EFFECTS OF STEROIDS ON LIVER LIPOGENIC AND GLUCONEOGENIC ENZYMES; ROLE OF INSULIN AND GLUCAGON

THESIS

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Dedicated to:

My husband Aziz

My daughter Dina

My son Ahmed

C O N T E N T S

C H A P T E R I

INTRODUCTION AND REVIEW OF LITERATURE

C H A P T E R I I

MATERIALS AND METHODS

A C K N O W L E D G E M E N T S

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A B S T R A C T

The effects of 17-β-oestradiol, progesterone and corticosterone on liver lipogenic and gluconeogenic enzymes have been examined in female ovariectomised rats. The activities of these enzymes were correlated with alterations in portal vein insulin and glucagon levels and with changes in responsiveness of the liver to pancreatic hormones.

Long term treatment of rats with 17-β-oestradiol induced the activity of liver lipogenic enzymes, acetyl CoA carboxylase and fatty acid synthetase. This effect was dose dependent and correlated with the rise in plasma triglyceride concentration caused by the administration of oestrogen.

Oestradiol inhibited liver gluconeogenic enzyme, phosphoenolpyruvate carboxykinase and reduced the flow of gluconeogenic intermediates through this step. These changes were associated with an increase in insulin : glucagon molar concentration ratio (I/G) in portal vein blood secondary to marked reductions in glucagon secretion. Oestradiol also impaired the sensitivity of liver cells to glucagon. The altered secretion and action of the pancreatic hormones, therefore, could be held responsible for the inhibited activity of phosphoenolpyruvate carboxykinase and the reduction in fasting plasma glucose concentration observed in oestrogen

treated rats.

Progesterone increased the secretion of both insulin and glucagon equally and hence the ratio between these two hormones remained constant. No significant changes were observed in the sensitivity of liver cells to these hormones, nor was there any alteration in the activity of lipogenic and gluconeogenic enzymes. Progesterone, therefore, did not affect the fasting plasma glucose or triglyceride concentrations.

Bilateral adrenalectomy in rats reduced the activity of liver lipogenic enzymes, while pharmacological doses of corticosterone induced their activity. These changes were not related to alterations either in the secretion of insulin and glucagon or in the sensitivity of liver cells to these hormones. The results suggest that the effects of glucocorticoids on plasma triglyceride concentration might be due to the direct actions of the steroid on liver lipogenic enzymes.

Though bilateral adrenalectomy lowered the insulin : glucagon molar concentration ratio in portal vein blood, the liver of these animals was resistant to the action of glucagon. Consequently, hepatic phosphoenolpyruvate carboxykinase activity was inhibited resulting in a fall in fasting plasma glucose level.

Moderate doses of corticosterone administered to rats increased the insulin : glucagon molar concentration

ratio, reduced phosphoenolpyruvate carboxykinase and inhibited gluconeogenesis. The fasting plasma glucose in these animals was lower than that of pair-fed control rats. Large doses of corticosterone, on the other hand, impaired the hepatic response to insulin and consequently enhanced phosphoenolpyruvate carboxykinase activity despite the marked hyperinsulinaemia. Fasting plasma glucose concentration was, therefore, increased in these animals.

In conclusion, the results indicate that part of the effects of steroids on lipid metabolism is due to their direct action stimulating the activity of liver lipogenic enzymes. The changes in gluconeogenic enzymes and hepatic carbohydrate metabolism, on the other hand, are in part mediated via alterations in pancreatic alpha and beta cell function with changes in portal vein insulin and glucagon levels. Moreover, some of the steroids appear to modify the hepatocyte response and hence modulate the biological effectiveness of the pancreatic hormones.

C H A P T E R I

INTRODUCTION AND REVIEW OF LITERATURE

I N T R O D U C T I O N

The use of steroid hormones and their synthetic analogues as pharmacological agents has profoundly influenced medical practice during the past thirty years. Female gonadal steroids have provided a convenient and reversible means of contraception for many females during child bearing years and they are alleviating many of the symptoms associated with the menopause. Glucocorticoids have proved to be effective as immunosuppressive agents and have been used successfully in the management of many diseases previously refractory to medical treatment. Unfortunately, the long term administration of these steroids induces several metabolic abnormalities which might be responsible for the increased cardiovascular complications occurring in these patients (Stern et al 1973 and Mann et al 1975).

Changes in fasting plasma triglycerides associated with an increase in the hepatic production of these molecules have been reported to occur in women and in experimental animals receiving the oestrogen-progestogen preparations (Doar & Wynn 1969; Larsson-Cohn et al 1970; Stokes & Wynn 1971; Kissebah et al 1973; and Afolabi 1974). Changes in hepatic output of glucose sometimes leading to deterioration of glucose tolerance have also been observed during the intake of these steroids (Doar & Wynn 1969; Larsson-Cohn et al 1970).

Similarly, an increase in plasma triglyceride synthesis and in glucose output leading to hypertriglyceridaemia and hyperglycaemia are common findings in patients on long term glucocorticoid therapy (Perly & Kipnis 1966 and Stern et al 1973). The conclusions from these studies suggest that steroids greatly influence the liver and that the altered metabolism of this organ could indeed be the major factor in genesis of the metabolic changes induced by these agents.

It is not certain whether the changes in liver metabolism are the direct effects of the steroids themselves or secondary to some other hormonal changes associated with the intake of these agents. Of particular importance in this respect are insulin and glucagon, since these hormones are known to play an important role in the control of hepatic metabolism and their concentration in blood seem to be influenced by the administration of steroids.

The aim of this study was to explore the role of altered pancreatic beta and alpha cell functions in the metabolic changes associated with the therapeutic intake of steroids. For this purpose, we have examined the effects of 17-β oestradiol, progesterone and corticosterone on some of the liver enzymes active in the control of lipogenesis and gluconeogenesis in the rat. Concurrently we have measured the portal vein blood levels of insulin and glucagon and tested the biological effectiveness of

these hormones in livers of steroid treated animals. We were then able to correlate the changes in hepatic activity induced by the steroids to alterations either in the secretion and/or in the actions of insulin and glucagon.

A. REGULATION OF HEPATIC TRIGLYCERIDE TRANSPORT; EFFECTS OF STEROIDS

I. General Outline

In the postabsorptive state when no exogenous fat is ingested, almost all circulatory triglycerides are derived from hepatic synthesis. Most of the endogenous triglycerides are transported in plasma as the major lipid component of very low density lipoproteins (VLDL). About thirty per cent of the total fasting plasma triglycerides are transported with other lipoprotein fractions (LDL & HDL).

Triglycerides are synthesised in the liver by a complex process which leads to the esterification of glycerophosphate with long chain fatty acids. Figure (1) shows a schematic representation of the possible sources from which the fatty acid portion could be derived. Thus, triglyceride fatty acids could be synthesised de novo from acetyl CoA units resulting from the catabolism of glucose and other carbohydrate sources (lipogenesis). Alternatively, fatty acids could be extracted by the liver from plasma free fatty acids (FFA) derived from the breakdown of adipose tissue triglycerides during the process of lipolysis. Finally, the fatty acid portion could be derived from a hepatic store of triglycerides which could be hydrolysed by the activity of a hepatic lipase.

The relative contribution of each of the above sources seems to vary both with the animal species and with the

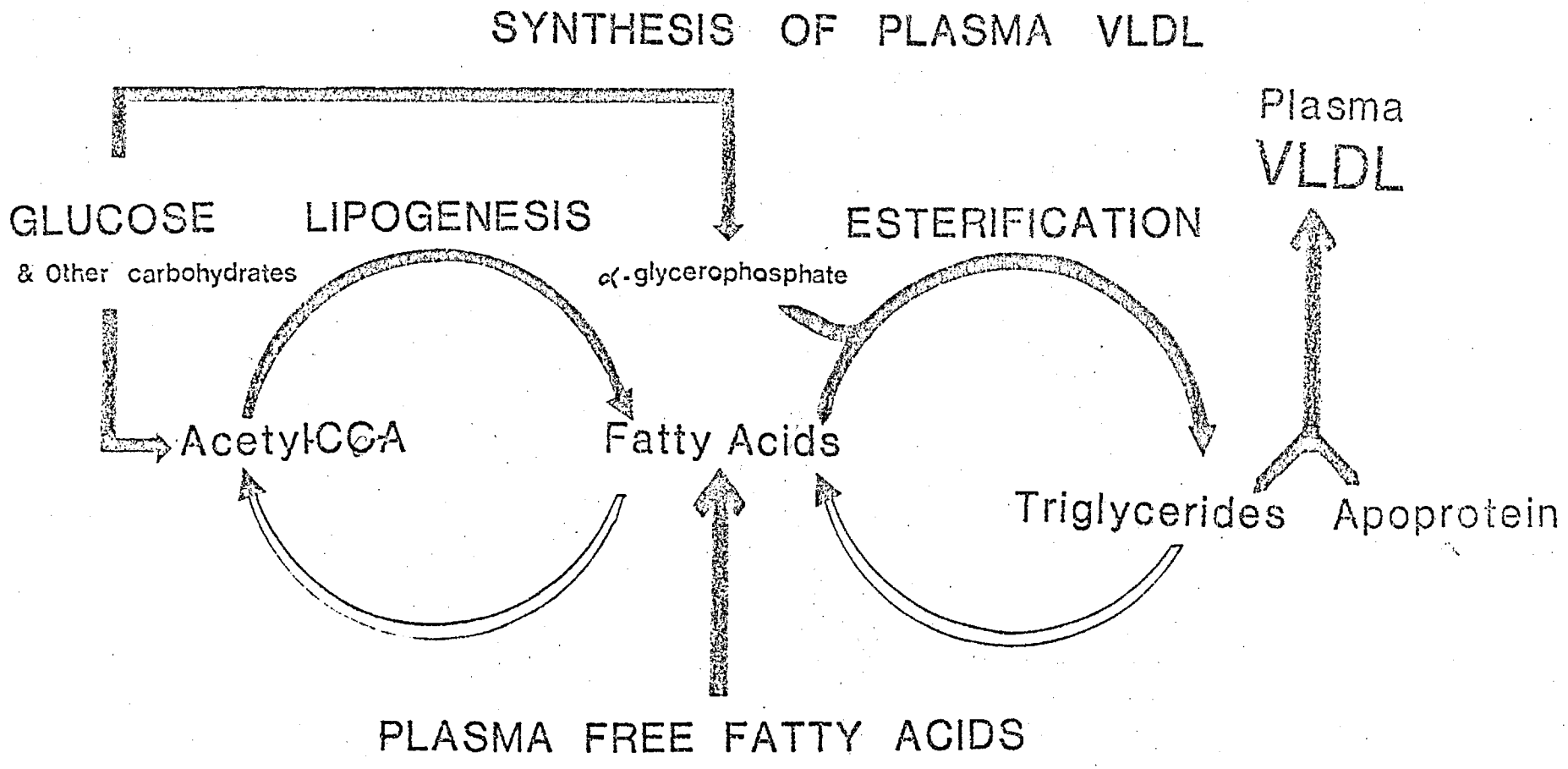

SYNTHESIS OF PLASMA VLDL
Plasma
VLDL
GLUCOSE
& Other carbohydrates
LIPOGENESIS
α-glycerophosphate
ESTERIFICATION
Acetyl-CoA
Fatty Acids
Triglycerides
Apoprotein
PLASMA FREE FATTY ACIDS

FIGURE (1)

dietary condition. In man, after an overnight fast, a major source of plasma triglyceride fatty acids is plasma FFA (Havel 1961; Friedberg et al 1961; Carlson & Ekelund 1963 and Eaton et al 1969). During carbohydrate feeding, however, hepatic lipogenesis from glucose becomes the main immediate precursor for the triglyceride fatty acids (Barter et al 1972; Barter & Nestel 1973). In rats, a minor fraction of triglyceride fatty acids is derived from plasma FFA and hepatic lipogenesis from glucose represents the major source (Baker and Schotz 1964). The fatty acids released during the hydrolysis of hepatic triglyceride stores could be reassimilated into plasma triglycerides only in special conditions such as chronic alcoholism (Barter et al 1972), obese subjects (Barter & Nestel 1973) and diabetic dogs with fatty liver (Basso & Havel 1970).

