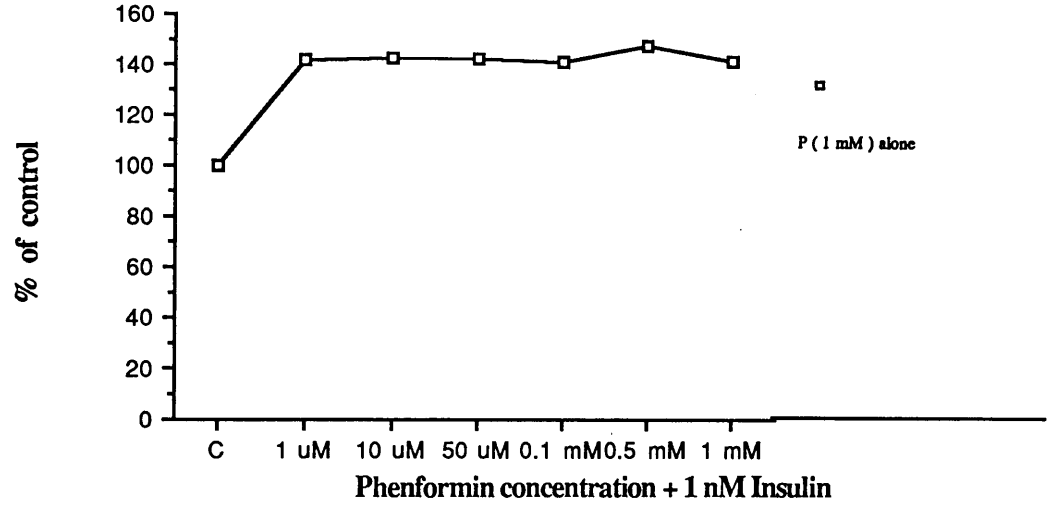
B)



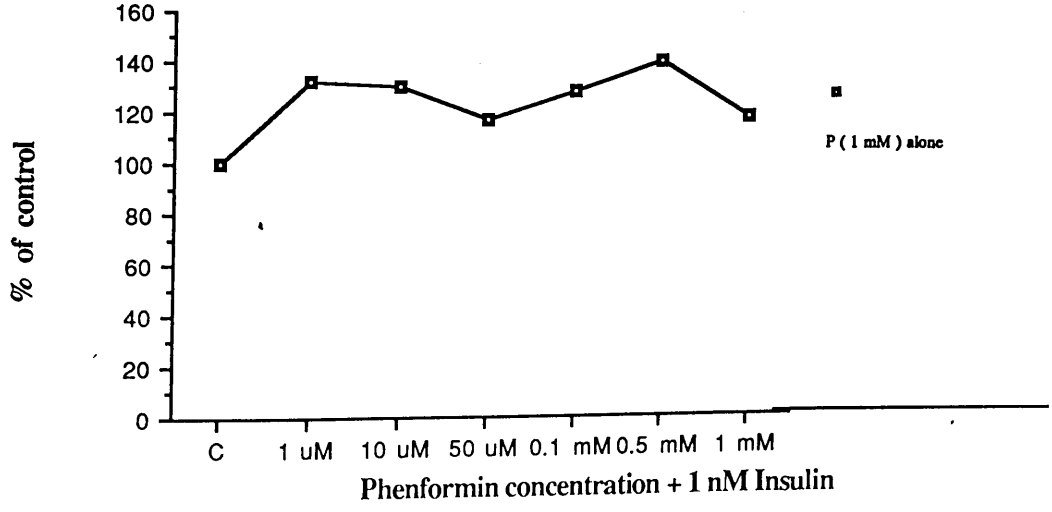
C)


160
140
120
100
80
60
40
20
0
% of control
P (1 mM) alone
C
1 uM
10 uM
50 uM
0.1 mM
0.5 mM
1 mM
Phenformin concentration + 1 nM Insulin

D)


160
140
120
100
80
60
40
20
0
% of control
P (1 mM) alone
C
1 uM
10 uM
50 uM
0.1 mM
0.5 mM
1 mM
Phenformin concentration + 1 nM Insulin

E)

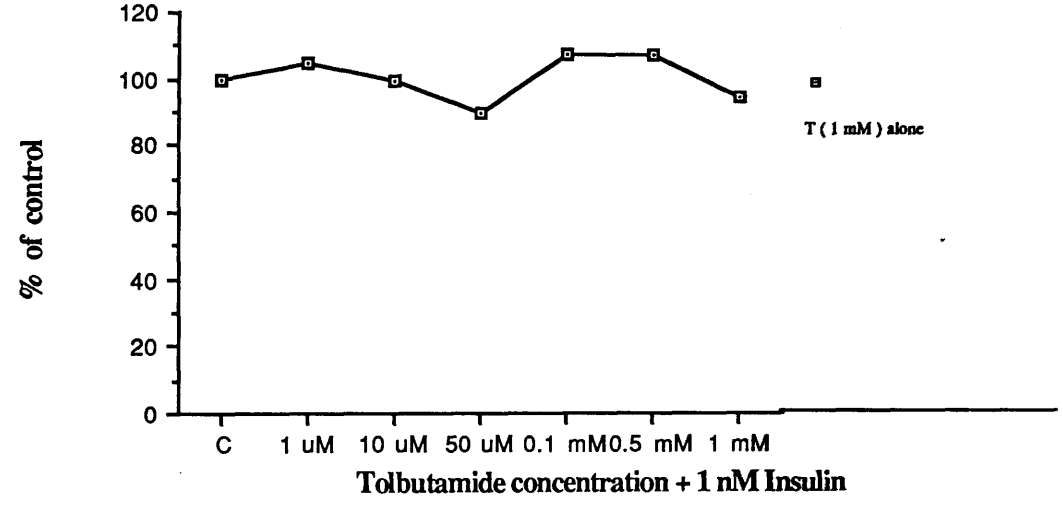

160
140
120
100
80
60
40
20
0
% of control
P (1 mM) alone
C
1 uM
10 uM
50 uM
0.1 mM
0.5 mM
1 mM
Phenformin concentration + 1 nM Insulin

Drug concentration	Enzyme activities (pmoles / min / million cells)				
	7α - OHase	6β - OHase	16 α -OHase	17 - OHSD	5 α - reductase
Control	25 ± 3	29 ± 1	20 ± 1	128 ± 6	46 ± 9
1 nM I alone §	40 ± 5	33 ± 3	31 ± 1	164 ± 8	70 ± 5
1 mM T alone	40 ± 1	47 ± 0	32 ± 1	168 ± 4	69 ± 6
1 uM T + I	42 ± 2	43 ± 1 *	31 ± 1	175 ± 2	79 ± 4
10 uM T + I	40 ± 5	50 ± 5 *	31 ± 3	182 ± 7	74 ± 3
50 uM T + I	36 ± 2	38 ± 2	29 ± 4	164 ± 7	72 ± 3
0.1 mM T + I	43 ± 4	54 ± 6 *	33 ± 5	193 ± 5 *	81 ± 7
0.5 mM T + I	43 ± 4	49 ± 2 *	33 ± 2	183 ± 5 *	79 ± 8
1 mM T + I	38 ± 1	42 ± 0 *	31 ± 4	184 ± 3 *	77 ± 5

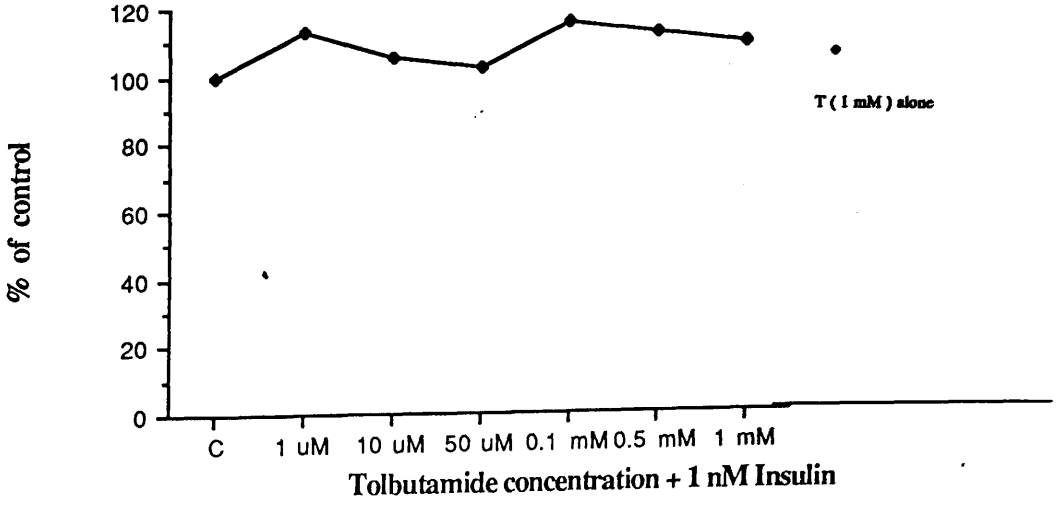
Table 53 . Dose-response effects of 7 α -, 6 β -, 16 α -hydroxylases (OHases), 17-oxosteroid oxidoreductase (OHSD) and 5 α -reductase activities to tolbutamide (T) after 24 hour preincubation followed by another 1/2 hour exposure to 1 nM insulin (I) in hepatocytes obtained from 3-days STZ-treated diabetic male rat. Results expressed as mean ± S.D (N = 3); * $P < 0.05$ with respect to § .

Figure 57 . Dose-response effects of (A) 7 α -hydroxylase [▫], (B) 5 α -reductase [•], (C) 6 β - [▲] and (D) 16 α -hydroxylases [▫] and (E) 17-OHSD [▫] activities to tolbutamide (T) after 24 hour preincubation followed by another 1/2 hour exposure to 1 nM insulin (I) in hepatocytes obtained from 3-days STZ-treated diabetic male rat.
Results are expressed as percentage of the relevant control and mean ± S.D (N = 3). Absolute data is given in Table 53 .
C = control (1 nM Insulin alone)

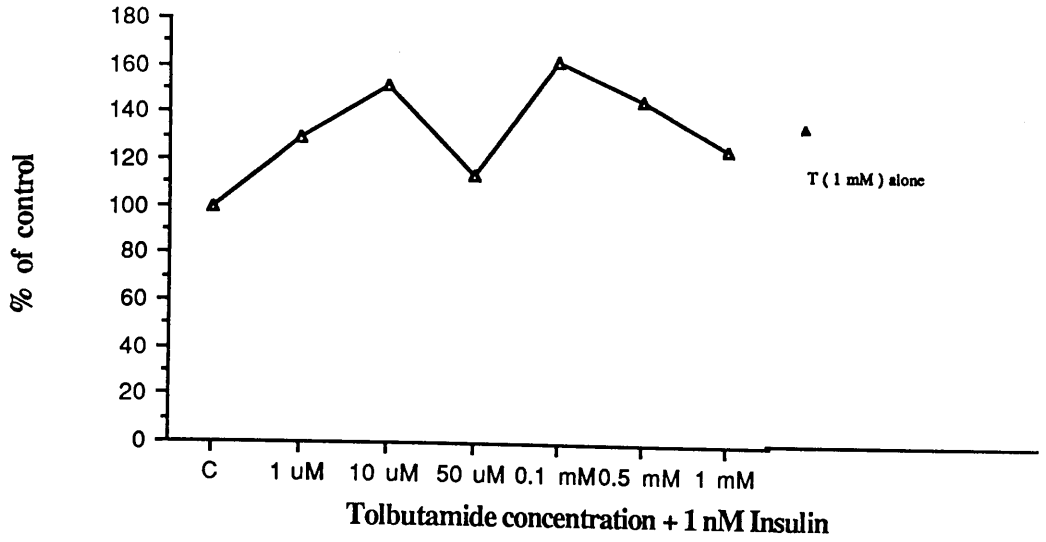
A)



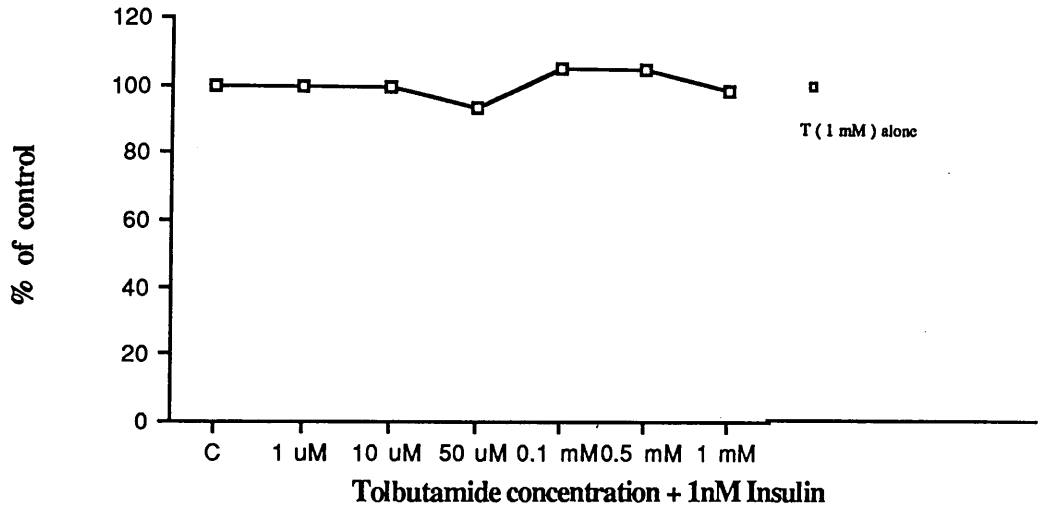
B)



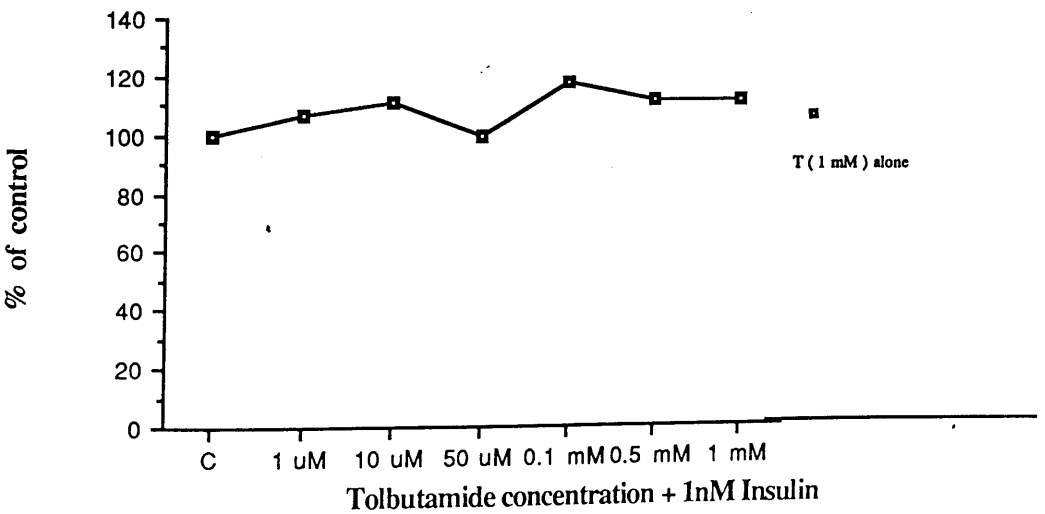
C)