Figure (2) is a schematic diagram showing the important steps involved in the process of hepatic lipogenesis and which can be summarised as follows: the pyruvate units (I) resulting from the breakdown of glucose, fructose and other sources are first transported to the cell mitochondria where they are converted to acetyl CoA units (II). The latter could also be derived from the partial degradation of fatty acids in the liver. Acetyl CoA condenses with oxaloacetate (III) to form citrate (IV). The latter is then transported to the cytoplasm and acetyl CoA is subsequently released by the action of the citrate cleavage enzyme. Acetyl CoA is carboxylated in the presence of adenosine triphosphate (ATP) and Mn^{++} or Mg^{++} to produce malonyl CoA (V). This latter

PATHWAY OF HEPATIC LIPOGENESIS

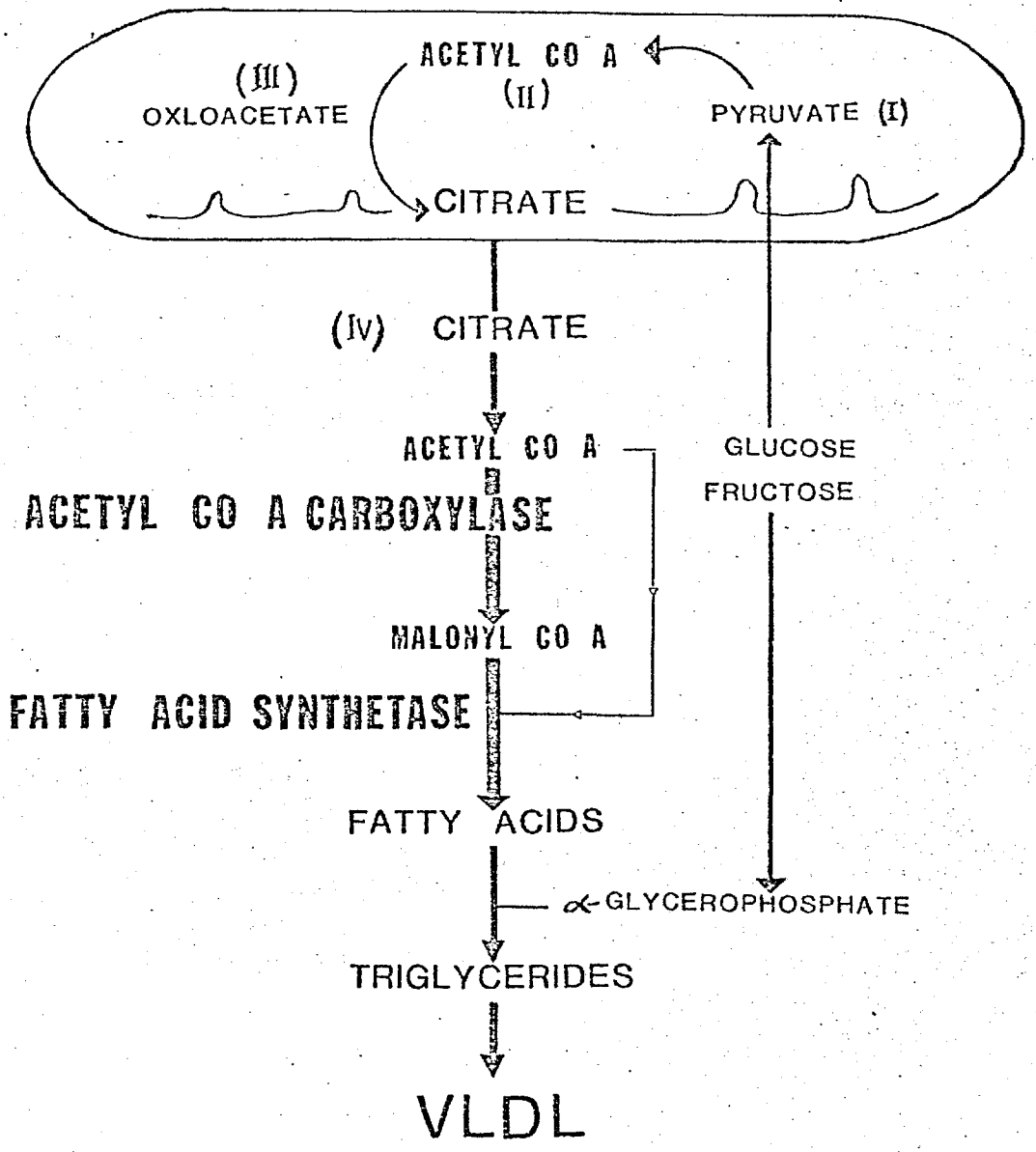


FIGURE (2)

step is catalysed by the enzyme acetyl CoA carboxylase. Malonyl CoA then condenses with other acetyl CoA units to produce long chain fatty acyl CoA (VI) via the activity of the fatty acid synthetase.

It is currently believed that the activities of acetyl CoA carboxylase and fatty acid synthetase enzymes represent the key points or rate limiting steps in the control of hepatic lipogenesis (Numa et al 1965; and Vagelos et al 1966). There is also evidence to suggest that the rate of hepatic triglyceride secretion is determined by the availability of fatty acyl CoA suggesting that no rate limiting reactions are situated between the synthesis of fatty acyl CoA and the release of triglycerides. Indeed Windmuller & Spaeth (1967) have reported a close correlation between the rate of fatty acid synthesis and the rate of triglyceride secretion from rat livers. Under special circumstances, however, the rate of hepatic fatty acid esterification and the rate of synthesis of the lipoprotein peptides might play an important role. Thus, in fasting and in diabetic animals the hepatic supply of fatty acids is high, while triglyceride secretion is low (Basso & Havel 1970). On the other hand, the chronic ingestion of ethanol decreases plasma FFA concentration, inhibits hepatic lipogenesis from glucose, and is associated with increased hepatic triglyceride production (Barter et al 1972).

II. Effects of Insulin and Glucagon

There is no doubt that in the rat, insulin stimulates

the hepatic release of triglyceride. Thus, Brady & Gurin (1950) and Altman et al (1951) have observed an enhancement in the synthesis of triglyceride from acetate in rat liver preparations incubated with the hormone in vitro. Chernick and Chaikoff (1950) have also reported an increase in the incorporation of glucose into triglycerides in livers obtained from animals treated with insulin. Furthermore, insulin added to liver slices in vitro increased the synthesis of triglyceride from glucose (Tulloch et al 1972).

Glucagon seems to exert opposite effects on hepatic lipid synthesis. The administration of cobalt chloride to destroy the glucagon secreting cells in rats caused an elevation in pre-β-lipoproteins and serum triglyceride concentration (Eaton; 1970). Also in dogs, surgical removal of body and tail of pancreas containing mainly the alpha cells resulted in marked hyperlipidaemia which could be reversed by the administration of glucagon (Paloyan & Harper 1961). The intravenous injection of glucagon on the other hand caused significant reduction in plasma lipids in dogs and in patients with hypertriglyceridaemia (Caren & Corbo 1960 & 1970). Furthermore, in isolated liver in vitro glucagon inhibits triglyceride synthesis (Vaughan et al 1964).

In principle, insulin and glucagon may influence hepatic lipogenesis and consequently triglyceride synthesis in at least three different ways, viz:

(1) By direct action on the amount of enzymes concerned with the production of acetyl CoA and its subsequent incorporation into long chain fatty acids.

(2) Through changes in FFA and glycerol mobilisation from adipose tissue resulting in a decrease or an increase in fatty acyl CoA and α-glycerophosphate concentration in the liver cell, thus modifying the feedback regulatory devices of hepatic lipogenesis.

(3) By altering hepatic glucose utilisation and/or release and consequently modifying the supply of building materials and necessary cofactors for optimal enzyme activity.

Which of these mechanisms is in operation depends much on whether one is dealing with short term or long term effects of these hormones, and whether their actions are observed in a diabetic or non-diabetic situation. From the point of view of the present thesis, the main interest is to learn to what extent insulin or glucagon plasma concentration, and particularly their long term changes, can influence hepatic lipogenesis in the non-diabetic state.

In pioneering studies, Bloch & Kramer (1948) showed that insulin increased the incorporation of acetate into fatty acids of rat liver slices incubated in vitro. This effect was seen in the absence of glucose but only if the liver was taken from a fed animal (Medes et al 1952). The presence of glucose in the medium slightly stimulated

lipogenesis in the liver slices obtained from fasted rats (Masri et al 1952). Haft (1967), in a more thorough study, has shown that insulin increased the incorporation of both ^{3}H-acetate and ^{14}C-glucose into total hepatic fatty acids when rat liver was perfused with physiological glucose concentrations whether the test animal was fasted or fed. In these experiments glucose oxidation was slightly increased by insulin but definitely much less than lipogenesis. These investigations seem to provide sufficient evidence that insulin is able to increase hepatic fatty acid synthesis even when this is basically stimulated by the normal fed state and by endogenous insulin. The results also indicate that the insulin effects on lipogenesis are independent of its action on glucose metabolism.

The mechanism of glucagon-induced hypolipidaemia is still uncertain, though several lines of evidence suggest that the hormone influences hepatic lipogenesis. Thus, glucagon has been found to inhibit the synthesis of fatty acids de novo by liver slices in vitro (Haugaard & Stadie 1953 and Berthet 1959). Similarly Heimberg et al (1969), using the isolated perfused rat liver, have demonstrated that glucagon significantly decreased the triglyceride fatty acid output, without any significant change in the rate of hepatic free fatty acid uptake. They therefore concluded that one important action of glucagon is to inhibit the synthetic pathways which lead to the formation and release of hepatic triglycerides.

Insulin and glucagon seem to influence the activity of several lipogenic enzymes in rat liver. Thus the state of insulin deficiency and glucagon abundance seen in diabetic rat leads to a decrease in the activity of acetyl CoA carboxylase (Wieland et al 1963 a) and fatty acid synthetase (Gibson & Hubbard 1960). In these animals acetyl CoA carboxylase activity was reduced approximately to the same extent as the decrease in fatty acids synthetising capacity. Furthermore, the addition of this enzyme to the supernatant of diabetic livers completely restored the lipogenic activity to normal (Wieland et al 1963).

In contrast to the finding with the diabetic animals, the hyperinsulinaemia and the hypoglucagonaemia seen in obese mice are associated with increased activity of acetyl CoA carboxylase and fatty acid synthetase (Chang et al 1967). The incorporation of acetate into hepatic fatty acids is also much increased in these animals (Winand et al 1968). The results, thus, suggest that insulin and glucagon modulate hepatic lipogenic enzymes to favour an increase or decrease in the rate of lipogenesis respectively.

Insulin is also known to increase the availability of NADPH necessary for the accelerated lipogenesis as a result of stimulating the pentose shunt pathway (Haft 1967). Insulin enhances the supply of substrate in the form of acetate and cofactors such as ATP resulting from the stimulation of glucose metabolism. Glucagon, on the other

hand, favours the breakdown of hepatic triglycerides and its release to the plasma (De Oya et al 1971, Heimberg et al 1969, and Penhos et al 1966) which subsequently may lead to the accumulation of fatty acyl CoA which could effectively inhibit lipogenesis.

Another important mechanism for the regulation of hepatic lipogenesis which might be modified by insulin and glucagon is related to the effects of these hormones on fatty acid esterification and on the synthesis of the apolipoprotein carrier. Thus insulin has been shown to enhance the esterification of hepatic fatty acids and to stimulate the production of VLDL apoprotein peptides (Nikkila, 1969). Consequently, the newly synthetised fatty acids immediately incorporated into triglycerides and released to the plasma and hence lipogenesis could continue uninhibited. On the other hand, glucagon decreases the fraction of fatty acids esterified by the liver (Heimberg et al 1969) and reduces the synthesis of the carrier apoprotein (Eaton 1970 & 1973). These effects would result in the hepatic accumulation of fatty acyl CoA which could feedback and inhibit the synthesis of new fatty acid molecules.

III. Effects of Steroids

1. Oestrogens

Several prospective and retrospective studies have reported a rise in plasma triglycerides in young women

receiving oestrogen containing oral contraceptive preparations. Doar & Wynn (1969) have examined the effect of oral contraceptives on the fasting plasma triglyceride concentrations in 128 young females and found a significant increase during therapy which was independent of age, and degree of obesity. In another 52 women who were tested initially during therapy and again after this had been discontinued, the mean plasma triglyceride concentration fell significantly after withdrawal of the steroid. In this study it was observed, however, that the magnitude of the plasma triglyceride increase in response to oestrogen containing preparations varied and that some subjects were affected more than others. Only 13% of all the patients receiving these preparations had serum triglyceride concentrations above the normal range.

Further studies by Stokes & Wynn (1971) have indicated that the increase in serum triglycerides seen during oral contraceptive therapy is proportionate to the dose of oestrogen in the preparation, suggesting that this effect could be due to the oestrogenic component. Indeed, several other studies have confirmed that the administration of oestrogen alone to either postmenopausal women (Furman et al 1967) or young females (Gustafson & Svanborg 1972) produces a significant rise in plasma triglyceride concentration.

Studies in experimental animals have also shown that

administration of oestrogens to ovariectomised female rats produces a significant elevation in plasma triglyceride concentration (Hill & Dvornik 1969; Schillinger & Gerhards 1973). Afolabi (1974), using the same animal model, has also demonstrated that the effects of oestrogens on plasma triglycerides concentration were dependent on the dose and duration of oestrogen therapy.

Both in women and in experimental animals the increase in serum triglyceride concentration was found to be associated with an increase in the plasma concentration of very low density lipoprotein VLDL (Wynn et al, 1966; Aurell et al 1966; Wynn & Doar, 1969; Gershberg et al 1968 and Afolabi 1974). No significant changes were seen in the triglyceride content of plasma low density or high density (LDL or HDL) lipoproteins. These results suggest that hypertriglyceridaemia induced by oestrogen was of endogenous origin and could result from a derangement in the metabolism of plasma very low density lipoproteins (VLDL).

Ham & Rose (1969) and Adams et al (1970) suggested that impaired removal of serum triglycerides might be the main determining factor in the oestrogen-induced hyper-triglyceridaemia. In their studies they reported that oestrogens impair the activity of the enzyme lipoprotein lipase, since the post-heparin plasma lipolytic activity was decreased in oestrogen treated subjects. This conclusion has been challenged, since in these patients

the removal of triglycerides was found to be within normal limits (Kissebah et al, 1973).

Kekki and Nikkila (1971) labelled the plasma triglycerides endogenously with ^{3}H glycerol and examined the turnover rate of these molecules in women receiving the combined oestrogen-progestogen oral contraceptive agents. They have reported that in these women the turnover of plasma triglycerides was much higher than in controls. Since these studies were performed while the subjects were in steady state situations the increase in turnover in women receiving these preparations was indicative of enhanced plasma triglyceride production. In a similar study Kissebah et al (1973) have pointed out that the increase in triglyceride production was due to the oestrogenic component and that this effect was demonstrable in women receiving oestrogens alone. They have also indicated that the increase in plasma triglyceride production during oestrogen therapy was not simply the result of enhanced supply of plasma FFA. It was, therefore, concluded that the excess fatty acids necessary for the increased triglyceride secretion in oestrogen treated women must have been derived from hepatic sources and that oestrogens might have increased the de novo synthesis of these molecules.