180
160
140
120
100
80
60
40
20
0
% of control
T (1 mM) alone
C
1 uM
10 uM
50 uM
0.1 mM
0.5 mM
1 mM
Tolbutamide concentration + 1 nM Insulin

D)


120
100
80
60
40
20
0
% of control
T (1 mM) alone
C
1 uM
10 uM
50 uM
0.1 mM
0.5 mM
1 mM
Tolbutamide concentration + 1nM Insulin

E)


140
120
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% of control
T (1 mM) alone
C
1 uM
10 uM
50 uM
0.1 mM
0.5 mM
1 mM
Tolbutamide concentration + 1nM Insulin

DISCUSSION

9.0 DEVELOPMENT AND CHARACTERIZATION OF A HORMONE-FREE HEPATOCYTE CULTURE SYSTEM

The biotransformation of a wide variety of endogenous and exogenous compounds such as drugs, hormones, chemical carcinogens and mutagens in the body is affected by many factors ranging from environmental pollutants to the pathological state of the body (Gibson and Skett, 1986). One major mechanism by which the body regulates the biotransformation of exogenous compounds is by hormonal control. Sex-dependent hepatic metabolism of steroids and xenobiotics in rats is now well recognized. Studies on the metabolism of 5α-androstane-3α,17β-diol (Berg and Gustafsson, 1973) androst-4-ene-3,17-dione, 4-pregnene-3,20-dione and 7α-hydroxy-4-cholesten-3-one (Skett, 1986; Einarsson et.al., 1973) all show the existence of sex-related differences. Sexual dimorphism is also shown in the field of drug metabolism. Such sex differences in hepatic metabolism of drugs have been shown for lidocaine and imipramine (Skett et.al., 1980), aminopyrine, antipyrine and hexobarbitone (Quinn et.al., 1958). These sex-specific differences in hepatic metabolism have been shown to be related to the steroid environment in the body during development. It was initially thought that, at least in the rat, that sex differences in xenobiotic metabolism was maintained by androgens. Later studies indicated that neonatal exposure to androgens is responsible for the appearance of sex differences in xenobiotic and steroid metabolism at puberty in the rat (for a review, see Skett and Gustafsson, 1979). Hypophysectomy of male and female animals abolished the sex differences in steroid metabolism (Gustafsson and Stenberg, 1974) and treatment of the hypophysectomized animals with androgens does not restore the condition to normal (Gustafsson and Stenberg, 1976).The emergence of the hypothalamo-pituitary axis hypothesis, forwarded by Gustafsson and co-workers (Gustafsson et.al., 1983), in the regulation of sex differences in hepatic steroid and

xenobiotic metabolism, has provided a different approach to this area of research. Denef (1974) suggested that the sex difference in the rat resided in a secretion from the pituitary gland and this pituitary factor, termed ' feminizing factor ', was later identified to be the growth hormone (Skett et.al., 1978). The sex differences in xenobiotic metabolism are thought to be due to the differences in the secretory patterns of growth hormone in the adult female and male rat (Skett, 1987). This is substantiated by the fact that treatment of rats with growth hormone by intermittent injection to mimic the male pattern and by minipump to mimic the female pattern does, indeed, account for the sex differences in drug and steroid metabolism in the rat liver.

The effect of diabetes mellitus on hepatic drug and steroid metabolism is well recognized and has been shown to be sex-dependent (Dixon et.al., 1961; Skett, 1986). It was originally thought that diabetes in some way interfered with the androgenic stimulation of drug metabolism in the male and, thus, exerted its sex-dependent effect. Indeed, it was found that diabetes interferes with the production of testosterone in the male rat (Murray et.al., 1981) and testosterone can reverse the effect of diabetes on drug metabolism (Skett et.al., 1984). However, it was confirmed by the latter group that there is a complete lack of correlation between androgens and drug metabolism in STZ-treated animals. Skett and Gustafsson (1979) have shown that androgens do not exert a direct effect on the liver but have an indirect action via the hypothalamo-pituitary axis. It is also known that the diabetic state can greatly influence growth hormone secretory patterns in the rat (Tannenbaum, 1981) and STZ-diabetes induces different neuroendocrine and morphological alterations in the hypothalamo-pituitary axis of male and female rats (Bestetti et.al., 1985). Diabetes, thus, could affect drug metabolism by its action on testosterone secretion. Another possible mechanism is by a direct effect of insulin on the liver since insulin is the principal hormone affected in the diabetic condition. Therefore, a suitable method or system is needed that would enable us to study

the direct action of insulin in the rat liver.

The hormonal control of xenobiotic metabolism represents a complex and intricate system whereby interaction of hormones are always involved. Due to this complex interactive system , it is not possible to evaluate or assess the effect of one particular hormone in an intact animal without changing the secretion of other hormones, thus making it impossible to ascribe a certain functional effect to that particular hormone. This project aims at dissecting that intricate hormonal system by testing the effect of two pancreatic hormones, glucagon and insulin, in an *in-vitro* animal model system cultured in a well defined hormone- and serum-free medium.

Mixed-function monooxygenase enzymes function not only in the metabolism of drugs, but also in the metabolic conversion of a wide variety of endogenous substrates, such as steroid hormones, fatty acids, cholesterol, and vitamin D (Conney and Kuntzman, 1971). The breakdown of steroids as well as drugs by the liver and other tissues are, to a great extent, dependent on the mixed-function oxidase system.

Previous studies using rat liver microsomes, have indicated that diabetes mellitus can affect steroid metabolism in the rat and this effect can be reversed by insulin administration to the diabetic animals (Skett, 1986). As steroid metabolism is closely related to drug metabolism in the rat liver by the enzymes involved and by an apparent common control mechanism (Skett et al., 1984), it was of interest to study the effect of insulin on androst-4-ene-3,17-dione metabolism in primary cultures of rat hepatocytes. The culture medium employed enabled us to examine the effect of insulin alone without the interference of other hormones. The biological effects of insulin vary in their time of onset from very rapid responses of enzymes (seconds to minutes) to quite slow modulations of cell proliferation that may take many hours to be expressed. Therefore in our protocol , we have conducted the experiments ranging from 1/2 hour to 72 hours. Only male rats were used in all the experiments because previous study had shown that

the effect of diabetes mellitus on steroid metabolism is sex-dependent i.e. only seen in the male and unaffected by the same treatment in the female (Skett, 1986).

Early studies on drug metabolism and the effect of diabetes have mainly involved the use of microsomal preparations of the animal tissues (Dixon et.al., 1961; Dixon et.al., 1963; Kato and Gillette, 1965b). Although microsomes are easy to prepare and can be stored for weeks under optimal conditions with minimal loss of enzyme activities, they suffer from a non-physiological nature with a loss of the tissue interaction as well as cell integrity. Alternative studies of drug metabolism were carried out in both hepatic and nonhepatic tissues in culture. Alfred and Gelboin (1967) first demonstrated that benzpyrene hydroxylase activity was induced by polycyclic hydrocarbons in hamster embryonic cells grown *in-vitro*. However, because of the relatively limited drug-metabolizing capacity of these easily cultured mesenchymal tissues, other methods were sought. Later, isolated liver perfusion was used in drug metabolism studies where drugs were administered *in-situ*. Although a good correlation was found to exist between the drug metabolism *in-vivo* (Bickel and Minder, 1970) and in the perfused liver, the length of time that the isolated liver will continue to perform its normal metabolic functions is a question of obvious concern (Kesters and Lambotte, 1973). Moreover, only one or two preparations may be run at one time, the setting up of the isolated perfused liver requires a high degree of skill and they can only be kept viable for a few hours. This makes this method unsuitable for long-term experiments on the effects of hormones on steroid metabolism Thus, newer systems were explored. *In-vitro* preparations of whole cells were used as alternatives and these include tissue slices, isolated hepatocytes and cells in culture. At present the most promising of these systems in the mammal is the hepatocyte in culture.

The development of techniques for the isolation and cultivation of hepatocytes from adult rats provided a useful intermediate model system for studies on hepatic

function and differentiation. Although tissue integrity is destroyed and, thus, the potentially important cell-cell contacts, the cellular structure is maintained (for comparison of different liver preparations used in assessing drug metabolism, refer to Table 54). Many studies have demonstrated the short-term maintenance and induction of microsomal cytochromes and / or monooxygenase activities by various xenobiotics and cofactors (Michalopoulos et.al., 1976a) in primary cultures of hepatocytes prepared from normal or regenerated adult rat liver (Sirica and Pitot, 1980). Monolayer cultures of rat hepatocytes have also been shown to be responsive to hormones like insulin and glucagon and to demonstrate several major metabolic functions characteristic of liver *in-vivo* (Bissell et.al., 1973).

The rapid loss of cytochrome P-450 in culture is a major problem, however, and attempts to maintain the concentration of this protein at initial or *in-vivo* levels has till now remained elusive. Many methods have been tried to prevent the loss of cytochromes including modifications of the culture medium, such as the addition of hormones (Dich et.al., 1988), nicotinamide or other pyridines (Paine et.al., 1979), or omission of certain amino acids (Paine et.al., 1982) or selenium and other trace elements (Engelmann et.al., 1985). None of these, however, have succeeded in more than partly preventing the decline. Earlier attempts to extend the lifespan of primary cultures of hepatocytes included coculturing of hepatocytes with a rat liver epithelial cell line (Begue et.al., 1984) and culturing on an extracellular matrix (Michalopoulos et.al., 1976b; Rojkind et.al., 1980). Numerous modifications of the culture medium have been formulated to attempt to retain the *in-vivo* levels of cytochrome P-450 and in most cultivation techniques for hepatocytes from adult rats, media with high concentrations of rat or calf serum or hormones are used (Bissell et.al., 1973; Stenberg et.al., 1978; Varandani et.al., 1985). Despite its drawback in lacking surface contact and rapid loss of cytochrome P-450 *in-vitro,* we have decided to use hepatocytes in our studies because

Table 54 . Comparison of liver preparations in assessing drug metabolism

Method	**Degree of difficulty**	***In-vivo* relevance**	**Reproducibility**
Perfused liver	High	Good	Poor
Liver slices	Moderate	Fair	Fair
Liver cubes	Moderate	Fair	Fair
Liver cells	Moderate	Fair	Fair
Subcellular fraction	Low	Fair-poor	Good

[Taken from Gibson and Skett (1986), Introduction to Drug Metabolism, Chapman and Hall, pp 217]

they can be obtained in high yields allowing us to conduct more experiments and long term hormonal studies.

The initial purpose of the present investigation has been to develop a simple technique for the cultivation of rat hepatocytes under maximally controlled conditions and thus to make it possible to study hormonal influences on hepatic steroid metabolism. Androst-4-ene-3,17-dione was chosen as a substrate because it has a sex-dependent pathway of metabolism which is well characterized (Stenberg, 1976) and also because it has commonly been used in our laboratory. Its metabolites are well defined and are easily separated. Multiple enzymes have been identified to be involved in its metabolism and they have been shown to consist of cytochrome P-450-dependent and cytochrome P-450-independent enzymes. The enzymes involved in its metabolism have been shown to be sexually-specific i.e. male- and female-specific. Moreover, insulin has been demonstrated to affect its metabolism *in-vivo* (Skett, 1986) and its labelled and unlabelled forms are easily available. All these criteria indicate that androst-4-ene-3,17-dione is an excellent substrate to be used in the study of hormonal regulation of steroid metabolism in isolated hepatocytes from the rat liver.

Initially, we developed a system whereby primary cultures of hepatocytes were maintained in Ham's F-10 culture medium supplemented with 2.5 % foetal calf serum and 15 % horse serum. The purpose of culturing the cells in a medium with serum supplements was to test the sensitivity of the system i.e. whether the steroid metabolizing enzyme activities are seen in the cell culture system or not. After 3 days in culture, all of the enzymes in the **male** rats showed a decrease in activity to less than half the values measured in the freshly prepared cells (Table 1 -top panel). Our data are consistent with previously reported work where Stenberg et.al. (1978) had reported a reduction of 16α-hydroxylase and 5α-reductase activities to less than 50 % of initial values on day 3 when the liver cells were cultured in a medium containing 1 % rat serum. In the **female**

rat, selective alterations in enzyme activities were seen (Table 2 -top panel). The activity of the female-specific enzymes, 7α-hydroxylase and 5α-reductase were selectively reduced while the activities of the male-specific enzymes, 6β-hydroxylase and 17-OHSD were significantly increased. 16α-hydroxylase activity was not altered. The difference seen could possibly be attributed to the marked alteration in hormonal environment of the cells in culture compared to *in-vivo*. In particular, growth hormone has been postulated to be a 'feminizing factor' and in the intact animal it maintains the female-type metabolism in the liver (Gustafsson et.al., 1983; Skett, 1987). The decrease in the female-specific and increase in the male-specific enzyme activities in the female rat is in agreement with this hypothesis since the influence of growth hormone was removed *in-vitro*. The maintenance of the 16α-hydroxylase activity is, however, inconsistent with this theory and alternative control mechanisms may have to be sought.

After six days in culture, the female-specific enzyme activities (7α-hydroxylase and 5α-reductase) in the male rat were restored to 90 % and 80 % of control respectively. Of the male-specific enzymes, only 17-OHSD activity returned to control level while the 6β- and 16α-hydroxylase activities only improved slightly. In the female rat, all of the enzyme activities except 5α-reductase, exceeded the activities of the freshly prepared cells. It is interesting to note that a recent study has reported an analogous observation on the cytochrome P-450 content in male and female rat hepatocytes (Vind et.al., 1988); in hepatocytes from female rat, the cytochrome P-450 content decreased by 65 % at day 3 of culture but addition of 1 μM dexamethasone or 1 mM phenobarbital gave rise to an induction of cytochrome P-450 (285 %) which was maintained for 3 weeks. In hepatocytes from male rat, however, the cytochrome P-450 content decreased by 45 % at day 3 of culture and the decrease continued irrespective of the presence of dexamethasone and/or phenobarbital. Could the observed alteration in the steroid enzyme activities seen in our study be related to the changes in the cytochrome P-450 isoenzymes concentration ? This remains to be investigated.