Afolabi (1974) has recently examined the effect of gonadal steroids on hepatic triglyceride production in ovariectomised female rats using two main approaches:

In the first technique, the clearance of plasma VLDL triglycerides was suppressed by the administration of Triton W.R.1339, which inhibits the lipoprotein lipase, and measured the post-triton rise in plasma triglycerides as an index of hepatic triglyceride secretion. From the rate of post-triton accumulation of triglycerides he was able to conclude that, in the oestrogen treated rats, the rate of entry of triglyceride into the plasma was three to four times higher than in control animals. In the second approach the hepatic release of triglyceride was blocked by colchicine and the rate of accumulation of newly synthesised hepatic triglycerides was assessed during a continuous infusion of ^{14}C-glucose. After colchicine administration hepatic triglyceride concentration was significantly higher and the incorporation of ^{14}C-glucose into triglyceride much greater in the oestrogen treated rats than in controls. These observations provide evidence of the enhanced hepatic triglyceride synthesis and secretion during the period of oestradiol administration.

Using liver slices in vitro Afolabi (1974) has also demonstrated an increase in the synthesis of triglyceride fatty acids from acetate and glucose together with an enhancement in the release of plasma VLDL carrier apoproteins. These results suggest that oestrogens stimulate the de novo synthesis of fatty acids (lipogenesis) and at the same time enhance the synthesis of the apolipoprotein carrier. These effects could be the main mechanism in the increased production of endogenous triglycerides

and hypertriglyceridaemia associated with oestrogen therapy.

2. Progestogens

The effects of progestogens on plasma triglycerides vary with the chemical structure of the analogue used, the pretreatment plasma triglyceride concentration and the concurrent administration of other steroids.

Beck (1969) and Beck (1970 & 1973) have reported that when progesterone derivatives were used as the only means of contraception the plasma triglyceride concentration was reduced. Similarly progesterone or its analogue megestrol administered to healthy young females produced a small but significant reduction in plasma triglyceride concentration (Kissebah 1974). This hypotriglyceridaemic effect was most apparent in patients with pre-existing hyperlipoproteinaemia (Glueck et al 1971 a), but was not discernable when used in combination with oestrogen in the birth control pill (Stokes & Wynn 1971).

Very few studies have been performed to evaluate the effects of progesterone on plasma triglyceride transport in humans. Kissebah et al (1973) have suggested that the presence of progesterone derivatives in combined pill preparations lowers the Km of plasma triglyceride clearance. Since the Km represents the triglyceride concentration at which the clearing mechanism is operating at half maximal capacity, the decrease seen with progesterone indicates an improvement in plasma triglyceride removal. Moreover,

the administration of progesterone or megestrol alone to seven healthy women decreased Km of plasma triglyceride clearance suggesting that the improvement seen with the combined preparation was primarily due to the progesterone component. The improvement in plasma triglyceride clearance was sufficient to explain the magnitude of reduction in serum triglyceride level and hence it was suggested that progesterone derivatives do not influence the hepatic production of these molecules.

Glueck et al (1969) and Glueck & Fallat (1974) have suggested that progesterone derivatives enhance the activity of the enzyme lipoprotein lipase since the plasma post-heparin lipolytic activity was increased in subjects treated with these agents. These results could provide an explanation of the increased triglyceride removal seen with these drugs.

Afolabi (1974) re-investigated this problem in female ovariectomised rats. In these animals it was observed that the administration of progesterone (5 mg/kg/day) increased the activity of adipose tissue lipoprotein lipase in fed rats and enhanced the skeletal muscle lipase in fasted rats. The hepatic production of fatty acids and triglycerides was not significantly influenced by the administration of progesterone to rats. Similarly the incorporation of labelled acetate and glucose into triglycerides of liver slices obtained from progesterone

treated rats was not significantly different from normal.

In contrast to the above findings Kim & Kalkhoff (1975) have reported that the administration of large doses of progesterone (25 mg/kg/day) to rats increased the post-triton rise in plasma triglyceride concentration suggesting that the steroid enhanced the hepatic release of these components. The reason for this discrepancy is uncertain though the difference between the progesterone doses used in the two studies might be a factor.

3. Glucocorticoids

During the past twenty years several groups have studied the effects of adrenocortical hormones on plasma lipid transport in several animal species. Adrenocortical hormones were generally found to cause a significant rise in plasma triglyceride concentration in rabbits (Moran 1962 and Mahley et al 1968) and in rats (Hill & Droke 1963 and Friedman et al 1965).

The effects of glucocorticoids on plasma triglycerides in man were initially reported by Adlesberg et al (1950) who indicated that treatment with cortisone actually lowered the plasma concentration of neutral fat. Stern et al (1973) have re-investigated this problem and reported that the administration of glucocorticoids led to an increase in plasma triglycerides associated with elevated plasma VLDL concentration. The anomalous results of Adlesberg et al, could be simply attributed to the

fact that in the majority of their patients the dose of corticosteroids was substantially less than that commonly used for therapeutic purposes. Indeed Kissebah et al (1974) have recently suggested that the plasma triglyceride response to the administration of adrenocortical hormones is variable and is dependent on such factors as the dose of the steroid, the duration of treatment, the age and sex of patients as well as the presence or absence of compensatory hyperinsulinaemia.

Stern et al (1973) estimated the turnover rate of plasma VLDL triglycerides in twelve patients before and after adrenocorticoid therapy. In all patients the turnover of VLDL triglyceride was increased with a parallel rise in plasma triglyceride concentration. These findings suggested that during adrenocorticoid therapy the production rate of endogenously synthesised triglyceride was increased to account for the increase in plasma triglyceride concentration. The plasma post-heparin lipolytic activity which indicates the efficiency of the lipoprotein lipase clearance system was higher than normal, suggesting that the steroid did not interfere with the removal of triglycerides from the plasma. Similar conclusions have been reported recently by Kissebah (1974) who also found an increase in the turnover of plasma triglyceride in some patients with Cushing's Syndrome.

The mechanism of the glucocorticoid effects on hepatic lipid metabolism and lipoprotein synthesis has been explored in several other animal species. Thus Friedman

et al (1965) observed that the administration of corticosterone to fed rats and rabbits stimulated the synthesis of hepatic triglycerides to induce a rise in plasma triglyceride concentration. Hill et al (1965) have also shown that the post-triton rise in plasma triglycerides was higher in cortisone-treated rats than in control animals. Similarly, Diluzio et al (1954) and Rudman & Di Girolman (1971) have shown an increase in the release of hepatic triglyceride in chickens and dogs given glucocorticoids.

In an electron microscopic study Mahley & co-workers (1968) have described an increase in the number and size of VLDL particles in the hepatocyte Golgi apparatus of rabbits given cortisone for 4-6 days. Reaven et al (1974) have studied hepatic lipoprotein metabolism in two other species of animals (rats and mice) by the ultrastructural and physiological approach. Their results have shown that glucocorticoids induce an increase in the size and number of VLDL particles present in hepatocyte Golgi apparatus and enhance the post-triton rise in plasma triglyceride concentration. These observations when taken together, strongly suggest that the increase in hepatic triglycerides and lipoprotein production is the mechanism responsible for the corticosteroid-induced hypertriglyceridaemia.

In contrast to the above finding, Bagdade et al (1970) have described two patients with rheumatic disease in whom severe hyperlipidaemia was associated with subnormal PHLA during treatment with adrenocorticoids suggesting that

impaired plasma lipoprotein clearance could be an additional mechanism. This abnormality, however, is not always demonstrable in patients treated with moderate doses of glucocorticosteroids and only when insulin deficiency is also present, as in the two patients studied by Bagdade, do the changes in the clearance rate become important.

B. REGULATION OF HEPATIC GLUCOSE TRANSPORT; EFFECTS OF STEROIDS

I. General Outline

The maintenance of a stable concentration of glucose in blood is one of the most finely regulated of all homeostatic mechanisms, and one in which the liver plays an important role. In contrast to extra hepatic tissue, the liver cells appear to be freely permeable to glucose and as a result the activity of certain key enzymes and their hormonal control appears to direct either the uptake or the output of glucose from the liver (Cahill et al 1959).

In the fasting state with blood glucose concentration between 80 & 100 mg/100 ml the liver appears to be a net producer of glucose. In the fed state, however, as the glucose concentration rises the output of glucose decreases and at higher concentrations there is a net uptake. In the rat, it has been estimated that the rates of uptake and output of glucose are equal at a hepatic portal vein blood glucose concentration of 150 mg/100 ml. These concentrations, however, do not apply to other species, and in some the level varies with the type of diet. Infusion of glucose into dogs maintained on a high protein diet resulted in a rise of blood glucose with a cessation of net hepatic glucose production only at hyperglycaemic concentrations. In contrast, in carbohydrate fed dogs,

very little increase in blood glucose concentration during a glucose infusion produced an immediate net glucose uptake by the liver (Landau et al 1961).

Gluconeogenesis provides the body with a source of glucose when carbohydrates are not available in sufficient amount from the diet. In this process, glucose and glycogen are synthesised from lactate, pyruvate and glycerol. This glucose source is necessary for energy supply of the nervous system and erythrocytes, and is also required for re-esterification of fatty acids in the adipose tissue. Glucose is also important for the anaerobic glycolysis in skeletal muscles, synthesis of lactose in mammary gland and in maintaining the concentration of intermediates of the citric acid cycle in many other tissues. It is not surprising, therefore, to find that even under conditions where fat might be supplying most of the caloric requirements of the organism there is always a basal requirement for glucose production via gluconeogenesis.

In most mammals the liver serves as the principle organ responsible for glucose production while the kidney becomes an important site during prolonged starvation and acidosis. In these organs, the conversion of gluconeogenic amino acids, lactate and glycerol to glucose occurs via a process which is essentially the reversal of glycolysis. Figure (3) shows the sequence of reactions by which lactate, pyruvate, glycerol and the major gluconeogenic amino acids are converted to glucose in the rat liver cell. The amino

acids and other substrates enter the cell by membrane transport systems. Lactate,alanine, serine and glycerol are converted to pyruvate in the cytosol. This substrate enters the mitochondria and is converted to oxaloacetate by pyruvate carboxylase enzyme. Oxaloacetate is then converted to malate and aspartate, which leaves the mitochondria.

In rat and mouse liver, malate and aspartate are reconverted to oxaloacetate in the cytosol and the product is converted to phosphoenol pyruvate by the phosphoenol-pyruvate carboxykinase enzyme (PEPCK) which is located almost exclusively in the cytosol. In pigeon, chicken and rabbit liver, this latter enzyme is present in the mitochondria and phosphoenol pyruvate is synthetised in these organelles. In humans, the enzyme is present both in the mitochondrial and in the cytoplasmic compartments, but the relative contribution of extra- and intra-mitochondrial phosphoenol pyruvate to glucose synthesis is not known.

Phosphoenol pyruvate is converted to fructose 1,6-diphosphate by the same cytoplasmic enzymes that are involved in the Emboden-Meyerhof glycolytic pathway. Fructose 1,6-diphosphate is hydrolysed to fructose 6-phosphate by fructose 1,6-diphosphatase, an enzyme present in large amounts only in tissues actively engaged in gluconeogenesis. Fructose-6-phosphate is converted to glucose-6-phosphate which is dephosphorylated to produce glucose by glucose-6-phosphatase, another enzyme specific for gluconeogenesis.