Since the foetal calf and horse serum used contained indefinite quantities of various hormones, growth factors and peptides, it was impossible to attribute the maintenance of the steroid metabolizing enzyme activities to any of the serum constituents. We therefore replaced the serum with 2 % Ultroser G, a multihormone serum substitute. The culture medium used was, therefore, well-defined (for composition of Ultroser G - see Table 55) and promoted cell attachment. At day 3, the fall in the enzyme activities was more pronounced in the male rat (Table 1 - bottom panel) than in the female rat (Table 2 -bottom panel). As seen with cells cultured in Ham's F-10 supplemented with horse and foetal calf serum, the reduction in enzyme activities was greater with the female-specific than the male-specific enzymes. The explanation behind this effect is probably the same as discussed above. At day 6, the enzyme activities in the female rat hepatocytes had returned to or exceeded the control level. On the other hand, in the male rat hepatocytes, improvement of the enzyme activities was very slight irrespective of the enzyme studied. The reason for the differential effect between the male and female hepatocytes is unclear but could be due to a poorer adaptation of the steroid metabolizing enzymes of the male rat to *in-vitro* environment or the lack (or the lack of effect) of ' masculinizing factor/s ' in the culture medium. As is seen from the composition of the serum substitute, we are still not working with a hormone-free medium. Indeed it would be impossible to investigate the effect of physiological concentrations of insulin in this system as the final concentration of insulin in the medium is in the order of 1 μM. Thus, although Ultroser G maintains enzyme activities as well as serum, it is of little use for our investigations.

In a further set of experiment, designed to develop a totally hormone-free hepatocyte culture system, we have modified the cultivation of the rat hepatocytes by initially plating the cells in Ham's F-10 supplemented with 2 % Ultroser G for 24 hour (Table 3 -top panel). The cells were then washed and cultured for a further 24,48 and

Table 55 . Composition of the synthetic multi-hormone culture medium, **Ultroser G** (LKB) [content / vial]

Calcium	Bovine serum albumin
Selenium	Traces of sulphates, inositol, thyroxine
Transferrin	and calmodulin.
Insulin (~ 1μM)	β-2 macroglobulin not used in
Oestradiol	manufacture but may be present.
Testosterone	Glucose none added but may be
Progesterone	some endogenous.
Triiodothyronine	Glutamine 1.6 μM
Dexamethasone	

(Information obtained from LKB, Sweden)

72 hours in Ham's F-10 containing only 0.1 % bovine serum albumin. In these experiments, we used only hepatocytes from *male* rats as we intended to examine the role of insulin in regulating hepatic steroid metabolism and insulin's effect is only seen in the male rat *in-vivo* (Skett, 1986).

No difference between the male-specific and female-specific enzyme activities was observed. All the enzyme activities fell after 1 day in culture. Apparently, the cytochrome P-450 enzymes were more prone to changes in activity than the non-cytochrome P-450 when cultured in this culture medium. At day 2, partial restoration of the cytochrome P-450 enzyme activities were observed while the activity of the non-cytochrome P-450 enzymes exceeded the control values. This level of enzyme activity was maintained for at least 3 days in culture. Hepatic steroid metabolism could, thus, be maintained in cultures of male rat hepatocytes in the complete absence of hormones or serum and, indeed, was maintained better than if hormones were present.

We have further extended the work by modifying the above method by replacing Ultroser G with 0.1 % bovine serum albumin (Table 3 - bottom panel). We have discovered that the enzyme activities were better maintained in this medium than in the other cell culture systems and that steroid metabolizing enzyme activities were returned to control level after 2 days in culture. The mechanism by which albumin maintained the enzyme activities is not well understood though it has been reported that human serum albumin caused protoporphyrin, that is generated in the cultured liver cells, to be transferred to the culture medium (Granick et.al., 1975) and, thus, relieves the feed-back inhibition on haem biosynthesis and/or decreases the activation of haem oxygenase by the porphyrin. We, therefore, have a hepatocyte culture method to evaluate the effects of insulin and glucagon on hepatic steroid metabolism in serum- and hormone-free, chemically defined medium.

We have further characterized the metabolism of androst-4-ene-3,17-dione by

finding the optimum number of hepatocytes (Figure 5) and period of incubation (Figure 6) to be used in the assay. Since the relationship between the number of cells used and increasing period of incubation and the amount of metabolite formed are linear, we have chosen 30 minutes of incubation time and 3 x 10^6 cells per incubation in the steroid assay throughout the study. We recognize the limitation of the cell culture system used but such a system is essential in order to evaluate, without interference, the effect of a single hormone on androst--ene-3,17-dione metabolism.

9.1 THE EFFECT OF INSULIN ON ANDROST-4-ENE-3,17-DIONE METABOLISM IN NORMAL MALE RAT HEPATOCYTES

The role of insulin in rat hepatic drug and steroid metabolism is known indirectly from the effect of diabetes mellitus on both the above liver parameters since insulin is the principal hormone altered in the diabetic state. This was first demonstrated by Dixon et.al., (1961) where they found that the metabolism of hexobarbitone, chlorpromazine and codeine was impaired in the alloxan-treated diabetic animals. Later, the alloxan-induced diabetes was replaced by the STZ-diabetes model system to investigate the role of insulin in the regulation of drug and steroid metabolism since the former was criticized for its overt toxicity (Hoftiezer and Carpenter, 1973). Early studies indicated that the effect of STZ-induced diabetes on drug and steroid metabolism is sex-dependent in the rat (Kato, 1974; Warren et.al., 1983). Investigation from our laboratory later indicated that the effects of STZ-induced diabetes are time-dependent i.e. the effects of acute diabetes on drug metabolism are not seen in animals left diabetic for 20 days (Skett and Joels, 1985). All of these effects of STZ are reversed by insulin treatment. Diabetes mellitus is associated with a decrease in serum androgen levels though there was no direct correlation between the latter and the effect of diabetes on drug metabolism (Skett et.al., 1984). A suggestion has been put forward that insulin and androgens affect drug metabolism in the rat liver indirectly by stimulating the pituitary gland to release growth hormone (Skett, 1987). However, up to date, no study has been conducted to investigate the possible direct effect of insulin on the rat liver pertaining to steroid metabolism. The success in the development of a hormone- and serum-free cell culture system which is responsive to insulin will enable us to assess the effect of insulin alone without the interference of other hormones.

After **1/2 hour** exposure to insulin, there was a significant increase in all of the

enzyme activities at physiological concentrations (10^{-10} - 10^{-9} M) of the hormone (Figure 7). This is in contrast to the selective effects of insulin reported by Skett (1986) in the intact rat. In this latter study, STZ-induced diabetes caused a reduction in 16α- and 6β-hydroxylase activities and 17-oxosteroid oxidoreductase, all of which are male-specific i.e. found higher in male than female rats but 7α-hydroxylase activity, which is higher in the female, is increased by diabetes. Insulin was able to reverse all of these effects. However, the experiment was carried out using liver microsomes, a system different from that used in this study . The variation observed could possibly be explained by the complex continuous interactive hormonal system *in-vivo*. The maintenance of different hormones at their respective physiological concentrations *in-vivo* is a consequence of mutual hormonal modulation and interaction. For example, in the diabetic state, not only the serum insulin and glucose levels are affected but also the serum glucagon, growth hormone and testosterone levels are among those altered and thus, this may explain the sex-dependent effect of insulin on drug and steroid metabolism observed in rats but not in isolated liver cells. At supraphysiological concentrations (10^{-7} and 10^{-6} M), insulin further increased the enzyme activities without reaching a maximal response, an effect also seen with insulin's effect on lipogenesis in primary cultures of hepatocytes (Amatruda and Chang, 1983b).

In order to discuss the possible mechanism of action of insulin in increasing the activity of the steroid-metabolizing enzymes, it is necessary first to look at the nature of the enzymes themselves. Many of the enzymes are monooxygenases (the hydroxylating enzymes) and cytochrome P-450 is the terminal oxidase component of the monooxygenase system responsible for many xenobiotic oxidation reactions including those of steroids. In view of this, it is extremely important for us to understand how it is regulated. Cytochrome P-450 isolated from phenobarbital treated rats has been shown to be phosphorylated by the catalytic subunit of cyclic AMP-dependent protein kinase in its

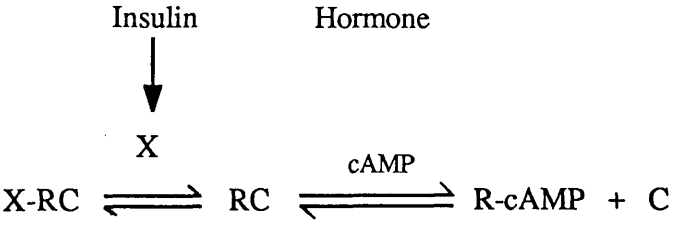
isolated form as well as in microsome- and liposome-bound states (Pyerin et.al., 1983 ; Pyerin et.al., 1984 ; Pyerin et.al. , 1986). This phosphorylation has been demonstrated to result in the conversion of cytochrome P-450, by the incorporation of a phosphoryl group into the cytochrome P-450 molecule at serine residue 128, to enzymatically inactive cytochrome P-420 followed by the loss of heme (Taniguchi et.al. , 1985) with consequent decrease in the monooxygenase activity (Pyerin et.al., 1984). A recent study has demonstrated that cytochromes P-450 are phosphorylated in an isoenzyme-specific manner (Pyerin et.al., 1987) where some isoenzymes were phosphorylated either by cyclic AMP-dependent protein kinase (protein kinase A) or calcium/phospholipid-dependent protein kinase (protein kinase C) or both and some by none of these two kinases. Thus, in our study, the effect of any drugs or hormones in the regulation of protein kinase activity would be crucial in the outcome of the amount of metabolites produced; at least for the cytochrome P-450 driven reactions.

The molecular basis for insulin action on cellular metabolism involves the modulation of the phosphorylation state of many key regulated enzymes (Krebs and Beavo, 1979 ; Cohen, 1982). Insulin action involves a cascade of events and has been shown to modulate various components of the effector systems distal to receptor binding. Below is a hypothetical description of the post-receptor effects of insulin and how it may bring about the observed increase in the enzyme activities studied. Stimulation of the intrinsic receptor tyrosine kinase results in the activation of a putative G-protein known as G_{ins} (Houslay, 1985) resulting in the activation of low K_m , membrane-bound cyclic AMP phosphodiesterase (Heyworth et.al., 1983a) and inhibition of adenylate cyclase (Heyworth and Houslay, 1983) culminating in the decrease of intracellular cyclic AMP concentration below basal level. Recent experiments by Irvine and Houslay (1988) suggested that insulin attenuates the functioning of ADP-ribosylated stimulatory guanine nucleotide regulatory protein G_s by acting as a substrate for the

insulin receptor, resulting in the attenuation of cholera-toxin stimulated adenylate cyclase. It is well established that under appropriate conditions, insulin can cause a diminution in the concentration of cyclic AMP in fat and liver cells. Convincing decreases in cyclic AMP concentrations can be observed when these cell are incubated with insulin in the presence of other hormones (e.g. glucagon) which increase cyclic AMP (Kiss, 1978). However, under basal conditions i.e. in the absence of other hormones, little or no effect of insulin on cyclic AMP levels in liver and fat cells is found. As indicated in our study, no significant changes in intracellular cyclic AMP concentration was seen after 1/2, 1 and 2 hours exposure to insulin (Figure 21). Insulin has been shown to inhibit hepatic cyclic AMP-dependent protein kinase in the absence of any changes in cyclic nucleotide concentrations (Miller and Larner, 1973 ; Mor et.al., 1981 ; Gabbay and Lardy, 1984). Similar inhibition of protein kinase activity has been observed in diaphragm (Walkenbach et.al., 1978), and skeletal muscle (Walaas et.al., 1973). However, the mechanism of this response has remained obscure. The effect of insulin to activate liver glycogen synthase without increasing cyclic AMP concentration shown by Walkenbach et.al. (1980) and Mor et.al. (1981) is analogous to the effect of insulin on androst-4-ene-3,17-dione metabolism seen in this study. Insulin here has been suggested to decrease the protein kinase activity by decreasing the sensitivity of the enzyme to its positive modulator cyclic AMP. Mor et.al. (1981) have reported that insulin caused a small shift in the affinity of protein kinase A for cyclic AMP in isolated hepatocytes. Gabbay and Lardy (1987) reported that insulin decreased the affinity of protein kinase A for cyclic AMP, raising the K_a without influencing the V_{max} , and therefore decreasing the capacity of the protein kinase to be dissociated. The holoenzyme of the cyclic AMP-dependent protein kinase is dissociated by cyclic AMP according to the following equation :

$$R_2C_2 + 4\,cAMP \longrightarrow R_2cAMP_4 + 2C$$

Based on Gabbay and Lardy's data, it appears that insulin shifts the equilibrium of this reaction to the left by decreasing the affinity of the regulatory subunit for cyclic AMP. Rannels and Corbin (1980) and Corbin et.al. (1981) have discovered that the regulatory subunit of the cyclic AMP-dependent protein kinase contains two intrachain cyclic AMP binding domains, termed site 1 and 2 and both binding sites appear to be involved in protein kinase activation (Robinson-Steiner and Corbin, 1983 ; Ogreid et.al., 1983). Gabbay and Lardy (1987) in their experiments suggested that insulin may act to decrease the affinity of protein kinases for cyclic AMP through a possible regulation of intrachain site 2 binding. Walkenbach et.al. (1978) proposed that an insulin- generated protein kinase inhibitor (X) may be directly associated with the type 1 holoenzyme (RC) as a result of insulin treatment, forming an altered protein kinase species (X-RC), which has a reduced ability to bind cyclic AMP.



It was recently demonstrated that the interaction of insulin with its receptors is followed by the hydrolysis of a novel glycophospholipid, resulting in the release of 1-alkyl-2-acylglycerol and phospho-oligosaccharide (Saltiel and Cuatrecasas, 1986 ; Saltiel et.al., 1986 ; Mato et.al., 1987a). The phospho-oligosaccharide, termed inositol phosphate-glycan (IPG) by Saltiel was generated by phosphodiesteratic hydrolysis with a phosphatidylinositol-specific phospholipase C from *Staphylococcus aureus* (Kelly et.al., 1986 ; Kelly et.al., 1987a) and was found to contain inositol, glucosamine, galactose and phosphate (Mato et.al., 1987b). This inositol phosphate- glycan was shown to mimic the effect of insulin to stimulate the high-affinity cyclic AMP

phosphodiesterase (Kiechle and Jarett, 1981 ; Saltiel et.al., 1986) and pyruvate dehydrogenase (Saltiel, 1987), and to inhibit adenylate cyclase (Saltiel, 1987 ; Malchoff et.al., 1987) and cyclic AMP-dependent protein kinase (Malchoff et.al., 1987 ; Villalba et.al., 1988). However, it had no effect on the calcium/phospholipid-dependent protein kinase (Villalba et.al., 1988). The ability of this inositol phosphate-glycan to modulate this long list of insulin-sensitive enzymes suggests that it functions as a second messenger for insulin action. In human serum, increased mediator concentrations (molecular weight ~ 1000-2000) have been observed with increased insulin concentrations during glucose tolerance tests (Sinha and Caro, 1985). This glycophospholipid could be derived from the glycosyl-phosphatidyl inositol structure which is thought to anchor proteins to the plasma membrane (Low, 1987). The insulin-like effects of this inositol phosphate-glycan were shown to be attributed to dephosphorylation of insulin-sensitive enzymes. As demonstrated by Villalba et.al. (1988), the phosphorylation of both phospholipid methyltransferase and histone IIA by the catalytic subunit of cyclic AMP-dependent protein kinase was almost completely inhibited by the addition of the inositol phosphate-glycan. In relation to the effect of insulin on steroid metabolism, the overall inhibition of the cyclic AMP-dependent protein kinase and the adenylate cyclase activity and stimulation of the cyclic AMP-dependent phosphodiesterase by this inositol phosphate-glycan would theoretically decrease the phosphorylation of cytochrome P-450 to its inactive cytochrome P-420 form, therefore, would explain the increased amount of substrate (i.e. androst-4-ene-3,17-dione) being metabolized. The mechanism by which the inositol phosphate-glycan inhibits the cyclic AMP protein kinase activity still remains unresolved. It had no effect on cyclic AMP binding by the cyclic AMP-dependent protein kinase and does not compete with ATP for the same binding site in the catalytic subunit of the cyclic AMP-dependent protein kinase (Malchoff et.al., 1987 ; Villalba et.al., 1988) and has been suggested by the latter group

to act by binding to a different site of the catalytic subunit of the cyclic AMP-dependent protein kinase.

Another possible explanation for the observed increase in enzyme activity is the ability of insulin to regulate the protein phosphatase(s). An ATP/Mg - dependent multifunctional protein phosphatase has been identified in mammalian nervous and non-nervous tissues (Yang et.al., 1980 ; Yang and Fong, 1985). This enzyme can be activated in the presence of ATP/Mg and an activating factor termed FA, a cyclic AMP - and Ca^{2+} - independent protein kinase (Vandenheede et.al., 1980 ; Hemmings et.al., 1981). This protein phosphatase activating factor, FA, has been isolated from the brain, liver, heart, smooth and skeletal muscles (Yang et.al., 1987). A recent report indicated that insulin can activate and cause the translocation of protein kinase FA in human platelets (Yang et.al., 1988). The FA exists mostly in an inactive form in the particulate fractions of the human platelet. The exposure of the platelets to physiological concentrations of insulin (10^{-9} - 10^{-8} M) resulted in an increase in cytosolic FA activity (molecular weight ~45 kDa) as assayed as an activator of ATP.Mg-dependent protein phosphatase, to about 300 % of control values in the absence of insulin and in a corresponding decrease in FA activity in the membrane. A 46-kDa membrane phosphoprotein of the fat cells was recently shown to be phosphorylated by insulin exclusively on tyrosine residues. Insulin stimulates the tyrosine phosphorylation 3- to 4-fold within 150 seconds in the intact cell in a dose-dependent way at insulin concentrations between 0.5 nM and 100nM. This protein is not immunoprecipitated by antibodies against different regions of the insulin receptor and its HPLC tryptic peptide map is different from the tryptic map of the insulin receptor, suggesting that it is not derived from the receptor β-subunit (Haring et.al., 1987). It still remain to be elucidated whether phosphoprotein discovered by Haring and colleagues (1987) is similar to the protein kinase FA isolated by Yang et.al. (1987). Interestingly, the increase in all the

enzyme activities active on androst-4-ene-3,17-dione following 1/2 hour exposure to insulin varies from 200 to 300 % of control. In addition to ATP/Mg-dependent protein phosphatase as its substrate, FA is capable of acting on many substrate proteins including glycogen synthase (Vandenheede et.al., 1980 ; Hemmings et.al., 1981), the regulatory subunit of cyclic AMP-dependent protein kinase (Hemmings et.al., 1982), the nerve growth factor receptor proteins (Taniuchi et.al., 1986) and myelin basic protein (Yang, 1986). FA has been shown to promote dephosphorylation of myelin basic protein in the central nervous system (Yang and Fong, 1985 ; Yang, 1986) however, its possible physiological function in regulating the monooxygenase enzyme system remains to be elucidated.

It is apparent that insulin's effect on androst-4-ene-3,17-dione metabolism involves a phosphorylation reaction since the protein kinase inhibitor, K-252a, completely inhibited the effect of insulin on androst-4-ene-3,17-dione metabolism (Figure 24 and 25). Paradoxically, if protein kinase A and C activity are inhibited, it might be expected that insulin would further increase the enzyme activities as both of these kinases are known to decrease steroid metabolism in isolated hepatocytes (Berry and Skett, 1988; Allan and Skett, 1988) and to phosphorylate cytochrome P-450 (Pyerin et.al., 1987). K-252a has been shown to competitively inhibit the protein kinases with respect to ATP and this inhibition is reversed by ATP indicating that K-252a directly interacts with the catalytic site of the kinase competing with ATP (Kase et.al., 1987). If the protein kinase A activity is inhibited, it is anticipated that the equilibrium will be shifted from cytochrome P-420 to P-450 leading to increase in enzyme activities. As explained above, the insulin mediator, inositol phosphate-glycan acts at a different site from the protein kinase inhibitor, K-252a. A recent study (Hagiwara et.al., 1988) has reported that bioflavonoids (eg. quercitin and myricetin) which have a similar mode of action to K-252a, by competing with ATP at the catalytic fragment of the protein kinases, also

inhibited insulin receptor tyrosine kinase. The tyrosine kinase activity could be responsible for the initial effects of insulin where the self-phosphorylation of the receptor is essential for the transmission of the signal (Espinal, 1987), therefore it is assumed that inhibition of the insulin receptor tyrosine kinase could also alter the activity of the protein kinases on phosphorylation. It is interesting to note that Hagiwara and co-workers (1988) demonstrated that the K_i values of myricetin for insulin receptor tyrosine kinase, cyclic AMP-dependent protein kinase and protein kinase C were 2.6 μM, 27.5 μM and 12.1 μM respectively. Until now the effect of K-252a on insulin receptor tyrosine kinase has not been reported. However, at this stage there is insufficient evidence to do more than to speculate that K-252a inhibition of insulin effect with respect to androst-4-ene-3,17-dione metabolism is due to its inhibition of the tyrosine kinase analogous to the effect of the bioflavonoids.

Protein kinase C (PK_C) has been demonstrated to be an important transmembrane signalling system in a number of cell responses to various effectors (Nishizuka, 1986). Recently hepatic steroid metabolism was shown to be inhibited by activation of protein kinase C suggesting that it may be one of the mechanisms by which the regulatory hormones affect steroid metabolism in the liver (Allan and Skett, 1988). Insulin enhances *de novo* synthesis of phosphoinositides in fat cells (Pennington and Martin, 1985) and provokes, through rapid activation of phospholipase C (Farese et.al., 1986), increases of diacylglycerol, which is the physiological activator of PK_C. However, the role of PK_C in insulin action has been questioned, in particular by the recent finding (Glynn et.al., 1986) that the hormone proved unable to provoke, as does 4β-phorbol-12β-myristate-13α-acetate (PMA), a translocation of PK_C from soluble to particulate fractions. The results from several laboratories indicated that the rate of diacylglycerol and phosphatidic acid turnover in response to insulin is tissue specific. In myocytes (Farese et.al., 1985b) and adipocytes (Pennington and Martin, 1985),

insulin has been reported to increase *de novo* synthesis of phosphoinositides, diacylglycerol and phosphatidic acid. In rat liver, insulin has no effect on the turnover of the major phospholipids, including the polyphosphoinositides, in several subcellular compartments including plasma membranes, microsomes, lysosomes, mitochondria and nuclei (Sakai and Wells, 1986). The result we have obtained are in accord with those above where no significant increase in incorporation of ^{32}P into phosphatidic acid and phosphatidylinositol between control and insulin-treated liver cells were observed (Figures 22 and 23). The tyrosine kinase of certain viral oncogenes has been suggested to phosphorylate phosphatidylinositol and phosphatidylinositol-4-P to the corresponding polyphosphoinositides (Sugimoto et.al., 1984; Macara et.al., 1984). Taylor et.al. (1985) have investigated the role of insulin in phosphoinositide metabolism using rat liver plasma membranes and isolated hepatocytes. Their findings suggested that the tyrosine kinase activity of liver insulin receptors is not important in phosphoinositide formation.

If the investigation of insulin's effect on hepatic steroid metabolism is extended, an attenuation of insulin's effect is observed after **1 hour** of insulin pre-incubation. At concentrations higher than 10^{-8} M, the enzyme activities returned to basal level (Figure 8). A return of all enzyme activities within the range of insulin concentration used (10^{-10} - 10^{-9} M) to basal level occurred after **2 hours** of insulin incubation (Figure 9). As previously reported, acute exposure (between 0 - 2 hours) of intact cells to insulin has no effect on receptor concentration (Gavin et. al., 1974). The decrease in insulin's effect could possibly be due to the desensitization of the second messenger system/s as occurs after a transient rise in the activity of the second messenger system with the effect of insulin on the activation and translocation of protein kinase FA (an activator of the ATP. Mg-dependent multifunctional protein phosphatase) as reported by Yang et.al.(1988). However, no direct relationship between translocation of

protein kinase FA and steroid metabolism has been studied. The modulation of the cyclic AMP level involving the adenylate cyclase or the phosphodiesterase can be ruled out since no significant changes in cyclic AMP content was observed after 1 and 2 hours of insulin preincubation. Another possible explanation for the attenuated insulin response is related to the degradation of insulin *in-vitro*. Varandani and Nafz, (1976), have reported that about 30 to 50 % of insulin is degraded after 15 minutes of incubation in isolated liver cells. The dissociation of insulin from receptor sites on liver membranes is biphasic and can be resolved into rapid and slow components (McCaleb and Donner, 1981; Corin and Donner, 1982). The fraction of the rapidly dissociating hormone decreases as the incubation time between liver cells or membranes is lengthened and this is accompanied by a proportionate increase in the amount of slow dissociating insulin (Donner and Corin, 1980). The dissociation of insulin from its receptors could also possibly explain the gradual decrease in insulin effect from 1/2 to 2 hours of incubation.

A further increase in enzyme activities is seen after **24 hour** insulin incubation. However, a notable difference between the effect of insulin at 1/2 hour and 24 hour is that the former showed a dose-related increase in enzyme activities which had not reached a maximum at 10^{-6} M insulin (compare Figure 7 and 10). However, at 24 hours, the enzyme activities fell significantly below control level as the insulin concentrations were increased above 10^{-8} M. Thus it is apparent that the effect of insulin observed at 1/2 and 24 hours could be mediated by two different mechanisms, and differentiated by time. Chronic exposure of cells to high concentrations of insulin (10^{-8} and 10^{-6} M) *in-vitro* has been reported to produce a decrease in insulin receptor concentrations i.e. insulin-induced receptor down-regulation (Gavin et. al.,1974). A recent study by McClain and Olefsky (1988) indicated that the rate and extent in internalization of insulin receptors in a human hepatoma cell line is dependent on the

insulin concentration. Their study showed that cells exposed to lower concentrations of insulin internalise fewer receptors and slower than cells exposed to a high insulin concentration. They concluded that internalization of insulin receptors can be mediated by two independent pathways depending upon the concentration of insulin used. It is not known whether the effect of insulin observed at 24 hour is linked to these two pathways. It is known that high concentrations of insulin do cause a greater degree of receptor down-regulation in adipocytes when exposed for a prolong period to insulin (Marshall et.al., 1984). However recycling rates of the insulin receptor were two fold higher in cells treated with high insulin concentration than in lower concentration (McClain and Olefsky, 1988). Its relevance to insulin effect on androst-4-ene-3,17-dione metabolism after 24 hour of exposure is again unclear.

Specific binding sites for insulin have been described in different intracellular organelles from liver (Goldfine, 1981). Recently intracellular insulin has been demonstrated to stimulate RNA and protein synthesis in oocytes from Xenopus laevis (Miller, 1988). However with regards to protein synthesis, the level of cytochrome P-450 was found not to be significantly altered after 24 hour of incubation with insulin, an effect similarly observed at 1/2 hour (Table 18)*. Another similar effect seen at 1/2 and 24 hours was the effect of the non-specific protein kinase inhibitor, K-252a, on the metabolism of androst-4-ene-3,17-dione by insulin (refer to Figure 24 and 25). Resembling the effect seen at 1/2 hour, preincubation of the liver cells with K-252a for 24 hours abolished the effect of insulin to increase the enzyme activities implying that the insulin effect observed at 24 hours is mediated by protein phosphorylation.

The effect of insulin observed at **48** and **72 hours** was further investigated to identify whether the attenuation or disappearance of insulin effect seen is due to the degradation of insulin or to down-regulation of insulin receptors. To determine this, insulin was added at 24 hour intervals from 0 to 72 hours (Figure 14). If the fall in

* Although the total cytochrome P-450 content did not change, it is possible that alteration in the level of specific isoenzymes could occur.

activity (at 48 and 72 hours) is due to breakdown of insulin molecules, we should expect a maintenance of increased enzyme activities at 24 hours as well as at 48 and 72 hours. However the cumulative addition of insulin at 48 and 72 hours did not sustained the increased enzyme activity seen at 24 hours indicating that the fall in activity at 48 and 72 hours could probably be attributed to the down-regulating effect of insulin on its receptors which is consistent with the documented chronic effect of insulin on its receptor in intact cells (Gavin et.al., 1974).

In summary, the effect of insulin on steroid metabolism over the period studied was characterized by the presence of two increases in enzyme activities i.e. at 1/2 and 24 hours. These effects of insulin are observed within the physiological range of insulin concentrations. The dose-response curves indicated that the acute effect (at 1/2 hour) of insulin on steroid metabolism could be mediated by a mechanism distinct from the chronic effect (24 hours) of insulin. The data gathered in this study suggest that insulin acts as a general stimulator of the enzymes in the liver metabolizing androst-4-ene-3,17-dione. We have presented data indicating the direct effect of insulin on steroid metabolism in the rat liver supporting our contention that insulin is a major factor in the regulation of xenobiotic metabolism in the rat liver. However, it is important to consider other possible interactions with other hormonal systems, since insulin also interferes with the secretion of other hormones and vice-versa.