HEPATIC GLUCONEOGENESIS

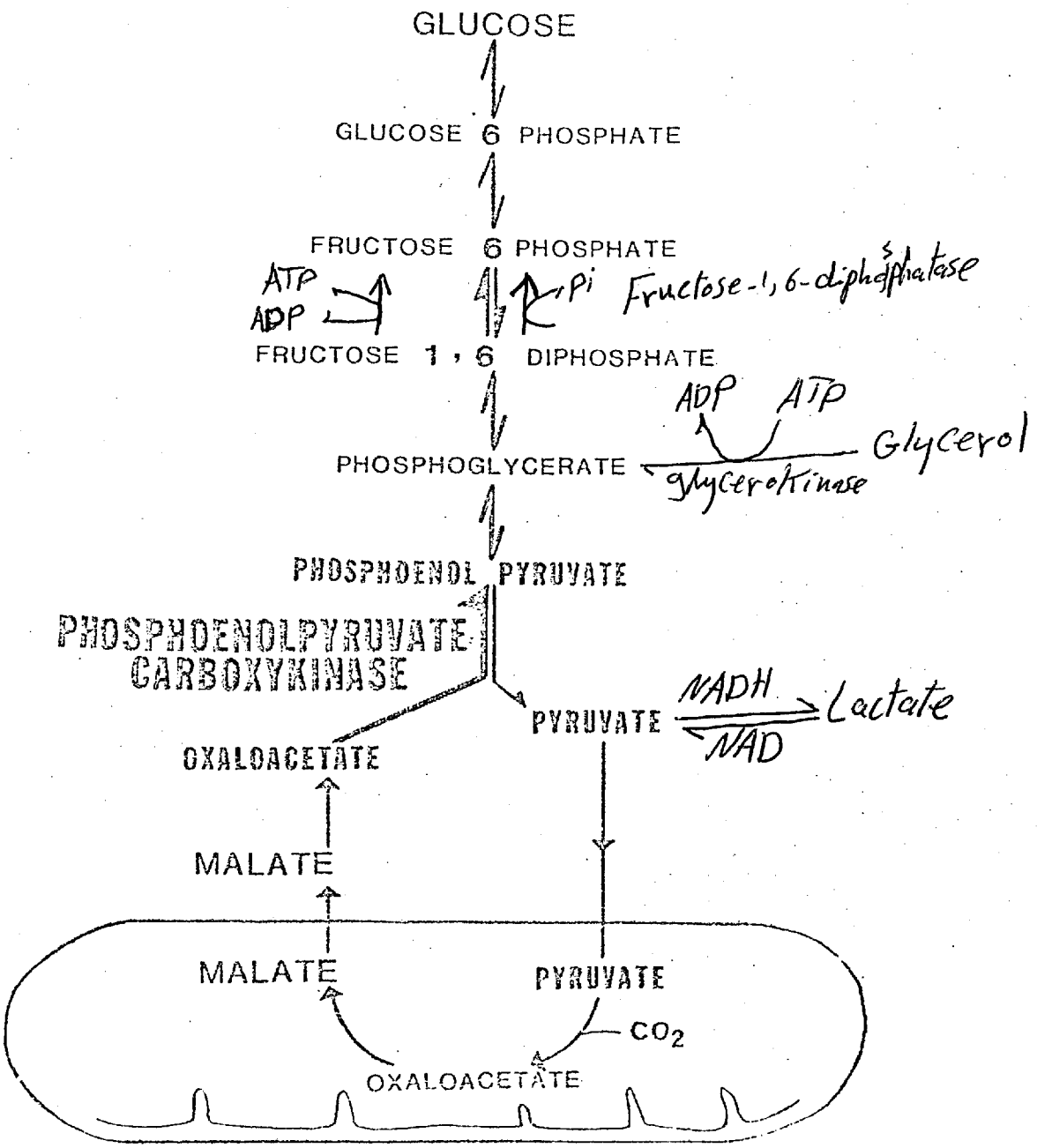


FIGURE (3)

The mechanism by which gluconeogenesis may be regulated by hormones can be discussed under two main headings: firstly, control by substrate supply, and secondly, control by enzyme activity.

Regulation of the supply of substrates to the liver is undoubtedly a major factor in the control of gluconeogenesis in vivo (Exton et al 1970). The blood concentrations of all the physiologic gluconeogenic substrates are subject to elaborate control and vary greatly in response to hormone action, and to metabolic state. Thus, the plasma concentrations of amino acids, lactate, pyruvate and glycerol are certainly influenced by insulin, glucagon and possibly directly by some steroid hormones. More significantly, however, some hormones seem to influence the hepatic extraction of some amino acids from the plasma. As pointed out by Mallette et al (1969), changes in the hepatic uptake of amino acids may be caused by changes either in their plasma concentration or their intracellular utilisation.

As regards the enzyme control there appears to be a number of energy barriers in the process of gluconeogenesis which require key regulatory enzymes to achieve glucose synthesis. These barriers are located between pyruvate and phosphoenol pyruvate, between fructose-1,6-diphosphate and fructose-6-phosphate and between glucose-6-phosphate and glucose. The enzymes which regulate these steps, namely; phosphoenolpyruvate carboxykinase, fructose-1,6-diphosphatase and glucose-6-phosphatase may be under

hormonal and dietary control.

II. Effects of Glucagon and Insulin

The possibility that glucagon may serve as a physiologic glucose-regulator has been suspected since the discovery of this hormone in 1923. The classic cross-circulation experiments of Foa and his co-workers (1952) provided the first experimental evidence for this supposition by demonstrating the presence of a hyperglycaemic factor in the pancreatic venous effluent of dogs made hypoglycaemic by insulin administration.

With the availability of radioimmunoassay techniques for glucagon Unger et al (1962) introduced the concept of a feedback relationship between plasma glucose concentration and glucagon secretion by the pancreatic alpha cells. Hypoglycaemia, whether induced actively by infusion of glucagon-free insulin, or biochemically by the administration of phloridzin, is associated with a considerable increase in the concentration of immunoreactive glucagon in the pancreatico-duodenal venous effluent of dogs. Such increases in glucagon were promptly reversed by raising the plasma glucose concentration by means of intravenous administration.

The quantitative aspects of such a complex feedback relationship were subsequently explored by Ohneda et al (1969). According to their findings, an increase in glucagon secretion occurs whenever the plasma glucose

concentration is lowered to 50 mg per 100 ml or less. Conversely, a decline in glucagon secretion was observed when the plasma glucose concentration was maintained in excess of 160 mg per 100 ml by intravenous glucose infusion.

The previous findings in vivo have also been validated by studies in vitro in which a reduction in glucagon release from isolated Islets of Langerhans has been observed at high concentrations of glucose in the incubation media, while increased glucagon release was observed at low glucose concentration (Vance et al 1968; Chesney & Schofield 1969; Edwards & Taylor 1970). The same inverse relationship between glucose concentration and glucagon secretion has been demonstrated in the isolated perfused rat pancreas by Luyckx & Lefevre (1971).

In all the experiments cited above, the reduction of plasma glucose concentration has been achieved by highly unphysiologic means. In nature, however, glucose lack occurs as a consequence of starvation, while exogenous glucose enters the circulation only via the gastrointestinal tract. In order to assess the importance of glucose-glucagon feedback relationship under physiological extremes of glucose availability, studies have also been performed on the effects of starvation and carbohydrate feeding on this relationship. Thus Aguilar-Parada (1969) have shown that, when normal subjects were fasted for 72 h. or more, a highly

significant rise in glucagon concentration was observed at the end of 48 h. and this rise was reciprocally related to a reduction in plasma glucose concentration. The period of maximal glucagon concentration occurred during the first week of starvation. According to Cahill et al (1966) this is the period of maximal gluconeogensis, during which the brain gradually adjusts to the shortage of glucose by adapting to ketones as its major source of energy.

The stimulatory effect of glucagon on hepatic glucose release and gluconeogenesis has been demonstrated as early as 1954 when Kalant found that the administration of this hormone to intact animals increased urinary nitrogen excretion. Van-Italie (1960) has subsequently confirmed these findings in vivo when he demonstrated an increase in amino acid uptake and glucose release by the liver in dogs infused with glucagon. This stimulatory effect of glucagon on gluconeogenesis has also been demonstrated in perfused liver in vitro. Miller (1961) and Miller (1965) have shown that glucagon in physiological concentrations stimulated protein catabolism and glucose production by perfused rat liver.

Exton and Park (1969) using livers from fasted rats perfused with a mixture of amino acids at several times their normal plasma concentration, have shown that glucose production was half maximal at normal plasma amino acid concentration and approached saturation at three times normal concentrations. They concluded that the rate of

release of amino acids from peripheral tissues can be an important factor in controlling hepatic gluconeogenesis. Glucagon, however, seems to influence the hepatic fate of amino acids rather than their concentration in plasma or their uptake by the liver. In livers perfused with physiological concentrations of amino acids, glucagon increased alanine conversion to glucose 3-5 fold and produced changes in the steady state concentration of gluconeogenic intermediates to indicate the stimulation of this process at the stage of conversion of pyruvate to phosphoenol pyruvate. No further stimulation with glucagon was obtained when the alanine concentration in the medium was raised as much as twenty times the physiological level. It was, therefore, proposed that glucagon plays a significant role in the regulation of gluconeogensis from amino acids by promoting their enzymatic conversion to glucose. Recent studies (Chiasson et al 1975) have confirmed these findings in man.

In 1956 Levine & Fritz (1956) summarised the data which suggest that insulin inhibits the synthesis of glucose from non-carbohydrate precursors. Mortimore (1963) using rat liver perfusion has subsequently demonstrated a significant inhibitory effect of insulin on glucose production with maximal responses at insulin concentration as low as 135 μU/ml (1×10^{-9}M). Though the inhibition of glucose production seen with insulin is in part due to inhibition of glycogenolysis, Mortimore et al (1967), in a subsequent study, have also demonstrated the

inhibition of gluconeogenesis by insulin.

Jefferson et al (1968) have confirmed the findings of Mortimore et al and have shown that the changes in radioactivity and specific radioactivity of ^{14}C-glucose added to the perfusion medium, confirm the hypothesis that insulin inhibits glucose production by suppression of glucose synthesis. These workers also found that livers obtained from rats made acutely insulin-deficient by intravenous injection of anti-insulin serum exhibited higher rates of gluconeogenesis from lactate than normal. Addition of insulin to the medium returned hepatic glucose output and gluconeogenesis to normal. Davidson et al (1974) using liver slices from fasted rats were able to demonstrate that insulin directly inhibits gluconeogenesis from glycerol and alanine.

Several reports are now available to suggest that the rate of gluconeogenesis is primarily determined by the ratio between the concentrations of insulin and glucagon reaching the liver (Mallette et al 1969; Mackrell & Skoll 1969; Cherrington & Varnic 1971). The opposing effects of the two hormones suggest that some index of the relationship of their concentrations to each other might constitute the most important signal of the hormonal influence of hepatic gluconeogenesis than the concentration of either hormone alone. A relatively modest increase in glucagon concentration, as for example, the 50% rise that occurs after 48 h. of starvation, may exert more glucagon - like biologic effect than the 200%

glucagon increase induced by the infusion of arginine. In the former circumstances, a reduction of insulin secretion accompanies the rise in glucagon, whereas in the latter case, a simulataneous increase in insulin secretion occurs. For this reason, the ratio of the molar concentrations of insulin : glucagon has been proposed as a more valid index of gluconeogenic activity.

Lewis et al (1970) have demonstrated that gluconeogenesis can be regulated from minute to minute by altering the ratio of insulin to glucagon concentrations in rat liver perfusion. Moreover, Parrilla et al (1974) have shown that a 100-1000 fold change in insulin and glucagon concentration at a constant glucagon : insulin ratio did not alter the rate of gluconeogenesis in perfused liver. Also calculations of the insulin : glucagon ratio based on the actual measurements of their concentrations under a varietyof physiological conditions indicate that the ratio does, indeed, correlate inversely with need for gluconeogenesis. Thus the ratio is lowest when such need is greatest, i.e., in total starvation, and highest when such a need is abolished as during loading with exogenous carbohydrate (Muller et al 1971). Similarly, infusion of the glucose precursor, alanine, which in the fasting state causes a fall in I/G ratio "catabolic response" increased I/G ratio during a glucose infusion an "anabolic response", which must spare alanine from gluconeogenesis. The same bihormonal control of glucose production has been observed after a protein load. Normally after an overnight

fast the I/G ratio rises in response to a beef meal - "an anabolic response" - unlike the carbohydrate deprived subject, in whom the I/G ratio does not rise, remaining at a catabolic level. During glucose ingestion, however, a beef meal induces a greatly exaggerated anabolic rise in I/G ratio.

Although the factor or factors responsible for the adjustment of the I/G ratio to meet the need for glucose cannot be identified with certainty, there is reason to believe that the rise in glucagon, which is in large part responsible for lowering the I/G ratio during a period of fast, is a consequence of an increase in alanine concentration. Alanine, which has been shown to be an important endogenous glucose precursor (Felig et al 1970), is a powerful stimulus of glucagon secretion but only during glucose need (Muller et al 1971). In the fasting state, the infusion of 1 mmol/kg of alanine into dogs over a fifteen-minute period causes a doubling in the plasma glucagon concentration without altering insulin concentration significantly so that the I/G ratio is reduced by 50%. When, however, the same alanine infusion is given during an infusion of glucose, which abolishes the need for gluconeogenesis, glucagon does not rise above the normal fasting concentration and insulin concentration almost doubles, i.e., the I/G ratio increases almost 100%. The former change, a reduction in I/G ratio, would tend to increase the conversion of alanine to glucose and this is, in fact, accompanied by

an increase in glucose concentration. The latter change, that is, an increase in I/G ratio, would tend to decrease conversion of alanine to glucose and increase glucose utilisation. It is also associated with a decline in glucose concentration (Muller et al, 1971).

There is now considerable evidence to suggest that the interaction between insulin and glucagon associated with gluconeogenesis are mediated by their opposing effects on hepatic phosphoenolpyruvate carboxykinase enzyme (PEPCK) (Lardy et al 1964; Weber et al 1965; Seubert & Huth 1965; Scrutton & Utter 1968; Ustenko 1970). In this connection Wicks et al (1969) have demonstrated that the hepatic PEPCK activity is increased by treatment of the intact rats with glucagon. This activation effect, however, is prevented by inhibitors of protein synthesis (Hager & Kenry 1968; Wicks 1968; Mallette et al 1973). On the other hand, Eaton et al (1974), using 24 h. fasted short term glucose fed rats were able to show an inhibition in the enzyme activity coinciding with the reduction in glucagon and the increase in insulin secretion.

The concept that PEPCK is the target for glucagon and insulin action on hepatic gluconeogenesis has been supported by the evidence of Exton & Park (1966). Measurement of intermediary metabolites in liver perfused with glucagon or insulin have shown changes consistent with the activation or inactivation respectively of an

enzyme or enzymes located in the gluconeogenic pathway between pyruvate and phosphoenolpyruvate; suggesting that this enzyme could well be PEPCK. This assumption has been confirmed and well established by other investigators (Weber et al 1965; Ballard & Harson 1967; Yeung & Oliver 1967; Ashmore & Weber 1968; Yeung & Oliver 1968; Philppichin & Ballard 1970 and Exton et al 1971b).