9.2 THE EFFECT OF INSULIN ON ANDROST-4-ENE-3,17-DIONE METABOLISM IN HEPATOCYTES ISOLATED FROM STREPTOZOTOCIN-DIABETIC MALE RATS

Streptozotocin was used as a diabetogen instead of alloxan because of its more selective β-cell toxic action (Arison and Feudale, 1967; Hoftiezer and Carpenter, 1973) and because its effect on the liver has been shown to be due to its diabetogenic action since treatment with insulin could reverse the effects seen (Reinke et.al., 1978; Reinke et.al., 1979; Favreau and Schenkman, 1987). On the other hand, alloxan has been criticized for its overt toxicity (Hoftiezer and Carpenter, 1973) and thus may not be a good model of diabetes. Other workers have used different doses of streptozotocin to induced diabetes; at a dose of 60 mg/kg, STZ has been shown to induce hyperglycaemia and hyperlipidaemia in adult rats (Skett, 1986) but we have chosen the dose of 100 mg/kg to ensure that the animals were ketotic since ketoacidosis is more commonly manifested in type 1 or insulin dependent diabetes mellitus than in type 2 or non-insulin dependent diabetes mellitus and since STZ-treated animals have been used as a model for type 1 diabetes (Junod et.al., 1969; Like and Rossini, 1976).

We have only used male rats in our study of the effect of the diabetic state on the response to insulin of steroid metabolism in hepatocytes because previous studies have indicated that the effect of diabetes on steroid and drug metabolism is sex-dependent i.e. only seen in the male rat (Skett, 1986; Skett and Joels, 1985). The activity of the enzymes studied were markedly reduced in the male but were unaffected in the female. The effect of STZ-induced diabetes on phase 1 **drug** metabolism has been found to be time-dependent (Skett and Joels, 1985). The effect of diabetes was conspicuous after 3 days of STZ-treatment but these effects were not observed in rats left diabetic for 20 days. With regard to this, it is of interest to investigate whether **steroid** metabolism is

similarly affected by STZ-induced diabetes since they share a common hepatic metabolism control mechanism.

As shown in Table 12, the animals lost weight and were hyperglycaemic, establishing that they were in fact diabetic. In this study it was shown that STZ-induced diabetes mellitus causes a change in the metabolism of androst-4-ene-3,17-dione. The changes in the enzyme activities were dependent on the enzyme being studied, with the basal activity of the male-specific enzymes (6β-hydroxylase and 17-OHSD) being decreased while the female-specific enzyme activities (7α-hydroxylase and 5α-reductase) were increased. This data is in general agreement with the results obtained from studies using hepatic microsomes (Reinke et.al., 1978; Skett, 1986) except that the 16α-hydroxylase and 5α-reductase activities were unexpectedly increased, whereas, Skett (1986) showed that 16α-hydroxylase and 5α-reductase activities were respectively decreased and unaffected in hepatic microsomes from acutely STZ-treated diabetic male rats. The variability of the observed effects may reflect the physiological status of the preparation used and the use of hepatocytes probably represent a more physiological situation. Abnormal testosterone metabolism by liver slices from alloxan-diabetic rats has been demonstrated and the formation of androst-4-ene-3,17-dione, dehydroepiandrostenedione and androsterone from testosterone was decreased in the diabetic animals (Demchenko and Tron'ko, 1975). Subbiah and Yunker (1984) have reported an increased 7α-hydroxylase activity in STZ-treated rats. The present report supports these previous observations and indicates altered steroid metabolism in diabetes.

Hepatocytes isolated from chronically diabetic rats (21 days post-STZ treatment), on the other hand, gave somewhat different results. There were no changes seen in the basal 7α- and 16α-hydroxylase activities (refer to Table 12). The basal activity of 6β-hydroxylase and 5α-reductase were decreased but the 17-OHSD activity was increased. Thus, it is clear that the effect of diabetes mellitus on phase 1 steroid metabolism in the rat

is time-dependent and is not the same in the acute and chronic phase. Also, the overall effect of chronic diabetes is much less than that seen in the acute phase, a result strikingly similar to that observed in microsomal drug metabolism (Dixon et.al., 1963; Skett and Joels, 1985). The hepatocyte system as used in this study is, thus, a good model for the effect of acute and chronic diabetes *in-vivo*.

The response of the cells to insulin is also markedly affected if the cells are derived from diabetic animals. Cells derived from an acutely diabetic (3 day) male rat did not respond to insulin at any time point or at any concentration (Figures 15 and 16), whereas the effect of insulin on the cells derived from the chronically diabetic (21 day) animals depended on the period of pre-incubation with insulin (Figures 17, 18 and 19). At **1/2 hour**, hepatocytes from normal rats responded in a dose-dependent fashion to insulin for all the enzyme activities measured with a significant increase in activity as low as 10^{-10} M insulin which had not reached a maximum at 10^{-6} M (Figure 7). However, primary cultures of hepatocytes from 3- and 21-days STZ-diabetic rats were not responsive to *in-vitro* insulin addition throughout the range of insulin concentrations used.

The effect of insulin after **1 hour** of exposure in hepatocytes from chronically diabetic rats resembles its effect in normal rat hepatocytes although the effect is not as marked (Figure 18). The bell-shape curves of enzyme activity after 1 hour exposure to insulin in normal rat hepatocytes were similarly exhibited in the chronic diabetic hepatocytes indicating that the phenomenon of insulin resistance in rats may be caused by a defect in the signal transduction system early in insulin action. The insulin resistance, similarly observed at 1 hour in acute diabetes, is different from the chronic phase in that the liver cells were still unresponsive to insulin in the former, and hence could possibly explain the differential effect of acute and chronic diabetes seen in this study and others (Skett and Joels, 1985; Dixon et.al., 1963). However, the ability of insulin to cause a

small but significant increase in all the enzyme activity after the liver cells from chronic diabetic rats were exposed to insulin for **2 hours** is unexpected and different from that in the normal rat (compare Figure 9 and 19). It seems, therefore, that in the chronically diabetic rat, the response to insulin is delayed and attenuated.

The incidence of insulin resistance is not restricted to liver alone. It has been shown to occur in adipocytes (Haring et.al., 1986), erythrocytes (Grigorescu et.al., 1984), lymphocytes (Kakehi et.al., 1988), denervated slow and fast muscles (Turinsky, 1987), duodenum and gastric-fundus (Altan et.al., 1987). In the human, insulin resistance occurs in many patients with diabetes mellitus, obesity, acromegaly, Cushing's syndrome (Berson and Yalow, 1970) and acanthosis nigricans (Kahn et.al., 1976). In other work, it has been shown that adrenaline, acting primarily through a β-adrenergic receptor, markedly impairs tissue sensitivity to an increase in plasma insulin levels (Deibert and DeFronzo, 1980). Thus, the phenomenon of insulin resistance is not restricted to one species only. However, whether the pathogenesis of insulin resistance in experimental animals and human is similar or not is still unsolved and is currently a subject of a great deal of research. Recently , Hussin and Skett (1988) have suggested that the 3 days STZ-diabetic rat may be a model for type 2 or non-insulin-dependent diabetes mellitus since insulin resistance has been shown to be a characteristic feature of type 2 diabetes mellitus (Kolterman et.al., 1983).

Insulin resistance may be said to exist whenever normal concentrations of insulin produce a less than normal biologic response (Kahn, 1978). In this study liver cells from acute- and chronically-treated STZ-diabetic rats were shown to be fully or partially resistant to the effect of insulin *in-vitro*. Resistance to the acute effects of insulin on lipogenesis has also been reported in primary cultures of hepatocytes from diabetic and fasted rats (Amatruda and Chang, 1983b) and this occurred in the presence of normal to increased insulin binding (Cech et.al., 1980) suggesting a postreceptor

defect in insulin's action. Following its synthesis and transport to target tissues, insulin exerts its biological effects by binding initially to its specific cell receptors. The overall target tissue insulin resistance can be subdivided into receptor and postreceptor defects. However, the currently available evidence indicates that resistance is *largely* related to postreceptor defects in insulin action. Since insulin action involves a cascade of events, it is apparent that abnormalities at any step distal to binding can cause or contribute to target tissue defects in insulin action. Insulin resistance can also be classified according to a decrease in sensitivity i.e. a rightward shift of the dose-response curve (receptor defect) or a decrease in responsiveness i.e. a decrease in the maximal response (postreceptor defect) or both. It is apparent that the insulin resistance with regards to steroid metabolism seen here could probably be attributed to both receptor and postreceptor defects since the liver cells were completely unresponsive to insulin addition up to 10^{-6} M concentration.

STZ-induced diabetes in experimental animals has been reported to be associated with numerous alterations of many insulin-modified intracellular biochemical steps at receptor and postreceptor levels. Recently, it was reported that the biosynthesis, the terminal glycosylation and the intracellular transport of the 190-kDa receptor precursor to the cell surface proceeded normally in a patient with extreme insulin resistance, but proteolytic maturation to α and β subunits does not occur (Kakehi et.al., 1988). They postulated that the defect results either from mutation(s) within the insulin-receptor gene or from a defect in the receptor processing gene. In another study, the lymphocytes from a patient with leprechaunism (inherited condition of severe insulin resistance) have been shown to have lower numbers of insulin receptors when compared to the normal population. The patient also exhibited a marked reduction in the level of lymphocyte insulin-receptor mRNA (Ojamaa et.al., 1988). Since the synthesis of the insulin receptor precursor proceeded at a normal rate in these patients, the authors suggested that

the defect in the posttranslational processing of the receptor or in its translocation to the plasma membrane could have accounted for the insulin resistance observed in these patients. Diabetes also has been demonstrated to induce structural and functional changes in insulin receptors (Burant et.al., 1986). The electrophoretic mobility of a subpopulation of β-subunits derived from diabetics was slightly decreased when compared to control. It appeared that excess sialidation may account, in part, for the altered mobility of the diabetic derived β-subunits since neuraminidase decreased or abolished the differences in electrophoretic mobility between controls and diabetics.

The insulin receptor is a tyrosine-specific protein kinase. Upon binding of insulin, the kinase is activated resulting in autophosphorylation of the receptor. Insulin-stimulated tyrosine-kinase activity has been demonstrated to decrease in erythrocyte and cultured fibroblast insulin receptors in patients with syndromes of severe insulin resistance (Grigorescu et.al., 1984; Grigorescu et.al., 1987), Cloudman S91 melanoma cell line (Haring et.al., 1984b), rat skeletal muscle (Burant et.al., 1986), skeletal muscle biopsies from obese and type 2 diabetic male patients (Arner et.al., 1987b), skeletal muscle of insulin-resistant obese mice (Le Marchand-Brustel et.al., 1985) and adipocytes of non-insulin-dependent diabetic patients (Freidenberg et.al., 1987). This long list of tissues affected in man and experimental animals indicates that defective tyrosine kinase activity may play a vital role in the pathogenesis of insulin resistance. Since the kinase activity has been shown to be an early step in the insulin transmembrane signalling, it is logical to see reduced insulin responses in patients and animals with insulin resistance.

Insulin resistance has also been associated with altered modulation of insulin action by protein kinase C (Van de Werve et.al., 1987). In lean rat hearts, phorbol myristate acetate provokes a translocation of the protein kinase C from a soluble to a particulate fraction. In obese rat hearts which are insulin resistant, the basal distribution

of protein kinase C is altered i.e. more activity found in the soluble and less in the particulate fraction. Moreover, phorbol esters have been demonstrated to modulate insulin receptor phosphorylation and insulin action (Takayama et.al., 1984) by increasing the tyrosine kinase K_m for ATP (Haring et.al., 1986). In this study we have discovered that insulin does not significantly affect the ^{32}P labelling of phosphatidic acid and phosphatidylinositol when compared to control (Figure 22). However the amount of ^{32}P labelling of the phospholipids were tremendously reduced in the diabetic rat (Figure 23) indicating a possible role of phospholipid in the appearance of diabetes. The importance of changes in phospholipid metabolism during the action of insulin is presently unknown but a recent report has indicated that phospholipid environment alters the hormone-sensitivity of the purified insulin receptor kinase (Lewis and Czech, 1987).

Insulin resistance in rats has also been associated with the failure of insulin to stimulate the release of a chemical modulator of pyruvate dehydrogenase (Amatruda and Chang, 1983a). Extracts of the mediator fractions were prepared from liver particulate fractions incubated in the absence or presence of insulin (2 nM). The liver particulate fraction from STZ-diabetic rats were resistant to the ability of insulin to generate the activator of pyruvate dehydrogenase activity. The situation could be rectified by treating the diabetic rats with insulin for 2 days. Recently, the chemical mediator was isolated and identified as the polar head group of an insulin-sensitive glycophospholipid (Gottschalk and Jarett, 1988). This polar head group, which was released by treating the glycophospholipid with a phosphatidylinositol-specific phospholipase C, stimulated pyruvate dehydrogenase in both subcellular and whole cell assays.

Experimental STZ-diabetes is associated with both a decrease in low K_m cyclic AMP phosphodiesterase and calmodulin as well as an apparent subcellular redistribution of these components (Smoake and Solomon, 1980; Solomon et.al., 1986b). STZ diabetes is also associated with a reduction in V_{max} of the low K_m cyclic AMP

phosphodiesterase in both liver and fat tissues (Solomon et.al., 1986b).

It has been reported that somatomedins (insulin-like growth factors, IGF's) are produced in the liver , based on observations using liver perfusions (Schalch et.al., 1979; Miller et.al., 1981) and monolayer cultures of rat hepatocytes (Kogawa et.al., 1983). STZ-induced diabetes mellitus has been associated with both a decrease in hepatic production and serum levels of IGF and its carrier protein in the rat and these can be restored towards normal by insulin replacement therapy (Miller et.al., 1981). The IGF 1 receptor resembles the insulin receptor in every aspect (Kasuga et.al., 1981a), the two peptides (insulin and IGF-1) have several biological activities in common and they crossreact with each others' receptors (Zapf et.al., 1978).

STZ-induced diabetes has also been associated with altered responsiveness of hormone-sensitive adenylate cyclase in liver (Soman and Felig, 1978), decreased guanylate cyclase activity (Vesely et.al., 1977), modification of a distinct species of guanine nucleotide regulatory protein [G_{ins}] (Houslay, 1985) and, recently, the loss of expression of inhibitory guanine nucleotide regulatory protein, G_i (Gawler et.al., 1987).

It still remain to be elucidated whether any one or a combination of these STZ-induced changes are associated with the insulin resistance seen in this study.

Insulin treatment of STZ-diabetic rats is known to reverse the effect of other measured parameters to control level. In the literature , a wide range of doses of insulin were used to treat diabetic animals. In this study we have used different doses of insulin (2, 12 and 16 units) purposely to study the efficacy of each dose in reversing the depressive effect of diabetes on androst-4-ene-3,17-dione metabolism. We found that both 2 and 12 units of insulin equally, partially restored the ability of the 3-days STZ-induced diabetic hepatocytes to respond to *in-vitro* insulin (Figure 20). The maximal effect of *in-vitro* insulin is seen at 10^{-8} M concentration in the treated diabetic rats,

whereas in untreated control rat, insulin response on the enzyme activities had not reached its maximum even at 10^{-6} M. *In-vivo* insulin treatment restores about 60 to 85 % of the enzymes' responsiveness of the normal rat at 10^{-8} M. Insulin treatment could restore the sensitivity of the cultured cells toward *in-vitro* insulin as can be seen by the effect of insulin at 10^{-10} M in the diabetic rat. Treatment with 16 units of insulin resulted in effects as seen in untreated diabetic rats (Figure 15). The absence of effect seen is probably due to the down-regulation of insulin receptors caused by the high dose of insulin administered (16 units/day for 3 days). However, we have no direct evidence to support this notion but others (Kahn et.al., 1972; Archer et.al., 1973) have found that the concentration of insulin receptors per cell fluctuates in response to altered conditions *in-vivo*. The partial restoration of other measured biological effects after *in-vivo* insulin treatment has also been reported by others (Favreau and Schenkman, 1987; Gawler et.al., 1987). *In-vivo* insulin treatment has been reported to restore liver microsomal drug metabolism and the ability of insulin to stimulate the release of a chemical modulator of pyruvate dehydrogenase to normal (Dixon et.al., 1963; Amatruda and Chang, 1983a). The restoration of responsiveness seen after *in-vivo* treatment with insulin but not *in-vitro* treatment suggests the presence of a factor/factors *in-vivo* which is/are absent *in-vitro*, which is/are important in reversing the hepatic insulin resistance in diabetes. Hepatocytes generally require insulin, corticosteroids and thyroxine to maintain differentiated functions in cell culture (Varandani et.al., 1982). It is interesting to note that recent work (Van der Hoeven and Galivan, 1987) has reported the effect of hormonal control of microsomal NADPH-cytochrome P-450 reductase in primary cultures of isolated hepatocytes. They reported that insulin and dexamethasone alone improved the retention of reductase activity and protein. Only when hepatocytes were cultured in insulin, triiodothyronine and dexamethasone could NADPH-cytochrome P-450 reductase activity and protein be maintained at the original level.

In summary, hepatocytes from diabetic rats (acutely and chronically treated) are not responsive to insulin *in-vitro* with respect to androst-4-ene-3,17-dione metabolism and the responsiveness is partially restored by *in-vivo* treatment with insulin. The need for insulin treatment in order to restore insulin responsiveness in the diabetic rat hepatocytes signifies the major role of insulin in the regulation of steroid metabolism in the rat liver.

9.3 THE EFFECT OF GLUCAGON AND TH-GLUCAGON ON ANDROST-4-ENE-3,17-DIONE METABOLISM IN NORMAL MALE RAT HEPATOCYTES

The liver plays a prominent role in the regulation of glucose and ketone homeostasis. The regulation of both of these parameters involves insulin and its counter-regulatory hormone, glucagon. Physiological changes in the diabetic rat include an increase in the ratio of glucagon to insulin production (Mackrell and Sokal, 1969) and a markedly diminished hepatic capacity for metabolizing drugs (Weiner et.al., 1972). Since starvation, diabetes, and glucagon have been shown to increase the level of cyclic AMP in the liver (Sutherland and Robison, 1966) and insulin has been demonstrated to have the opposite effect (Senft et.al., 1968), it is possible that the inhibition of drug metabolism produced by starvation and diabetes is mediated by an increase in intracellular cyclic AMP. With respect to the adenylate cyclase system, the acute activation could be due to changes in the insulin-glucagon ratio (Unger and Orci, 1975) and in the diabetic state an increased sensitivity to glucagon could be involved (Allgayer et.al., 1982). In fact after treatment of the diabetic rat with insulin this increased sensitivity to glucagon stimulation returned to normal values (Hepp, 1972). Up to date very little work has been done to investigate the effect of glucagon on xenobiotic metabolism. The many counter-regulatory effects of glucagon on the effects of insulin and the role of cyclic AMP-dependent phosphorylation in the control of xenobiotic metabolism in the liver (Banhegyi et.al., 1988) prompted us to investigate the effect of glucagon on steroid metabolism.

A time-course experiment indicated that there is a lag phase in glucagon effect on androst-4-ene-3,17-dione metabolism (Figure 26 - 30). The effects of glucagon began to be manifested at **1/2 hour** of preincubation (Figure 31). Glucagon decreases the

activity of the enzymes active on androst-4-ene-3,17-dione at physiological concentrations (10^{-10} - 10^{-8} M). Higher concentrations are less effective. The maximum effect of glucagon was always seen at 10^{-8} M for all enzymes. This effect of glucagon was exactly reproduced when the primary cultures of hepatocytes were preincubated with glucagon for **24 hours** (Figure 34). Recently, Wakelam et.al. (1986) have suggested that the effects of glucagon in the liver are mediated by its interaction with two distinct receptors, GR-1 (a receptor coupled to the stimulation of inositol phospholipid breakdown) and GR-2 (a receptor coupled to the stimulation of adenylate cyclase activity). The biphasic effect of glucagon on androst-4-ene-3,17-dione metabolism observed is similar to that seen for the glucagon-stimulated production of inositol phosphates, with inhibition occurring at higher glucagon concentrations. The inhibitory second phase of the glucagon-stimulated inositol phosphates production occurs at higher glucagon concentrations when adenylate cyclase activity is maximally stimulated (Wakelam et.al., 1986). The use of TH-glucagon, a glucagon analogue which stimulates the production of inositol phosphates in a dose-dependent manner but does not activate adenylate cyclase or cause any increase in cyclic AMP in hepatocytes, helped to place the puzzle into a better perspective. Recently, we have presented evidence showing the dose-dependent effect of TH-glucagon on androst-4-ene-3,17-dione metabolism, an analogous observation with the effect of TH-glucagon on inositol phosphate turnover (Hussin et.al., 1988) suggesting that the effect of glucagon on steroid metabolism is mediated via the GR-1 receptor linked to phosphatidylinositol-4,5-bisphosphate turnover. TH-glucagon is a potent antagonist of glucagon activation of the hepatic adenylate cyclase assay system (Johnson et.al., 1982) and is 7 % as active as glucagon in binding to glucagon receptors in the rat liver (Epand, et.al., 1976). We have carried out dose-response studies with TH-glucagon at different periods of preincubation in order to investigate the possible molecular mechanism of action of glucagon in mediating its effect

on steroid metabolism.

From our study, we have gathered that the effect of TH-glucagon on steroid metabolism is time-dependent. The acute effect (**1/2 hour**) of TH-glucagon was seen to increase all the enzyme activities (Figure 42) while its chronic effect (**24 hours**) was to decrease the activities (Figure 45). In isolated hepatocytes, TH-glucagon is an extremely weak partial agonist for cyclic AMP accumulation by having no ability to activate adenylate cyclase nor to stimulate protein kinase activity (Cote and Epand, 1979). However, TH-glucagon is a full agonist for the stimulation of glycogenolysis, gluconeogenesis and urea synthesis in rat hepatocytes (Corvera et.al., 1984), thus mimicking the effect of the endogenous hormone, glucagon. TH-glucagon causes a dose-dependent increase in the production of inositol 1,4,5-trisphosphate (Wakelam et.al., 1986) with concomitant release of diacylglycerol which stimulates the calcium/phospholipid-dependent protein kinase, protein kinase C. This is consistent with our observation of TH-glucagon's effect on androst-4-ene-3,17-dione metabolism at 24 hour of preincubation. We have also presented evidence that 4β-phorbol-12β-myristate-13α-acetate (PMA) but not A 23187, a calcium ionophore, was able to mimic the effect of glucagon and TH-glucagon on the enzyme activity suggesting that glucagon activated protein kinase C via generation of diacylglycerol. The dose-response curves to 10^{-8} M TH-glucagon at 24 hour is similar to that of glucagon at 1/2 and 24 hour implying that glucagon effects at both 1/2 and 24 hours are mediated by protein kinase C. The ability of protein kinase C to mediate the effect of glucagon on xenobiotic metabolism is not surprising since the phosphorylation of a number of cytochrome P-450 isoenzymes by protein kinase C has been demonstrated elsewhere (Pyerin et.al., 1987; Allan and Skett, 1988). Thus the inhibitory effect of glucagon on androst-4-ene-3,17-dione metabolism can be seen at 1/2 hour and 24 hour, a similar time-scale to the stimulatory effect of insulin (refer to Figure 31 and 34) as discussed in the earlier section. The physiological

relevance of the observed opposing effects of insulin and glucagon occurring at the same time in the cultured hepatocytes is not known and remains to be elucidated.

The paradoxical effect of TH-glucagon at 1/2 hour preincubation was not expected. This is surprising because of the ability of TH-glucagon to mimic the effect of glucagon on various liver parameters such as gluconeogenesis and glycogenolysis (Cote and Epand, 1979). However, TH-glucagon has been seen to reduce the blood sugar levels in streptozotocin-diabetic rats suggesting that it may be blocking the metabolic effects of glucagon in these hyperglycaemic animals (Johnson et.al., 1982). The maximal effect of TH-glucagon was observed after 5 minutes of drug administration and maintained for at least 105 minutes. Thus the effect of TH-glucagon does not always mimic the effect of glucagon. Following this, it is not surprising to see an increase in all the enzyme activities by TH-glucagon at 1/2 hour and to extend even up to 1 hour of preincubation (Figure 43). However the exact mechanism by which TH-glucagon increased the enzyme activities at the short time periods remains unclear.

As expected, the incubation of the cells with glucagon and the non-specific protein kinase inhibitor, K-252a, resulted in the returning of the enzyme activities to control, confirming our hypothesis that glucagon's effect on androst-4-ene-3,17-dione metabolism is mediated by phosphorylation. Preincubation with glucagon for 1/2 hour in the presence of K-252a resulted in the complete abolition of the glucagon dose-response curves (Figure 38). Longer period of preincubation with K-252a i.e. 24 hours, resulted in the inversion of the dose-response curves (Figure 39). This was not expected and the explanation for this is not known and remain to be elucidated.

The effect of glucagon on steroid metabolism at 1 and 48 hour and at 2 and 72 hour were similar. Therefore their results will be discussed together. At 1 and 48 hour (Figure 32 and 35 respectively) of preincubation, the effect of glucagon on the

enzyme activities was the reverse of that seen at 1/2 and 24 hour. Maximum stimulation of enzyme activities could be seen at 10^{-8} M glucagon. Glucagon has been shown to stimulate a low K_m membrane bound phosphodiesterase in the adipose tissue (Solomon, 1975) and liver (Loten et.al., 1978). By devising a technique, Heyworth and colleagues (1983b) managed to resolve the subcellular membrane fractions and identify cyclic AMP phosphodiesterase activity in both membrane and cytosol fractions in hepatocytes. They have discovered that glucagon is able to activate a fraction which they had called 'dense vesicle' phosphodiesterase, with criteria similar to the membrane bound low K_m phosphodiesterase described by Loten et.al. (1978). Both Loten and co-workers (1978) and Solomon (1975) have reported the ability of glucagon to activate the phosphodiesterase at physiological concentrations (10^{-10} - 10^{-8} M) with an absence of effect at higher concentrations. Interestingly, this effect of glucagon was found to be similar to the effect of glucagon on androst-4-ene-3,17-dione metabolism suggesting that the effect of glucagon on steroid metabolism at 1 hour could possibly be mediated via the activation of the low K_m phosphodiesterase.

At **2 and 72 hours** of preincubation, glucagon showed a sex-differentiation in its effects on enzyme activities (Figure 33 and 36 respectively). None of the female-specific enzyme activities were significantly altered by glucagon. On the other hand, the male-specific enzyme activity were significantly increased. The mechanism behind this differential effect of glucagon at 2 and 72 hours is not known and neither is its physiological relevance. It is worth mentioning that in man, significant increments in growth hormone levels were found within 2-3 hours of subcutaneous administration of glucagon (Mitchell et.al., 1969). There is a possibility that some of the effect of glucagon on xenobiotic metabolism could be mediated indirectly via the pituitary gland.

In summary, glucagon has a time- and concentration-dependent effect on androst-4-ene-3,17-dione metabolism by acting directly on the liver. Glucagon decreases the

steroid metabolism at 1/2 and 24 hour of preincubation. This effect of glucagon has been shown to be mediated by a phosphorylation reaction since its effect was abolished by a protein kinase inhibitor, K-252a. The phosphorylation reaction is thought to be brought about by the activation, by diacylglycerol, of protein kinase C subsequently leading to a decrease in the enzyme activities. This is supported by the observed effect of TH-glucagon at 24 hour of preincubation on androst-4-ene-3,17-dione metabolism and the fact that PMA mimicked the effect of glucagon in decreasing the enzyme activities. It should be noted though that at other periods of preincubation with glucagon, increased enzyme activities were also observed. The data gathered suggest that glucagon does have a role in the regulation of steroid metabolism in the rat liver. As for the TH-glucagon, its effect on androst-4-ene-3,17-dione metabolism is time-dependent. The enzyme activities were increased at 1/2 and decreased at 24 hour respectively.

9.4 THE EFFECT OF GLUCAGON ON ANDROST-4-ENE-3,17-DIONE METABOLISM IN HEPATOCYTES ISOLATED FROM STREPTOZOTOCIN-DIABETIC MALE RATS

Glucagon influences many metabolic parameters in the liver and among the important ones is the regulation of ketone and glucose homeostasis. In type 2 diabetes, glucose metabolism by the liver is altered partly due to the high serum glucagon level (Best et.al., 1982). Since hyperglucagonaemia is usually present in uncontrolled type 2 diabetes, this hormonal excess has been implicated in the overall pathophysiology of the disease (Unger and Orci, 1981). With regards to drug metabolism, glucagon has been shown to increase the metabolism of various drugs in the mouse (Rouer et.al., 1985). Since glucagon has been shown to have an influence on steroid metabolism (previous section) and has been implicated in the overall picture of the diabetic condition, it is pertinent for us to extend our investigation to the effect of glucagon in the diabetic rat hepatocytes.