Changes in the activity of PEPCK are mediated by alterations in the rate of synthesis of the enzyme and are probably related to the concentration of intracellular cyclic-AMP. The evidence to support this conclusion is as follows:

(1) Utilisation of labelled lactate or pyruvate in the perfused liver is stimulated by cyclic-AMP and is associated with increased production of labelled glucose. This establishes that flow through this part of the pathway is raised (Exton & Park 1968, 1969).

(2) In the steady state of stimulation the level of pyruvate in the tissue is decreased, whereas the level of phosphoenolpyruvate is increased. This means that some steps between pyruvate and phosphoenolpyruvate must have been activated since pyruvate disappeared at a faster rate in spite of lower concentration and PEP was produced at a faster rate in spite of higher concentration.

In 1968 Sutherland et al introduced the concept suggesting that c-AMP operates as the intracellular

mediator of the glucagon effect on liver gluconeogenesis. Their evidence was based on the following:

(1) glucagon causes a rise in c-AMP in intact liver tissue and broken cell preparations;

(2) this rise in c-AMP precedes or coincides with the stimulation of gluconeogenesis (Exton et al 1971a);

(3) the effects of the hormone at suboptimal concentration on gluconeogenesis are enhanced by theophylline (an inhibitor of the phosphodiestrase which enhances c-AMP);

(4) the metabolic effects of glucagon are duplicated by exogenous c-AMP, or by analogues of c-AMP such as the dibuturyl derivatives (Menahan & Wieland 1967) but not by other cyclic nucleotides.

The opposing effects of insulin on hepatic gluconeogenesis could also be explained on the basis of changes in c-AMP concentration. Thus it has been proposed (Exton et al 1971a and Jefferson et al 1968) that the insulin effect may be partly due to a decrease in liver c-AMP (Fig. 4). This proposal is based on the following observations:

(1) insulin produces a small but significant decrease in the concentration of c-AMP in perfused rat livers;

(2) depletion of insulin in vivo by treatment with insulin antiserum or alloxan results in increased liver c-AMP;

(3) exogenous c-AMP and glucagon which raise the concentration of c-AMP produce effects on gluconeogenesis which are opposite to those caused by insulin;

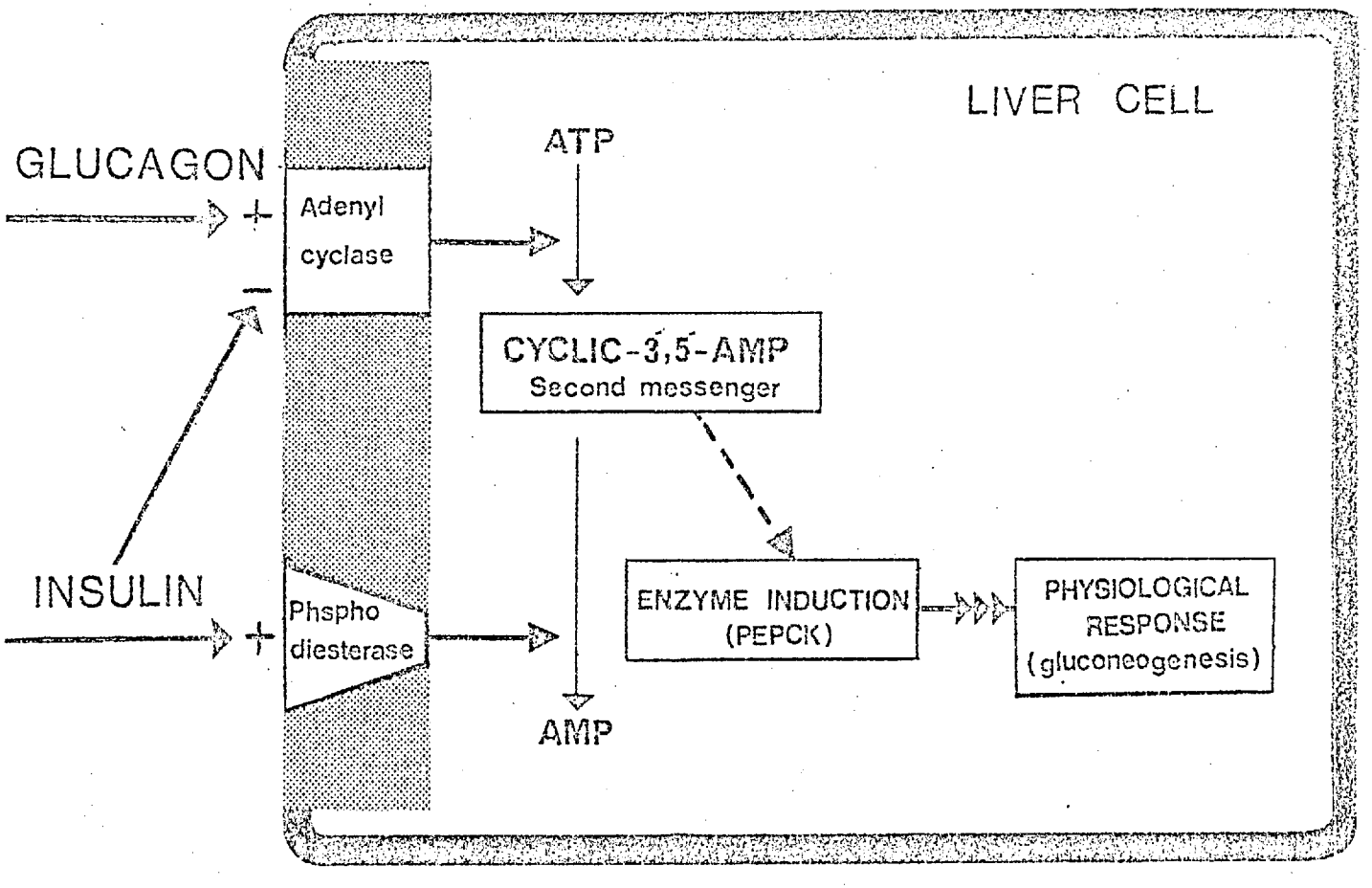

LIVER CELL
GLUCAGON
+
Adenyl
cyclase
−
ATP
CYCLIC-3,5-AMP
Second messenger
INSULIN
+
Phspho
diesterase
AMP
ENZYME INDUCTION
(PEPCK)
PHYSIOLOGICAL
RESPONSE
(gluconeogenesis)

FIGURE (4)

(4) insulin antagonises the action of c-AMP or glucagon on gluconeogenesis in the perfused liver; and
(5) insulin reduces the accumulation of liver c-AMP in the presence of glucagon.

The mechanism whereby c-AMP could regulate the synthesis of PEPCK, however, remains obscure. Langan (1969 a & b) has proposed that c-AMP might activate a protein kinase which phosphorylates a specific histone peptide in the nuclear chromatin complex which could have a role in gene repression and hence initiate the synthesis of the enzyme.

III. Effects of Steroids

1. Oestrogens

In spite of conflicting reports the balance of evidence indicates that oestrogen containing preparations alter glucose transport in women. The contribution of oestrogens to the changes in glucose transport is difficult to assess, since these preparations contain other steroids as well.

Most of the studies concerned with the effects of oestrogens alone on glucose transport were conducted in animals with and without diabetes. Foglia et al (1947) were the first to ascribe a significant role for gonads and gonadal hormones in the regulation of glucose metabolism in the rat. In their study they observed that in the sub-totally pancreatectomised rats, ovariectomy

greatly increased the frequency of diabetes, while the administration of oestradiol benzoate diminished the incidence of diabetes. These findings were similar to those of Ingle (1941) who was able to demonstrate that oestradiol benzoate ameliorated the hyperglycaemia and the severity of diabetes in alloxan diabetic rats. These results were in contrast to a subsequent publication by Ingle et al (1947) in which they demonstrated that the administration of diethyl stilboestrol to force-fed normal rats, to partially pancreatectomised rats, or to the alloxan diabetic rats caused an increase in glycaemia and glycosuria.

The controversy concerning the effects of oestrogens on glucose transport in partially diabetic rats has been considered by Rodriguez (1950). In his studies Rodriguez demonstrated that in vitro there was a biphasic response to oestrogen; firstly there was an increase in glycaemia and glycosuria, but after one month of continuous oestrogen treatment the glycaemia and glycosuria disappeared and there was some protection against the development of diabetes in these animals. This protective action of oestrogen was ascribed by Lewis et al (1950) and Costrini & Kalkhoff (1971) to be due to hypertrophy and hyperplasia of the Islets of Langerhans in animals on long term therapy.

Few studies have been performed in women receiving oestrogen preparations alone. Thus Talat et al (1965), Di Poola et al (1968), Yen & Vela (1968) and Spellacy,

Buhi & Birk (1972a) have demonstrated that glucose tolerance was unaffected or might be improved in normal women taking synthetic oestrogens. Furthermore, Gershberg et al (1967) found that the synthetic oestrogen mestranol improved the carbohydrate tolerance in a small group of patients with maturity-onset diabetes. Di Paola et al (1968), on the other hand, did not find any significant effect of mestranol on carbohydrate tolerance in a similar group of patients.

Recently Aitken et al (1973), in a comprehensive study of women, have reported that the only demonstrable effect of oestrogen alone is to produce a significant reduction in fasting plasma glucose. Oliver (1973), using the ovariectomised rat, has also demonstrated a consistent reduction in fasting plasma glucose during the long term adminstration of oestradiol to animals. It was also observed that the concentration of blood pyruvate is increased in rats treated with oestrogen. These results suggest that the effects of oestrogen alone may be due to the inhibition of hepatic gluconeogenesis. Experiments on rat liver slices have confirmed that oestrogen administration inhibits the conversion of ^{14}C-alanine to glucose indicating that the steroid suppresses the gluconeogenic pathway (Matute & Kalkhoff, 1973).

2. Progestogens

The effects of progestogens on plasma glucose transport depends on whether the compound is an analogue of

testosterone or progesterone. In many studies, deterioration of glucose tolerance has been observed in women receiving a combined contraceptive preparation in which a testosterone derivative has been incorporated as a progestational agent (see review by Beck; 1973). Progesterone derivatives, on the other hand, have no discernable effect on glucose tolerance in normal women whether taken alone as a megestrone derivative or when combined with oestrogen as in birth control pills. Also the use of 17-acetoxyprogesterone derivatives as the sole contraceptive agent generally caused no significant changes in glucose tolerance.

Beck (1970) has reported no change in glucose tolerance in either non-diabetic women or women with sub-clinical diabetes when treated with chlormadinone acetate over periods ranging from two weeks to six months. Larsson-Cohn et al (1969); Vermeulen et al (1970) also reported no abnormality in glucose tolerance in women treated with the same drug for up to one year. Similarly, Goldman et al (1968) observed no significant change in glucose transport in normal women treated with medroxy-progesterone acetate, another progesterone derivative.

Experiments in animals on the effects of progesterone on glucose transport have confirmed results in human subjects. Lewis et al (1950) showed that progesterone has no effects on alleviating the incidence of diabetes in the sub-totally pancreatectomised rat. Ingle et al (1953) have shown that progesterone administration to

force-fed sub-totally pancreatectomised rats was without effect at low doses. Later work by Beck (1969) using rhesus monkeys has confirmed that progesterone was without effect on fasting plasma glucose while its derivative chlormadinone acetate caused a significant increase in the glucose disappearance rate.

3. Glucocorticoids

It is now widely recognised that human subjects suffering from Addison's Disease, and adrenalectomised animals are liable to die in hypoglycaemia during starvation. Houssay & co-workers (1947) drew attention to the fact that the fall in fasting glucose in these animals was primarily due to the failure of gluconeogenesis. Such factors as lack of inhibition of peripheral glucose utilisation probably contribute to the severity of the hypoglycaemia.

Studies *in vitro* in perfused rat liver have confirmed this concept, for adrenalectomy abolishes the increase in gluconeogenesis caused by starvation, diabetes and glucagon. Since glucocorticoid replacement *in vivo*, or its addition to the perfusion medium *in vitro* reverses the effects of adrenal gland removal, it has been suggested that part of the steroid effect must be exerted directly on the liver (Exton et al 1970 and Exton & Harper 1972).

The evidence of effects of glucocorticoid excess on plasma glucose transport in man has been rather

conflicting; thus some subjects receiving therapeutic doses of adrenocorticoid steroid do not demonstrate significantly impaired glucose tolerance, and, indeed, may show a reduction in fasting plasma glucose (Perley & Kipnis 1966). On the other hand, in many patients receiving moderate or high pharmacological doses of glucocorticoids, a diabetogenic effect may be observed (Marco et al 1973 and Wise et al 1973).

Evidence from several studies summarised by Cahill (1971) indicates that glucocorticoids stimulate the production of glucose by the liver though impaired peripheral glucose utilisation may play a role. Kipnis & Stein (1964), Ashmore (1964), Klausner & Heimberg (1967) and Munck (1971) have confirmed these findings in perfused rat livers in vitro. Cortisol added to the perfusion medium stimulated the output of glucose by the liver. This effect has been attributed to the enhanced hepatic gluconeogenesis induced by glucocorticoids (Long et al 1940; Levine 1964 and Ashmore 1964).

The stimulatory effect of glucocorticoids on liver gluconeogenesis is probably related to the induction of gluconeogenic enzymes. The observation that the stimulation of gluconeogenesis induced by steroids in perfused livers is relatively slow and is blocked by cyclohexamide or actinomycin D is in accord with the involvement of enzyme synthesis (Exton & Harper 1972). Of particular importance in this respect is the enzyme (PEPCK) which is probably a major site of action for

glucocorticoids. Measurement of the steady state of gluconeogenic intermediates in perfused rat liver has shown increases in phosphoenolpyruvate associated with unchanged concentrations of malate and oxaloacetate after cortisol treatment (Shrago et al 1963 and Exton & Harper 1972).