In the diabetic (3 days STZ-treated) rats, the second phase of glucagon action normally seen at 1/2 hour in normal rat hepatocytes was not evident (Figure 37). In fact, the first depressive phase of glucagon, which reached a maximum at 10^{-9} M, was maintained at higher glucagon concentrations (about 70 to 80 % of control). These data indicate that the repressive effect of the adenylate cyclase system on inositol phosphate production had not occur, possibly due to a defect in the adenylate cyclase system. The ability of glucagon to stimulate liver membrane adenylate cyclase is, indeed, decreased in diabetic rats (Portha et.al., 1983). Dighe et.al. (1984) reported a 50 % reduction in glucagon stimulation of adenylate cyclase accompanied by a 67 % reduction of the glucagon receptor levels. This result is similarly seen in man; Arner et.al. (1987a) reported a reduction of adenylate cyclase activity of 35-50 % in membranes from type

2 diabetic patients. The activity of the low K_m phosphodiesterase was also decreased in plasma membranes from diabetic rats accounting for the increased tissue levels of cyclic AMP (Pilkis et.al., 1974). The reduced adenylate cyclase activity could probably be due to a decrease in the amount of adenylate cyclase in the liver plasma membranes (Portha et.al., 1983) or a modification of the guanine nucleotide regulatory protein (Gawler et.al., 1987). This could probably account for the maintenance of the first, inhibitory phase of glucagon's effect on androst-4-ene-3,17-dione metabolism in the diabetic rat hepatocytes seen in this study.

In summary, glucagon causes a dose-dependent decrease in androst-4-ene-3,17-dione metabolism in diabetic rat hepatocytes with maximum effect seen at 10^{-9} M concentration distinct from the V-shaped dose-response curves exhibited in normal rat hepatocytes. These differences could probably be attributed to a defect in the adenylate cyclase system in the diabetic hepatocytes.

9.5 THE EFFECT OF COMBINATIONS OF INSULIN AND GLUCAGON ON ANDROST-4-ENE-3,17-DIONE METABOLISM IN HEPATOCYTES FROM NORMAL AND STZ-DIABETIC MALE RATS

Numerous studies performed in animals and in man have shown the existence of peripheral plasma insulin and glucagon oscillations. The frequency of these oscillations is remarkably stable but their amplitude is increased after a meal and decreased by food deprivation. (Lefebvre et.al., 1987). The oscillatory pattern of plasma hormone levels may be physiologically important to reduce down-regulation of receptors and, consequently, to enhance hormone action. The interaction of these hormones and the feedback mechanism will determine the concentration of a particular hormone at one given time and hence influence the outcome of its physiological effects. For this reason we have decided to test the effect of insulin plus glucagon by adding them together in various combinations. In the result analysis we have decided to look at the influence of physiological (10^{-9} M) and supraphysiological (10^{-6} M) glucagon concentrations on insulin effect on androst-4-ene-3,17-dione metabolism due to the markedly different effects of the two concentrations of glucagon when given alone.

In the **normal** rat, glucagon at 10^{-9} M selectively decreased the effect of 10^{-9} M insulin on 16α-hydroxylase and 17-OHSD while having little effect on the other enzyme activities studied (Figure 40). A selective decrease in the activity of the male-specific enzymes is evident indicating, for the first time, the possible importance of hormonal interaction in expressing sexual differences in steroid metabolism. In the normal rat, many reports have indicated the ability of insulin to lower the cyclic AMP concentration when it is added in the presence of glucagon (Loten et.al., 1978; Heyworth et.al., 1983b) and the magnitude of this effect was dependent on insulin concentration (Heyworth et.al., 1983a). Insulin and glucagon together, each in a maximally effective

concentration have a greater effect on the low K_m phosphodiesterase than does either hormone alone (Loten et.al.,1978; Heyworth et.al., 1983b). Presumably at 10^{-9} M, the activation of protein kinase C by glucagon (and, thus, a decrease in enzyme activity) is dominant over the effect of insulin to increase the enzyme activities. Moreover, protein kinase C, which is activated by phorbol esters, has been shown to phosphorylate the serine residue of the insulin receptor, resulting in about 65 % reduction of the tyrosine kinase activity (Bollag et.al., 1986). However, it is not known why only the 16α-hydroxylase and 17-OHSD activity were selectively inhibited.

Again in the normal rat, glucagon at 10^{-9} M, selectively inhibited the effect of 10^{-6} M insulin on the 16α-hydroxylase while potentiating the effect of insulin on the 6β-hydroxylase and 5α-reductase activity. At both 10^{-9} and 10^{-6} M insulin, the physiological glucagon concentration was able to reduce the activity of 16α-hydroxylase below the control indicating the susceptibility of this enzyme to glucagon. At 10^{-6} M insulin, 6β-hydroxylase and 5α-reductase activity were preferentially increased while the effect of insulin was significantly suppressed by glucagon.

In the normal rat, a supraphysiological glucagon concentration (10^{-6} M) could selectively antagonize and potentiate the effect of 10^{-9} M insulin on 17-OHSD and 5α-reductase activity respectively. It is interesting to note that only the non-cytochrome P-450-dependent enzymes are involved. This possibly indicates that the two non-cytochrome P-450-dependent enzymes are more susceptible to alteration by these two hormones at the concentrations used. These effects were only observed *in vitro* and whether they have any significant relevance *in-vivo* is not known.

In normal rat, 10^{-6} M glucagon potentiated the effect of insulin (10^{-6} M) on 6β-hydroxylase and 5α-reductase while antagonizing the effect of insulin on 16α-hydroxylase and 17-OHSD. The activity of 7α-hydroxylase was suppressed by insulin

(10^{-6} M) in the presence of 10^{-6} M glucagon.

In the acutely **diabetic** rat, physiological concentration of glucagon (10^{-9} M) selectively increased the activity of 16α-hydroxylase only in the presence of insulin (10^{-9} M) whereas insulin alone has little effect (refer to Figure 41). Thus there is a differential effect seen in normal and diabetic rat. The insulin receptor tyrosine kinase activity has been found to decrease in the diabetic rat (see section **9.2**) but the glucagon-stimulatable protein kinase C activity is unaffected. It is expected that the insulin and glucagon combination (both at 10^{-9} M) would result in a decrease in the enzyme activity. In the diabetic rat it is not known why the 16α-hydroxylase activity was increased.

In the diabetic rat, all the enzyme activities were not significantly altered by other glucagon and insulin combination (10^{-9} and 10^{-6} M respectively) and no explanation could be given presuming that the protein kinase C could still be activated by glucagon in the diabetic rat.

Glucagon at 10^{-6} M, selectively increased the effect of 10^{-9} M insulin on the 6β-hydroxylase and 17-OHSD activities but had little effect on the remaining three enzymes.With glucagon (10^{-6} M) and insulin (10^{-6} M) combination, none of the enzyme activities were significantly altered in the presence of this hormonal combination. This is consistent with our hypothesis that the adenylate cyclase system was defective in the diabetic state. This together with the defective second messenger systems associated with insulin would result in the absence of effect seen in the diabetic hepatocytes by the insulin and glucagon combination.

In summary, differential effects on the enzyme activities are evident when different concentrations of insulin and glucagon are added together. The effects are variable depending on the combinations of insulin and glucagon added. Therefore it can be speculated that different plasma levels of insulin and glucagon could partly contribute to

the role of hormonal regulation in sex-differentiation seen in the rat liver.

9.6 THE EFFECT OF PHENFORMIN ON ANDROST-4-ENE-3,17-DIONE METABOLISM IN NORMAL AND STZ--DIABETIC MALE RAT HEPATOCYTES

Sulphonylureas as well as biguanides are widely used as hypoglycaemic agents in the treatment of diabetes mellitus. Both group of drugs are used for the treatment of type 2 or insulin-independent diabetic patients. Since both drugs are most effective in those patients with the ability to secrete insulin, it has been postulated that both may act to potentiate the effects of insulin at the cellular level (Shen and Bressler, 1977). The clinical use of these drugs is well reported on, but little is known about their mechanism of action at the cellular level up to date despite numerous reports from many laboratories. Our objective was to study the effect of these drugs, using phenformin and tolbutamide as representatives of biguanide and sulphonylurea respectively, and their possible interaction with insulin on steroid metabolism in rat liver.

In *normal* rat hepatocytes, phenformin has been demonstrated to cause a dose-dependent increase in all the enzyme activities with maximum response observed at 5×10^{-5} M (Figure 46). Many hypotheses have been put forward to explain the mechanism of action of phenformin and other biguanides. One possible mechanism is their ability to alter the function and physical structure of membranes by changing the electrostatic surface potential of the membrane. The perturbation of the membrane structure and physical membrane properties may then be transmitted to integral membrane proteins and their catalytic function (Schafer, 1976). Studies *in-vitro* have shown a rapid dose-dependent effect of biguanides on insulin binding to target cells (Cohen et.al., 1980; Vigneri et.al., 1982). They have been shown to increase insulin-receptor binding in a variety of cultured cells (Pezzino et.al., 1982) and cells from diabetic (Pagano et.al., 1983) and non-diabetic subjects (Bailey , 1988). However,

many studies have reported pharmacological and biochemical effects of biguanides to enhance insulin action without measurable effect on hepatocyte insulin-receptor binding (Lord et.al., 1985; Purrello et.al., 1988), suggesting that the biguanides can influence postreceptor sites of insulin action independently of insulin receptor binding. This idea was substantiated by a report (Jacobs et.al., 1986) showing the ability of metformin (another biguanide) to enhance hexose transport in the absence or presence of insulin despite seeing no changes in the insulin receptor number or affinity or the insulin-receptor tyrosine kinase activity. The effect of biguanide was not blocked by cycloheximide indicating that synthesis of new receptors is not required to increase insulin binding (Pezzino et.al., 1982).

Any influence (direct or indirect) by phenformin on the phosphorylation state of the cytochrome P-450 could affect the amount of metabolites being produced. In relation to this, a study has demonstrated that phenformin stimulated a high affinity cyclic AMP phosphodiesterase of isolated liver plasma membrane in a dose-dependent way, decreasing the intracellular cyclic AMP content of the isolated hepatocytes without being effective on plasma membrane-bound adenylate cyclase (Luly et.al., 1977). These effects on the high affinity cyclic AMP phosphodiesterase and on the intracellular cyclic AMP content could probably account for the observed increase in all the enzyme activities by phenformin.

In the *diabetic* hepatocytes , phenformin still exhibited a dose-dependent increase in all the enzymes activities but there was a decrease in the drug responsiveness and sensitivity (Figure 52) suggesting the existence of receptor and postreceptor defects. As for the latter, there was a slight shift in the dose-response curve. In diabetic rats, the hepatocytes were not responsive to insulin but were still able to respond to phenformin. Metformin has been reported to increase insulin-mediated glucose uptake by rat adipocytes without affecting the insulin receptor binding and insulin receptor β-subunit

phosphorylation (Jacobs et.al., 1986). Presuming that phenformin has a similar effect as metformin, the former could possibly exerts its effect on androst-4-ene-3,17-dione metabolism by acting through mechanisms that may be beyond insulin receptor binding that has yet to be determined. Phenformin has been shown to cause an increase in insulin binding in diabetic rat liver plasma membrane (Chaujar et.al., 1984). Biguanides have been suggested to ameliorate insulin resistance by causing a positive influence on tissue sensitivity to insulin by acting at the postreceptor level (Lord et.al., 1983; Lord et.al., 1985). Recently Gawler et.al. (1987) demonstrated that metformin was able to restore the ability of insulin to inhibit glucagon-stimulated adenylate cyclase activity and suggested an effect of metformin distal to insulin binding.

Many studies have demonstrated the potentiating effect of biguanides on insulin actions (Lord et.al., 1985; Jacobs et.al., 1986). Recently, metformin has been shown to enhance only some of the actions of insulin (Purrello et.al., 1988). Metformin potentiated insulin-stimulated [^{3}H] glucose incorporation into glycogen and tyrosine-aminotransferase activity but had no effect on insulin-stimulated [1-^{14}C] aminoisobutyric acid uptake. In our study using *normal* rat hepatocytes, insulin in the presence of phenformin (10^{-3} M) selectively produced greater effect on the 6β-hydroxylase, 17-OHSD and 5α-reductase activities than with insulin or phenformin alone (Table 44). This concentration of phenformin was used since it appears to be very close to the drug clinical serum level (Luly et.al., 1977). In the presence of phenformin (10^{-3} M) the maximum effect of insulin was achieved at 10^{-9} M concentration while the effect of insulin has not even reached its maximum at 10^{-6} M in the absence of phenformin (refer to Figure 46). It seems that phenformin may inhibit the ability of insulin to increase the activity of 7α- and 16α-hydroxylases. On the other hand, it is apparent that phenformin (10^{-3} M) markedly lowered the maximal response of the cells to insulin (compare to

dose-response effect of insulin in normal rat hepatocytes-Figure 7). Mutual inhibition of the stimulatory effect of insulin and phenformin on the high affinity cyclic AMP phosphodiesterase activity has also been reported by Luly et.al. (1977). They have reported that insulin in the presence of phenformin produced lower stimulation of the high affinity cyclic AMP phosphodiesterase activity than that produced by insulin or phenformin alone. The mechanism by which phenformin reduces the response of the liver cells to insulin is not known and further work is needed for its elucidation.

In the *diabetic* rat hepatocytes, phenformin (10^{-3} M) shows no potentiation of the effect of insulin on any of the enzymes except the 7α- and 6β-hydroxylase activity at physiological insulin concentrations (10^{-10} and 10^{-9} M) (Table 50). The high phenformin concentration (10^{-3} M) has no potentiating effect on insulin within the range of insulin concentrations used.

If the format of the experiment is changed to observe the influence of physiological insulin (10^{-9} M) on the effect of a range of concentrations of phenformin on the enzyme activities, it is seen, in *normal* rat, that insulin (10^{-9} M) potentiated phenformin effect on all the enzyme activities (Table 46). Again phenformin plus insulin produced higher enzyme activities than 10^{-9} M insulin or 10^{-9} M phenformin alone.

In the *diabetic* rat, a physiological insulin concentration (10^{-9} M) potentiated the effect of phenformin on all the enzyme activities with the exception of the 7α-hydroxylase (Table 52). Insulin only potentiated the effect on 16α-hydroxylase and 5α-reductase activity at high phenformin concentration i.e. 10^{-3} M which would account for the lack of effect of 10^{-3} M phenformin on increasing insulin concentration mentioned in the last paragraph. It is apparent that in the diabetic rat, that a physiological insulin concentration is only able to potentiate the effect of submaximal phenformin concentrations.