Administration of glucocorticoids to rats has also been shown to increase the activity of fructose-1,6-diphosphatase, another regulatory enzyme in the control of hepatic gluconeogenesis (Mokrasch et al 1956 and Weber & Singhal 1964). The importance of this enzyme, however, in the stimulation of gluconeogenesis by glucocorticoids remains uncertain in view of the following observations:

(1) The enzyme increase following glucocorticoids is of much slower onset than that of gluconeogenesis (Weber & Singhal 1964).

(2) Measurement of hepatic intermediary metabolites in glucocorticoid treated rats does not shown evidence of increased conversion of fructose-1,6-diphosphate to fructose-6-phosphate (Exton et al 1970 and Exton & Harper 1972).

(3) Gluconeogenesis from fructose is not impaired in liver from adrenalectomised rats, nor is fructose-1,6-diphosphate concentration increased.

The enzyme glucose-6-phosphatase which determines the hydrolysis of glucose-6-phosphate with subsequent release of glucose seems to be also stimulated in animals treated with glucocorticoids (Ashmore & Weber 1959).

However, the changes in glucose and glucose-6-phosphate concentration in livers from glucocorticoid treated rats seems to suggest that this enzyme might not be an important site for glucocorticoid action (Exton & Harper 1972).

Another important component in the increased gluconeogenesis caused by glucocorticoids in vivo is the increased mobilisation of amino acids from muscle and other extrahepatic tissues in glucocorticoid treated animals. These hormones are known to decrease the incorporation of labelled amino acids into proteins of skeletal muscle and other tissues (Wool & Weinshelbaum 1959 and Exton et al 1970). Also glucocorticoids increase the release of amino acids in eviscerated animals (Smith & Long 1967). It is not known, however, whether these effects are due to a primary action of the steroid or secondary to some other change associated with the intake of glucocorticoids.

C. EFFECTS OF STEROIDS ON INSULIN AND GLUCAGON SECRETION

Alteration in peripheral blood concentration of insulin in response to female gonadal steroid therapy has been widely reported in humans and animals. In these studies, insulin concentrations were found either to increase, decrease, or remain unchanged, depending on the nature and complexity of the steroid therapy and on the experimental model under investigation. An effect of glucocorticoids on circulating insulin concentration has been documented in patients with Cushing's Syndrome and in subjects on long term glucocorticoid therapy.

The development of specific radioimmunoassay techniques for measuring pancreatic glucagon has also revealed that steroids have dramatic effects on the circulating concentration of this hormone. Thus the administration of oestrogen and progestogen containing preparations to young females resulted in the suppression of glucagon response to arginine infusion suggesting that the secretion of this hormone was inhibited by the intake of these steroids. On the other hand, patients with Cushing's Disease and subjects receiving moderate or high doses of glucocorticoids show an increase both in basal and arginine-stimulated glucagon concentrations suggesting that glucocorticoids enhance the activity of the pancreatic alpha cell function (Jose Marco et al 1973; Wise et al 1973).

It appears, therefore, that the long term administration

of steroids modifies the secretory function of both the alpha and the beta cells of the pancreas, thus modulating the secretion of insulin and glucagon and that this effect may play an important role in the metabolic changes associated with the intake of these steroids. The effects of these agents upon the pancreatic alpha and beta cell functions are summarised below:

1. Oestrogens

The effects of oestrogen on pancreatic beta cell function has been studied primarily in rats. As early as 1941 it was demonstrated that the administration of oestrogen to rats increased the insulin content of the Islets of Langerhans (Fraenkal-Conrat et al 1941 and Griffiths Marks & Young 1941). Lewis et al (1950) have also observed that oestrogens have a trophic action of the pancreas and that the steroid produces hypertrophy and hyperplasia of the Islets of Langerhans. This effect of oestrogen does not require the presence of the adrenal or pituitary glands and probably is the result of direct action of the steroid on the islets (Foglia 1954). More recently, Costrini & Kalkhoff (1971) have demonstrated that pancreatic islets obtained from rats treated with oestrogen and incubated with glucose *in vitro* show an enhanced capacity to secrete insulin.

In contrast to the above finding, Beck (1969), using the rhesus monkey, observed that the administration of oestrogen decreased the fasting plasma insulin concentration and was without any effect on the insulin

response during glucose tolerance. Hager et al (1971) have also demonstrated in a similar study that the insulin release from pancreatic islets, obtained from oestrogen treated rats, was inhibited.

The effects of oestrogen containing preparations on plasma insulin levels in humans are also contradictory. Thus Spellacy (1969) and Beck (1969) have demonstrated an increase in insulin levels in women receiving combined oestrogen progestogen agents. Javier et al (1968) and Wynn & Doar (1969), on the other hand, found a decrease in fasting plasma insulin while Spellacy et al (1972b) reported that the plasma level of this hormone remained unchanged during oral contraceptive therapy.

No specific study has so far been made to evaluate the effects of oestrogen alone on the morphology and function of the pancreatic alpha cell. However, Beck et al (1975) have recently reported that oral contraceptive agents containing oestrogen suppress the activity of the alpha cells and decrease the basal and the amino acid stimulated glucagon concentrations in women. On the other hand, Saudek et al (1975) have suggested that in rats increased oestrogenic activity during late pregnancy could be an important factor in the hyperglucagonaemia associated with this condition. In this study, however, the effects of oestrogens were not segregated from those of other hormonal changes associated with pregnancy.

2. Progestogens

Beck (1969) using the rhesus monkey as an experimental animal has reported that progesterone and synthetic progestogens increase the insulin response during an intravenous glucose load. Similarly Costrini and Kalkhoff (1971) and Hager et al (1971) have demonstrated that the Islets of Langerhans obtained from rats treated with progesterone show increased insulin response when incubated with glucose in vitro. This effect was not produced acutely, suggesting that the long term administration of progesterone induces hypertrophy and enhanced secretory capacity of the beta cells. Indeed, Gobrena et al (1971) have demonstrated that the administration of synthetic progestogens to the sub-totally pancreatectomised rats decreased the incidence of diabetes and caused an increase in insulin response during glucose challenge.

Studies in the human with regard to the effect of progestogens on insulin secretion are rather scanty. Kalkhoff et al (1970) noted an increase in the insulin response to glucose and tolbutamide in women receiving progesterone derivatives. Spellacy et al (1970), also using progestogens, demonstrated an increase in insulin during fasting and after an oral glucose load. On the other hand, Adams & Wynn (1972) using megesterol acetate, a progesterone derivative, observed no change in plasma insulin response.

No studies have been performed to assess the influence

of progesterone on plasma glucagon levels.

3. Glucocorticoids

Elevations in plasma insulin concentrations have been observed in patients receiving glucocorticoids (Perley & Kipnis 1966; Campbell et al 1966 and Marco et al 1973) and in patients with Cushing's Syndrome (Klink & Estrich 1964).

Perley & Kipnis (1966) studied the acute effects of a large dose of glucocorticoid, administered to normal subjects, on insulin secretion. Within 48 h. the plasma insulin response to both orally administered glucose and intravenously infused tolbutamide were markedly increased above control values. Since there is no evidence that insulin degradation is influenced significantly by glucocorticoids (Elgee & Williams 1955 and Berson et al 1957) the elevated plasma insulin concentrations clearly indicate excessive pancreatic insulin secretion. Similarly an increase in insulin concentration has been observed in normal subjects receiving moderate doses of glucocorticoids for four days (Marco et al 1973).

Although adrenal steroids are generally thought of as protein catabolic agents, the administration of cortisol in vivo has been reported to result in hyperplasia of the Islets of Langerhans in some animal species (Kinash & Haist 1954; Volk & Lazarus 1959; Vranic 1965). Malaisse et al (1967) have re-investigated the effect of adrenalectomy and cortisol treatment on pancreatic tissue. Removal of the adrenal glands resulted in reduced responsiveness of

the pancreatic tissue to glucose stimulation *in vitro*. This effect was demonstrable three days after the induction of adrenocortical deficiency. On the other hand, cortisol when administered *in vivo* for two to five days increased the sensitivity of pancreatic tissue to glucose. When added to the incubation media *in vitro*, however, cortisol has no effect upon the glucose induced insulin secretion by pancreatic tissue from normal or adrenalectomised rats suggesting that the effects of glucocorticoids were not directly exerted on the beta cells and that some other factor must be involved.

The changes in insulin secretion induced by glucocorticoids are not simply the result of changes in insulin content of the pancreas. Rather the change is due to the increased sensitivity of the insulin secreting mechanism to glucose (Malaisse et al 1967). The explanation of this phenomenon is unknown, though there is evidence that glucocorticoids may influence the activity of enzymes regulating glucose metabolism in the Islets of Langerhans (Gepts & Toussaint 1964). This effect might, therefore, alter the islet metabolism of glucose and hence might influence the ability of glucose to enhance insulin secretion.

Marco et al (1973) were the earliest to demonstrate hyperglucagonaemia in patients treated with glucocorticoids. Both with respect to basal and arginine-stimulated glucagon; the concentrations were higher than in control subjects.

Wise et al (1973) have confirmed these findings in non-obese and obese subjects treated with dexamethasone and in patients with Cushing's Syndrome. These authors concluded that glucocorticoids increase plasma glucagon concentration in the basal state and in response to protein ingestion or aminogenic stimulation. This effect occurs in obesity and persists in chronic hypercorticoidism.

The secretion of glucagon from pancreatic islets in vitro was also enhanced when the islets were obtained from glucocorticoid treated rats (Calle et al, 1975).

As with insulin, however, the addition of glucocorticoids to the medium of isolated islets incubated in vitro did not influence the capacity of the alpha cells to secrete glucagon suggesting that the effects of glucocorticoids are not directly exerted on the islets.

Wise et al (1973) have also suggested that raised plasma alanine concentrations might be an important factor in the increased glucagon levels in patients with acute and chronic glucocorticoid excess. A direct linear correlation has been demonstrated between the rise in plasma alanine and the increase in plasma glucagon in glucocorticoid treated subjects. Alaternatively, Marco et al (1973) have proposed that the interference of glucocorticoids with the uptake of glucose by the alpha cells, an effect analogous to the insulin antagonism observed in other tissues, may increase the reactivity to aminogenic stimulation.

C H A P T E R I I

MATERIALS AND METHODS

M A T E R I A L S :

I. Animals

Experiments were performed on female Wistar rats with body weights varying between 120 and 140 g. The animals were housed in groups of six per cage and kept in rooms in which a temperature of 20-22^{o}C was maintained. Lighting was controlled to give alternate 12 h. of light and darkness.

Rats were fed ad libitum on a standard purina laboratory chow obtained commercially and were allowed free access to drinking water. Surgical procedures were performed under pentobarbitone anaesthesia, sodium pentobarbitone was diluted in 0.9% NaCl and was injected intraperitoneally in a dose of 45 µg/kg body weight. Two dorso-laterally placed incisions were made in each rat and the two ovaries were manipulated and excised within the pursa ovarica under direct vision. When required, bilateral adrenalectomy was carried out through the same incisions and appropriate haemostasis was secured. The rats were weighed every other day for the first ten days after the operation to ensure a normal gain in weight prior to the administration of steroids. Animals which gained weight at a rate less than 90% of the mean rate for unoperated weight-matched controls were excluded from the study.

During the period of treatment, some steroids were found to lead to a change in the feeding habits of rats. Thus, the oestradiol and corticosterone treated rats gained weight at a rate less than weight-matched controls. In order to minimise any error resulting from changes in feeding habits and differences in weight, pair fed controls were used in each experimental group to produce controls consuming the same amount of food as the experimental animals. This objective was achieved by weighing the food given daily to the steroid-treated rats. The calculated amount consumed per rat in each group was then offered to its pair fed control. Also, the adrenalectomised animals were allowed free access to drinking water as well as 0.9% NaCl to prevent depletion of the body fluid volume due to sodium loss in urine.

II. Steroids

Ten days after operation, animals were grouped so that:

(a) In each group the difference between the weight of the biggest and the smallest rat in the group did not exceed 10 g (<7% of mean body weight).

(b) The difference between the means of the body weights of animals in any of the groups did not exceed 15 g (<10% of mean body weight).

The dose of each steroid was calculated as follows: If the dose taken, expressed as µg or mg/kg body weight, was 'D', then the total dose injected was $\frac{'D \times W'}{1000}$ per rat where 'W' was the mean body weight in grams of the rats in the group of animals to which the particular steroid was to be administered. This starting dose was maintained for the whole experimental period. The dose of oestradiol administered ranged from 1 to 100 µg/kg body weight/day. Progesterone was given in a dose range of 0.05 to 5 mg/kg rat body weight/day. Corticosterone was administered in doses ranging between 0.5 and 50 mg/kg body weight/day.

Oestradiol, progesterone and corticosterone were purchased from Sigma (London) Chemical Co. Oestradiol and progesterone were dissolved in corn oil so that 0.2 ml contained the total daily dose of the steroid required per rat per day. Homogenous solubilisation was ensured by adding the required quantity of steroid powder to the appropriate volume of oil and stirring on a magnetic stirrer for 24 h. at room temperature. Corticosterone was given as an oily suspension.

The gonadal steroids were administered by daily subcutaneous injections using 25 g, 2 inches long hypodermic needles and 1 ml disposable plastic syringes. The sites of injection were alternated between the ventral aspects of the four quadrants of the animal. Corticosterone, on the other hand, was given intramuscularly. The duration of steroid administration ranged between 8 and 10 weeks.

In each experiment the last dose of steroid was administered 24 h. before the animals were sacrificed and samples taken.

METHODS :

I. Measurement of Hormones and Metabolites in Blood

The plasma concentration of immunoreactive insulin and glucagon were measured in samples of blood obtained from the main portal vein. Animals were anaesthetised with pentobarbitone and the portal vein was exposed with minimal trauma through a small abdominal incision. Samples of blood were withdrawn from the main portal vein and dispensed into either heparin tubes for measurement of insulin, or into tubes containing trasylol (1000 units) and EDTA (3 μmol) per 1 ml of blood for glucagon estimation. The plasma was immediately separated and frozen at -20°C until the time of assay. Blood was also obtained from the abdominal aorta for the estimation of plasma glucose and triglyceride concentrations.

(1) Radioimmunoassay of Glucagon

Principle

Glucagon was assayed in plasma samples using a modification of the method of Sauntensanio et al (1972). The antibody specific for pancreatic glucagon (30K) was purchased from Professor Unger, University of Texas South Western Medical School, U.S.A. The separation of the

antibody bound from the free hormone was performed using dextran coated charcoal.

(a) Preparation of I^{125} Glucagon

Crystalline beef-pork glucagon was purchased from Eli-Lilly Co. (Indianapolis, Indiana, U.S.A.). Two mg of this material was accurately weighed and solubilised in 8 ml of 0.01 M HCl. The solution was then divided into 10 µl portions each containing 2.5 µg of glucagon. This material was then stored in autoanalyser cups at -20°C. The iodination mixture contained 2.5 µg glucagon in 10 µl of 0.01 M HCl, 25 µl phosphate buffer (0.5 M, pH 7.4) and 2 mci of carrier free Na I^{125} obtained from Amersham Radiochemical Centre, Bucks., England. A solution (50 µl) containing 20 µg of chloramine T in phosphate buffer (50 mM, pH 7.4) was added and the reaction allowed to proceed for 15 s. It was terminated promptly by the addition of sodium metabisulphite ($Na_2S_2O_5$) solution (300 µl containing 144 µg in phosphate buffer).

The iodinated glucagon was separated from free I^{125} iodine using column chromatography on Sephadex G-25 fine. For this purpose about 10 g of Sephadex were washed with distilled water to remove the fine particles. The gel was allowed to swell overnight in 4-6 volumes of 0.2 M glycine buffer, pH 8.8 and then equilibrated with the same buffer containing 0.5% albumin. After this procedure the gel was packed into columns (1.2 x 15 cm) and the buffer allowed to drain.

The iodination mixture was transferred to the Sephadex column using a pasteur pipette. Elution was performed using glycine buffer, 0.2 M, pH 8.8 and containing 0.5% albumin. After the void volume fractions containing ten drops of the eluate were collected in separate vials. Initial experiments showed that fractions 5, 6 & 7 contained iodinated glucagon with the highest immunoreactivity. The specific radioactivity of these fractions was approximately 0.5 mci/µg. The chosen I^{125} glucagon fractions were combined and stored in aliquots frozen at $-20^{o}C$. This material was suitable to use for 4-6 weeks. On the day of the assay the label was further purified using a longer Sephadex column as described below.

(b) Preparation of Glucagon Standards

Crystalline glucagon (5-10 mg) was weighed and dissolved in 0.01 M HCl to exactly 0.4 mg/ml (Stock I). This was diluted with the diluent assay buffer to a concentration of 100 µg/ml (Stock II). Another solution was prepared by further dilution to contain 500 pg/ml (Stock III).

Working standards were then prepared as follows:

STANDARD	A	B	C	D	E	F	G
Diluent Buffer in ml	9.8	9.9	-	5	7.5	8.75	9.375
Glucagon Standard in ml (Stock II)	0.2	0.1	-	-	-	-	-
Glucagon Standard in ml (Stock III)	-	-	10	5	2.5	1.25	0.625
Glucagon Conc. pg/ml	2000	1000	500	250	125	62.5	31.25

Each standard was subdivided into amounts sufficient for one assay and stored at -20°C.

(c) Glucagon Antiserum

Glucagon antibody produced against beef-pork glucagon in rabbits, was supplied in lypholised form in 50 µl portions. To this amount the diluent buffer (5 ml) was added and the whole mixed gently by inversion. The vial was left to stand for 1 h. with frequent mixing by inversion to ensure complete solution. The diluted antibody was subdivided into 0.2 ml fractions and stored at -20°C.

(d) Other Assay Reagents

(i) Trasylol or Kallikrin inhibitor: trasylol (10,000/ml) was obtained commercially from Bayer, Germany.

(ii) Diluent or working buffer: the appropriate dilution of standard antibody and tracer was performed

using glycine buffer (0.2 M, pH 8.8) containing bovine crystalline albumin (0.25%) and 1% normal sheep serum. This buffer was also used in the assay mixture.

(e) The Glucagon Assay Procedure

(i) Preparation of Plasma Samples: stored samples were taken out of the deep freeze and thawed at room temperature. The tubes were mixed by inversion.

(ii) Purification and Dilution of I^{125} Glucagon: during the storage of I^{125} glucagon a significant amount of damage occurred. It was, therefore, necessary to repurify each batch of the labelled glucagon immediately before the assay. Purification was performed using a Sephadex G-25 fine column (1.2 x 50 cm). The adoption of this procedure made it possible to separate I^{125} immunoreactive glucagon from damaged material and from some residual free iodine.

The purified tracer was then diluted using the diluent buffer to produce 10,000 d.p.m./0.5 ml which is equivalent to approximately 15 pg of glucagon. This amount was used in each assay tube.

(iii) Dilution of the Antiserum: an aliquot of the antibody, stored at -20°C, was thawed at 4°C, and diluted using the diluent buffer to produce an antibody concentration which bound 50-60% of the tracer in the assay tube containing no glucagon standard. Initial experiments showed that a final dilution of 1/40000 was the most

suitable to achieve this binding ratio.

(iv) The Glucagon Assay Mixture: the assay mixture of glucagon was prepared in plastic tubes in duplicates as shown in the following table:

Tube	Buffer	Plasma Sample	Glucagon Standard (pg/ml)	Antibody	Tracer	Trasylol	Assay Volume
STD curve damage control	400	-	200	-	500	100	1200
Zero Standard	200	-	-	400	500	100	1200
STD A-G	-	-	200	400	500	100	1200
Unknown Plasma damage control	400	200	-	-	500	100	1200
Unknown Plasma Samples	-	200	-	400	500	100	1200

Tubes were placed in a cold tray during the addition of the assay ingredients and mixed by gentle shaking of the racks. The tubes were then incubated for 4 days at $4^{o}C$.

(v) Separation of Bound and Free Glucagon: A suspension of dextran coated charcoal (0.5% charcoal and 0.25% dextran) was prepared by mixing equal volumes of 1% Norite A in 0.2 M glycine buffer (pH 8.8) with 0.5% dextran 80 prepared in the same buffer. The mixture was thoroughly stirred magnetically for 15-20 min. Normal sheep serum (0.2 ml) was added to the tubes not containing plasma in

RADIOIMMUNOASSAY OF

RAT GLUCAGON STANDARD CURVE

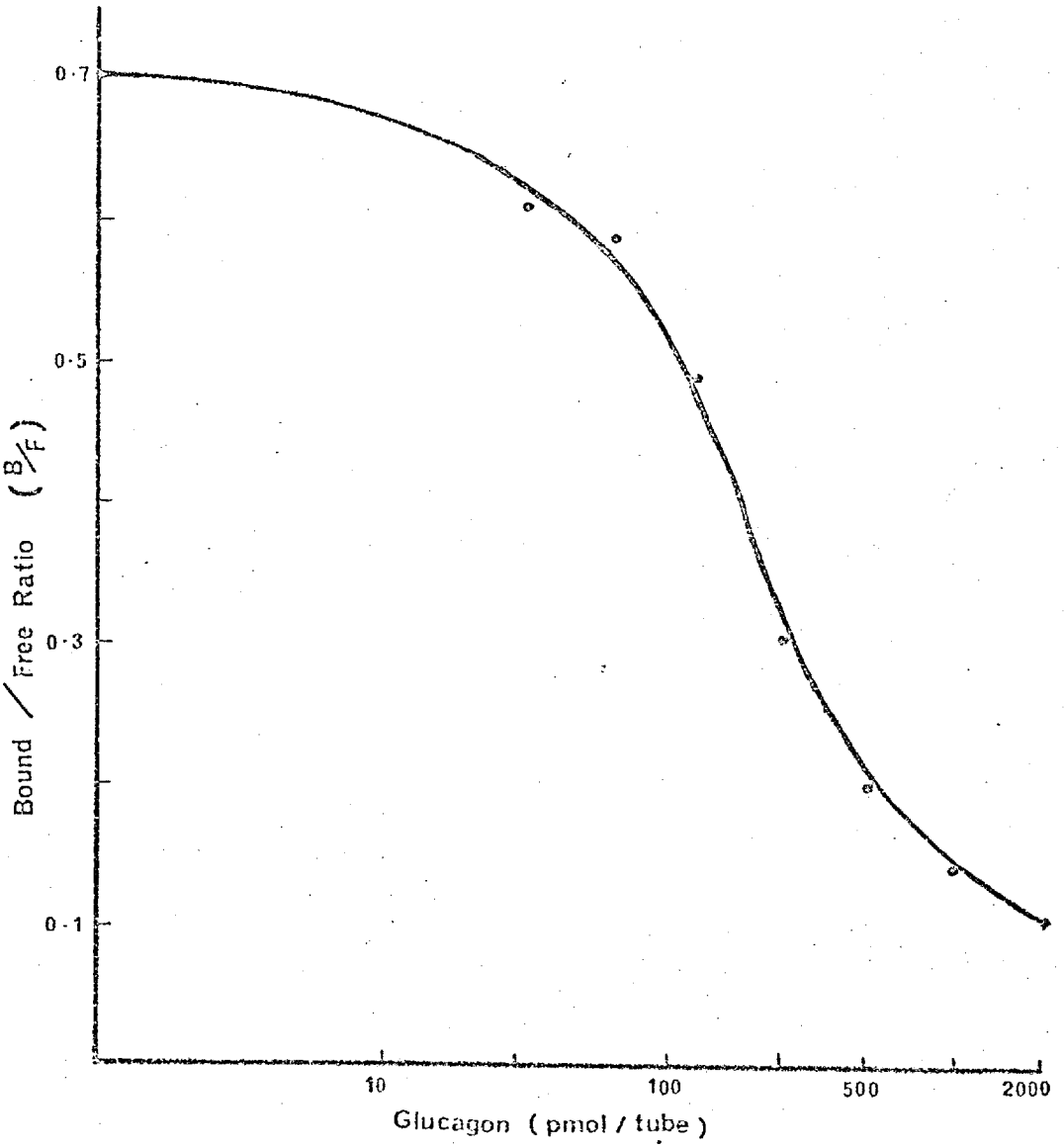


FIGURE (5)

order to compensate for the protein content of samples. Charcoal dextran mixture (0.5 ml) was added to each tube and the tubes were re-incubated for 45 min. The tubes were centrifuged at 4°C for 15 min. and the supernatant was aspirated. Both the supernatants and the charcoal pellets were counted for radioactivity in a Wallac gamma spectrometer.

The mean value of bound/free (B/F) ratios in duplicate determinations was plotted against standard amounts of glucagon to obtain a calibration curve with each assay and unknown concentrations were determined by reference to this curve (Figure 5).

(2) Radioimmunoassay of Insulin

Principle

Insulin was assayed by a modification of the method of Albano et al (1972) using rat insulin standard and insulin antiserum produced in guinea pigs. The antibody bound was separated from the free hormone using albumin coated charcoal.

(a) Materials: the assay was carried out in phosphate buffer (0.05 M) at pH 7.4. The buffer was prepared in concentrated form (7.8 g $NaH_2PO_4 2H_2O$ and 101.2 g Na_2HPO_4/litre) and was subsequently diluted 5-fold with water. To the diluted buffer crystalline bovine plasma albumin was added (0.3 g/100 ml). This working phosphate albumin buffer was used for subsequent dilution of standards, radioactive

tracer and antiserum, and was also used in the assay mixture. Insulin standards were prepared from crystalline rat insulin (Novo Industries, Copenhagen) and were kept as stock solutions containing 10 μU/ul in veronal acetate buffer pH 8.6 containing 0.5% albumin. Aliquots were stored frozen at -20°C. Dilutions of this standard were made to give the working range of the standards as shown below:

STANDARD	A	B	C	D	E	F	G	H
Working Buffer (ml)	0.95	0.9	0.8	0.7	0.6	0.4	0.2	-
Insulin Standard (ml)	0.05	0.1	0.2	0.3	0.4	0.6	0.8	1.0
Insulin Concentration in μU/ml	12.5	25	50	75	100	150	200	250

Insulin I^{125} (specific radioactivity, 100 to 200 μci/ug) was obtained from Amersham Radiochemical Centre. The antiserum used in the assay was purchased from the Wellcome Research Laboratories (anti-insulin serum code MR09/10). This antiserum produced in guinea pigs against pork-insulin demonstrated very good cross reaction with rat insulin standard (Oliver 1973).

The standard curve for the assay was performed in the presence of insulin free plasma so that approximately equivalent protein concentration was maintained in the standard and test tubes. The insulin-free plasma was prepared by adding fasting human plasma (10 ml) to

Norit OL activated charcoal (1 g) and incubating the mixture for 2 h. At the end of this period it was filtered and centrifuged and the clear plasma obtained was stored in 0.2 ml portions at -20°C.

(b) The Insulin Assay: Volumes of each reagent added to the assay tubes are shown in the table below.