In summary, phenformin was shown to have a direct effect on the liver in elevating androst-4-ene-3,17-dione metabolism. It is able to mimic the effect of insulin in normal and diabetic rat hepatocytes with respect to androst-4-ene-3,17-dione metabolism though there was a decrease in the liver cells responsiveness and sensitivity in the latter. Physiological insulin concentration potentiated the effect of phenformin on almost all of the enzyme activities. A direct effect of phenformin on cytochrome P-450 cannot be ruled out.

9.7 THE EFFECT OF TOLBUTAMIDE ON ANDROST-4-ENE-3,17-DIONE METABOLISM IN NORMAL AND STZ--DIABETIC MALE RAT HEPATOCYTES

In *normal* rat hepatocytes, tolbutamide elicited a dose-dependent increase in androst-4-ene-3,17-dione metabolism (Figure 47) similar to that exhibited by phenformin. The mechanism by which tolbutamide increased all the steroid enzyme activities is unclear at present. There is some evidence that part of the therapeutic effects of sulphonylureas on glucose metabolism are due to an extrapancreatic action of the drugs. It has been suggested that sulphonylureas , administered *in-vivo* (Olefsky and Reaven, 1976; Feinglos and Lebovitz, 1978) or employed *in-vitro* (Prince and Olefsky, 1980), may enhance insulin action through increasing the hormone binding to its receptors. However, exposure of cultured cells to sulphonylureas has yielded variable results. Various reports have reported no changes in insulin receptor binding (Maloff and Lockwood, 1981; Vigneri et.al., 1982; Dolais-Kitabgi et.al., 1983). The report of an enhancement of maximal insulin-stimulated glucose uptake (Maloff and Lockwood, 1981) and lipogenesis (Salhanick et.al., 1983) in the absence of any effect on insulin receptor binding, suggests a direct effect of sulphonylureas on the post-binding pathways of insulin action.

Specific binding of sulphonylureas to receptors on the β-cell membrane has been shown (Lebovitz, 1984) and it has been suggested that the sulphonylurea receptor may constitute part of an ATP-sensitive K^+ channel (Sturgess et.al., 1985). Tolbutamide has been suggested to decrease the K^+ permeability of the β-cell membrane (Henquin, 1980) by inhibiting ATP-sensitive K^+ channels (Sturgess et.al., 1985). The inhibition of an (Na^+-K^+)-ATPase by chlorpropamide has been reported in isolated liver plasma membrane (Luly et.al., 1977). It is not known whether part or all of tolbutamide effect on the steroid metabolism is mediated via an alteration of this enzyme.

Another possible mechanism through which tolbutamide could affect the steroid metabolism is its effect on cyclic AMP metabolism. Sulphonylureas have been shown to alter the activity of the adenylate cyclase. However, the changes induced in cyclic AMP level may be variable, depending upon the tissue studied and the experimental system used (Blumenthal, 1977; Leichter and Chase, 1978). Sulphonylureas have been demonstrated to increase adenylate cyclase activity in the heart (Lasseter et.al., 1972) but have the opposite effect in the kidney and liver (Leichter and Galasky, 1981) while having no effect on plasma membrane-bound adenylate cyclase in the liver (Luly et.al., 1977).

Another means through which tolbutamide could increase androst-4-ene-3,17-dione metabolism is by lowering intracellular cyclic AMP concentration via activation of the nucleotide degrading enzyme, phosphodiesterase. In fact, sulphonylureas have been shown to affect the activity of low K_m cyclic AMP phosphodiesterase but the effects reported have been inconsistent. Goldfine et.al.(1971) have reported an inhibition of the cyclic AMP phosphodiesterase activity by tolbutamide in rat liver, lung, kidney and brain tissues. Brooker and Fichman (1971) also reported an inhibition of the cyclic AMP phosphodiesterase by chlorpropamide and tolbutamide in rat kidney. However, these two studies were conducted using microsomal preparation and no measurement of cyclic AMP concentration were made. A more pertinent report to our study, and probably a more physiological study, was conducted by Luly et.al. (1977). They have found a chlorpropamide-stimulated low K_m cyclic AMP phosphodiesterase of isolated liver plasma membrane. The drug also decreased the intracellular cyclic AMP content of isolated hepatocytes without affecting the plasma membrane-bound adenylate cyclase indicating that chlorpropamide has no effect on the synthesis of cyclic AMP.

Recently, it was reported that tolbutamide inhibited the protein kinase A activity in rat liver cytosol by inhibiting the activity of the catalytic units of the protein kinase A in

a dose-dependent manner (Okuno et.al., 1988). Androst-4-ene-3,17-dione metabolism is controlled by specific enzymes, the activities of which seem to be altered by the phosphorylation states of the liver cells. Thus, the ability of tolbutamide, presumably having a similar mechanism of action, to inhibit the activity of protein kinase A is consistent with the observed dose-response increase in androst-4-ene-3,17-dione metabolism by tolbutamide.

Tolbutamide has also been reported to increase soluble and particulate guanylate cyclase activity in rat liver, lung, heart, spleen, colon, pancreas and kidney cortex (Vesely, 1986). Cyclic GMP has been suggested to play a role in glucose homeostasis (Vesely et.al., 1979). It still remain to be seen whether the Ying-Yang hypothesis with the observed increased cyclic GMP and reported decreased in cyclic AMP, has any role to play in steroid metabolism.

In the *diabetic* rat, tolbutamide was still able to increase all the enzyme activities despite a reduction in responsiveness (Figure 53). Selective decreases in the enzymes' sensitivity were also observed. The 16α-hydroxylase and 17-OHSD only responded to tolbutamide at higher concentrations (10^{-4} and 5 x 10^{-4} M respectively). Solomon et.al. (1986a) have reported a decrease in activity of cyclic AMP phosphodiesterase and calmodulin in the STZ-treated rat. Treatment with glyburide, a sulphonylurea, fully restored both activities to normal indicating both cyclic AMP phosphodiesterase and calmodulin as sites of action of glyburide, distal to the insulin receptor. A decrease in the cyclic AMP phosphodiesterase activity in the diabetic rat and the ability of tolbutamide to partially restore the enzyme's activity in the rat hepatocytes corresponds well with those of their reported antidiabetic effects (Melander, 1987).

In the *normal* rat, tolbutamide selectively inhibits the insulin-stimulated 5α-reductase activity (Table 45). With regard to the other enzymes, increasing insulin concentrations potentiated the effect of 10^{-3} M tolbutamide when compared to

tolbutamide (10^{-3} M) alone. Similar to phenformin, tolbutamide reduces the responsiveness of the insulin-stimulated steroid enzyme activities and the mechanism responsible for this is yet to be determined. In *diabetic* hepatocytes, increasing insulin concentrations in the presence of 10^{-3} M tolbutamide did not increase the enzyme activities except for the 16α-hydroxylase at 10^{-8} and 10^{-7} M insulin , when compared to tolbutamide 10^{-3} M alone (Table 51). When compared to the normal rat, there were fewer effects in the diabetic rat hepatocytes when insulin and tolbutamide 10^{-3} M were added together. This is possibly due to the postreceptor defects that exist in the diabetic state.

In the *normal* rat, increasing tolbutamide concentration selectively potentiated the insulin-stimulated activities of the non-cytochrome P-450 enzymes in a dose-dependent manner while the cytochrome P-450 enzyme activities were not significantly different from 10^{-9} M insulin alone (Table 47). In the *diabetic* rat, the drug and hormone combinations no longer selectively potentiated the non-cytochrome P-450 activities (Table 53). Only higher tolbutamide concentrations ($> 5 \times 10^{-5}$ M) potentiated 10^{-9} M insulin effect on 17-OHSD activity. The effect of insulin (10^{-9} M) on 16α-hydroxylase activity was potentiated by tolbutamide at concentrations as low as 10^{-6} M concentration. It is apparent that the combination of tolbutamide and insulin reduces the maximal response attained by tolbutamide or insulin alone in normal and diabetic rat hepatocytes.

In summary, tolbutamide has been shown to exert a direct effect on the liver *in-vitro* and mimicked the effect of insulin in stimulating the steroid enzyme activities in normal rat. Similar to phenformin, tolbutamide was still able to increase all the enzyme activities in diabetic hepatocytes with a shift in the drug responsiveness when compared

to its effect in normal rat hepatocytes. Although many biochemical actions have been reported on the potentiation of insulin action by tolbutamide and other sulphonylureas, we have found that, with respect to steroid metabolism, tolbutamide reduced the responsiveness of the liver cells towards insulin.

A direct effect of tolbutamide on cytochrome P-450 cannot be ruled out.

9.8 GENERAL DISCUSSION

All the results can be summarised as follows :

i) In *normal* rat hepatocytes, addition of **insulin** *in-vitro* caused an increase in steroid enzyme activities at 1/2 and 24 hour. The dose-response curves indicated that the effect of insulin at 1/2 hour could be mediated by a mechanism distinct from the effect of insulin at 24 hour pre-incubation. Our data suggested that insulin , *in-vitro*, acts as a general stimulator of the enzymes in the liver metabolizing androst-4-ene-3,17-dione substantiating our contention that insulin is a major factor in the regulation of xenobiotic metabolism in the rat liver.

ii) Hepatocytes from acutely STZ-treated *diabetic* rats are not responsive to insulin *in-vitro* with respect to androst-4-ene-3,17-dione metabolism and the responsiveness is partly restored by treating the diabetic rats with insulin. However, in the hepatocytes from chronically STZ-treated diabetic rats. the response to insulin is delayed and attenuated.

iii) In *normal* rat hepatocytes, the effect of **glucagon** on androst-4-ene-3,17-dione metabolism is time- and concentration-dependent.Glucagon decreases the steroid metabolism at 1/2 and 24 hours of pre-incubation and exhibited V-shaped dose-response curves. Our data indicated that the effect of glucagon is mediated by a phosphorylation reaction and is thought to be brought about by the activation by diacylglycerol of protein kinase C leading to a decrease in the enzyme activities.

iv) In the *diabetic* rats, glucagon causes a dose-dependent decrease in androst-4-ene-3,17-dione metabolism but no longer exhibit the V-shaped dose-response curves as seen in normal rat hepatocytes. It is suggested that these differences could probably be due to a defect in the adenylate cyclase system.

v) Differential effects on the enzyme activities are observed when different concentrations of **insulin** and **glucagon** are added together. Selective changes in the

activity of the male-specific and female-specific enzymes are evident indicating, for the first time, the possible importance of hormonal interaction in expressing sexual differences in steroid metabolism.

vi) **Phenformin** is able to mimic the effect of insulin in the normal and diabetic rat hepatocytes with respect to androst-4-ene-3,17-dione metabolism though there was a decrease in the liver cells responsiveness and sensitivity in the latter. Physiological insulin concentration potentiated the effect of phenformin on almost all of the enzyme activities.

viii) **Tolbutamide** is able to mimic the effect of insulin with respect to androst-4-ene-3,17-dione metabolism in normal rat hepatocytes. In the diabetic rat hepatocytes, there was a shift in the drug responsiveness. Unlike with phenformin, our data indicated that tolbutamide reduced the responsiveness of the liver cells towards insulin.

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APPENDIX I. abbreviations

The following abbreviations have been used :-

ADP	Adenosine-5'-diphosphate
ATP	Adenosine-5'-triphosphate
BSA	Bovine serum albumin
$[Ca^{2+}]_i$	Intracellular concentration of free calcium
c.p.m.	Counts per minute
Cyclic AMP	Cyclic adenosine-3',5'-monophosphate
Cylic GMP	Cyclic guanosine-3',5'-monophosphate
DMSO	Dimethylsulphoxide
EGF	Epidermal growth factor
FCS	Foetal calf serum
g	Gram
g	Centrifugal g-force
G	Glucagon
G_i	Inhibitory guanine nucleotide regulatory protein
G_{ins}	Guanine nucleotide regulatory protein specific for insulin.
G-protein	Guanine nucleotide regulatory protein
G_s	Stimulatory guanine nucleotide regulatory protein.
GTP	Guanosine-5'-triphosphate
HBSS	Hank's balanced salt solution
H.P.L.C	High performance liquid chromatography
HS	Horse serum
I	Insulin
IBMX	3-isobutyl-1-methylxanthine

IGF	Insulin-like growth factor
IPG	Inositol phosphate-glycan
K_a	Association constant
kDa	Kilodalton
K_i	Inhibitory constant
K_m	Substrate concentration producing half-maximal velocity.
K_s	Spectral dissociation constant
M_r	Relative mass
M.W.	Molecular weight
NADP	Nicotinamide adenine dinucleotide phosphate (oxidized).
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced).
NIDDM	Non-insulin-dependent diabetes mellitus
OHase	Hydroxylase
OHSD	Oxosteroid oxidoreductase
P	Phenformin
PI	Phosphatidylinositol
PLC	Phospholipase C
PMA	4β-phorbol-12β-myristate-13α-acetate
Protein kinase A	Cyclic AMP-dependent protein kinase
Protein kinase C / PK_C	Calcium phospholipid-dependent protein kinase
Protein kinase FA	A multifunctional protein phosphatase activator
RNA	Ribonucleic acid
S.D	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis.

STZ	Streptozotocin
T	Tolbutamide
TH-glucagon	[1-N$^{\alpha}$-trinitrophenylhistidine,12-homoarginine] glucagon.
t.l.c.	Thin layer chromatography
Tyr	Tyrosine
U	Unit
V_{max}	Maximal velocity
K-252a	(8R*, 9S*, 11S*)-(-)-9-hydroxy-9-methoxy-carbonyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1H,8H,11H-2,7b,11a-triazadibenzo (a,g) cycloocta (cde) trindene-1-one

APPENDIX II.

Publications.

i) Hussin, A.H. and Skett, P. (1986)
Maintenance of steroid metabolism in primary cultures of adult rat hepatocytes in serum-free medium. *Biochem. Soc. Trans.* **14**, 914-915

ii) Hussin, A.H. and Skett, P. (1987)
The effect of insulin on steroid metabolism in isolated rat hepatocytes. *Biochem. Pharmac.* **36**, 3155-3159

iii) Hussin, A.H., Allan, C.J., Hruby, V.J. and Skett, P. (1988)
The effects of glucagon and TH-glucagon on steroid metabolism in isolated rat hepatocytes. *Mol. Cell. Endoc.* **55**, 203-207

iv) Hussin, A.H. and Skett, P. (1988)
Lack of effect of insulin in hepatocytes isolated from streptozotocin-diabetic male rats. *Biochem. Pharmac.* **37**, 1683-1686