Tube	Buffer	Ins.free Plasma	Plasma Samples	Ins. STD	Antibody	Tracer	Total Volume
Standard Curve damage control	600	50	-	-	-	100	750
Zero Standard	550	50	-	-	50	100	750
Standards A-H	500	50	-	50	50	100	750
Unknown Plasma damage control	600	-	50	-	-	100	750
Unknown Plasma Samples	550	-	50	-	50	100	750

(All volumes are in µl)

At least five quality controls of stored plasma were run with each assay and treated in the same way as unknown plasma samples. Unknown samples which were considered to have a concentration greater than the highest standard were diluted with charcoaled (insulin-free) plasma. The plasma samples, quality controls, and appropriate standards, were thawed and mixed thoroughly. Portions (50 µl) were measured into each tube using a Hamilton

syringe. The I^{125} insulin tracer, antiserum and working buffer were added using "Repette" syringes for each component.

Blanks containing insulin-free plasma or test plasma were run simultaneously to allow for "damage" correction for both the standard curve and the test samples respectively. The "damage" fraction in this instance is defined as: that fraction of insulin which does not bind to charcoal in the absence of insulin antibody.

The insulin I^{125} tracer and the antiserum used in the assay were diluted as follows:

Each vial contained 5 µci of insulin I^{125} and approximately 0.1 µg insulin and was diluted in 120 ml of buffer. Each 100 µl of this solution would contain about 80 pg insulin and 10,000 d.p.m. The diluted radioactive tracer was then stored at -20°C and was used within four weeks. Insulin antiserum was diluted so that its final assay mixture would be 1/50,000-fold.

The assay mixture was incubated at 4°C for 65 h. and the incubation terminated by the addition of 100 µl of dextran coated charcoal suspension. The charcoal suspension was prepared by adding 1 g of charcoal (Norit OL) to 20 ml phosphate buffer containing 0.3 g% albumin and mixed for 1 h. After charcoal addition, the incubation mixture was allowed to stand for 30 min. and the tubes were then centrifuged 3000 g for 20 min. The supernatant containing the antibody-bound hormone was transferred to

RADIOIMMUNOASSAY OF

RAT INSULIN STANDARD CURVE

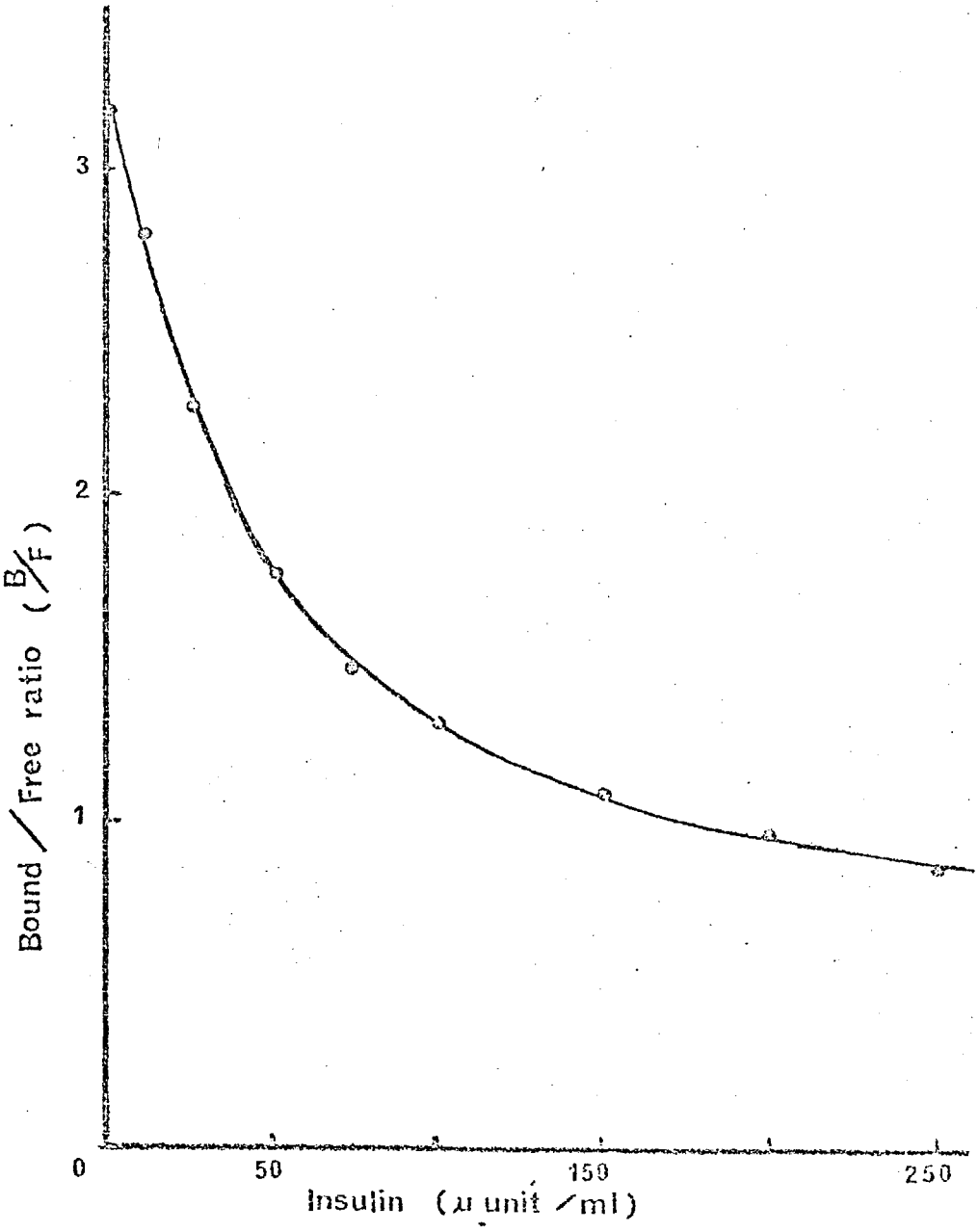


FIGURE (6)

a plastic vial, capped and counted in a Wallac gamma spectrometer. The radioactivity in the respective charcoal pellets was counted simultaneously.

(c) Calculations

The bound/free ratio was calculated and a calibration curve of standard concentration of insulin in μU/ml was plotted. Unknown concentrations were then determined by reference to this curve (Figure 6).

(3) Plasma Triglycerides Determination

Principle

Plasma triglycerides were extracted free from phospholipids and from free glycerol by the method of Laune (1966). The triglycerides were then saponified and the liberated glycerol was measured enzymatically as described by Garland and Randle (1962).

(a) Extraction and Saponification of Triglycerides:

Activated silicic acid (0.5 g; 100 mesh) was added to 1 ml of ethanol/propan-2-ol ether mixture (1:19 V/V) in 10 ml stoppered centrifuge tubes. The tubes were shaken briskly for 20 seconds and aliquots (0.1 ml) of either plasma, water blank or triolein standard were added. The tubes were again shaken for 10 s. A further 5 ml of ethanol:isopropyl ether mixture was added to each tube and the stoppered tubes were shaken for 30 s. every 10 minutes for the next hour. The tubes were centrifuged at 1000 x g for 5 min. and portions (4 ml) of the

supernatant were cautiously transferred to a second set of tubes each containing 0.6 ml ethanolic KOH (0.5 ml of 6 M KOH added to 9.5 ml absolute alcohol). The tubes were then stoppered, shaken to mix and left in a water bath at 70°C for 30 min. They were then left to cool to room temperature and 1 ml of 0.15 M magnesium sulphate was added. The tubes were briskly shaken and centrifuged at 1000 x g for 5 min. The organic supernatant layer was aspirated and discarded. Portions of the aqueous infranatant were used for the determination of glycerol.

(b) Glycerol Assay:

Materials: Stock solutions of the following materials were prepared.

(i) ATP, disodium salt (90 mM). This solution was adjusted to pH 7.6 with 6 M KOH and stored as 0.3 ml aliquots at -20°C.

(ii) NADH, disodium salt (4 mM) stored as 1.5 ml aliquots at -20°C.

(iii) Triethanolamine buffer (100 mM). The solution as pH 7.6 contained 6 mM magnesium sulphate, 2 mM potassium chloride and 7 mM sodium phosphoenol pyruvate. This buffer was stored in 10 ml portions at -20°C.

The assay medium was prepared by mixing the ATP stock solution (0.2 ml), the NADH stock solution (0.75 ml) pyruvate kinase (500 µg), lactate dehydrogenase (250 µg) and the triethanolamine buffer (10 ml).

Assay Procedure

The extinction of a 2.5 ml volume of the assay medium was read against a water blank in a UNICAM SP 800 spectrometer (with an expanded scale) at 340 nm, using 1 cm light path and silica cuvette. More NADH was added if necessary to give an extinction of apprximately 0.8. To this medium 0.2 ml of sample or glycerol standard was added and when there was no further change in extinction an initial reading was taken. Glycero-kinase (20 µg) was added and the reaction was allowed to proceed for 5 min. at 25°C and when this was complete a second reading of the extinction was made. A standard calibration curve was prepared with each batch of assays and unknowns were measured by reference to this calibration.

(4) Glucose Estimation

Glucose was assayed on Technicon autoanalyser using the method of Cramp (1967). In this method glucose is oxidised by glucose oxidase to gluconic acid. The hyrdogen peroxide which is formed in this reaction is broken down to water and oxygen by the enzyme peroxidase. The oxygen acceptor O-tolidine hydrochloride also present in the assay mixture is converted to a coloured compound which is determined colorimetrically.

Glucose standards (20-100 mg%) were made up freshly for each assay. Quality control serum obtained from Hyland (Travenol Labs.) was also run with each assay to check on variability between assays. Samples from control and steroid treated animals were run simultaneously.

This method was used for measuring glucose both in plasma samples and in liver extracts.

II. Assay of Hepatic Enzymes, Intermediates and Cyclic Nucleotide

(1) Liver Lipogenic Enzymes

(a) Preparation of Enzyme Extract

Ten days prior to sacrifice, the rats were given a special diet in order to reveal the maximal activity of liver lipogenic enzymes as suggested by Neuman et al (1961) and Allman et al (1965). Animals were fed a low-fat, high carbohydrate diet for five days followed by fasting for 48 h. and they then received the low-fat high-carbohydrate for another 48 h. On the day of the experiment rats were given the appropriate dose of pentobarbitone anaesthesia. The abdomen was opened and the liver immediately excised. Portions of the liver randomly selected from different lobes were rinsed in ice cold isotonic saline, lightly blotted and weighed. The liver tissue was then minced by scissors and homogenised in approximately two volumes of ice cold phosphate-sucrose buffer containing potassium phosphate buffer (100 mM, pH 7.5), EDTA (50 μM), sucrose (250 mM) and 2-mercapto ethanol (10 mM). The homogenate was centrifuged at 25,000 x g, at 4^{o}C for 15 min. and the floating fat cakewas removed. The infranatant was then separated and re-centrifuged at 100,000 x g at 4^{o}C for 1 h. to obtain the cytoplasmic fraction. The high speed supernatant, 100,000 g, served as the source of liver lipogenic enzymes. The enzyme preparation was stored at

-20°C in 1 ml portions. Under these storage conditions loss of enzymatic activity was less than 10% after two weeks. All assays were performed within three days of the date of sample.

(b) Assay of Acetyl CoA carboxylase activity

The activity of acetyl CoA carboxylase (Acetyl CoA : CO_2 ligase EC 6.4.1.2) was measured in the 100,000 x g supernatant of liver homogenate using a modification of the procedure described by Maragoudakis (1970). In this acetyl CoA is carboxylated to malonyl coenzyme A in the presence of optimal concentrations of ATP, citrate, Mg^{++} and $^{14}CO_2$ (provided as $NaH^{14}CO_3$). The quantity of CO_2 appearing in the acid stable material as malonyl coenzyme A was then determined (Vagelos et al, 1962).

The enzyme preparation was diluted 4-fold with phosphate-sucrose buffer. Aliquots (0.05 ml) from this material were mixed with 0.25 ml of triethanolamine assay buffer (triethanolamine 50 mM, tripotassium citrate 50 mM, Mg Cl_2, 20 mM, 2-mercaptoethanol, 10 mM, Mn Cl_2, 2 mM, 1% bovine plasma albumin and pH 7.5). The tubes were covered and pre-incubated at 37°C for 30 min. Acetyl CoA (0.25 µmol) and ATP (1 µmol), each in 50 µl of triethanolamine buffer, were then added. Under these conditions the reaction rate was linear for up to 10 min. and was proportionate to the enzyme concentration (Fig. 7 & 8).

The tubes were immediately covered and incubated at 37°C for a further 5 min. At the end of this incubation the reaction was terminated by the addition of 50 µl of 60% perchloric acid ($HClO_4$). The tubes were shaken and

centrifuged at (10,000 x g for 10 min.). Aliquots of the supernatant were then transferred to glass scintillation vials and were left uncovered under a stream of air at room temperature for 12 h. The residue in the vial was redissolved in 50% ethanol (0.2 ml). Scintillation fluid (10 ml) was added and the radioactivity measured. Quenching was corrected by recounting with internal standard of ^{14}C-hexadecane. The scintillation fluid consisted of 160g naphthalene, 10 g of 2.5 diphenyloxazole (PPO) and 100 mg of 2-(1-naphthyl)-5-phenyloxazole (α-NPO) dissolved in a mixture of 770 ml of xylene, 770 ml of 1-4, dioxan and 460 ml of absolute ethanol.

Enzyme Unit and Calculation of Enzyme Activity

One unit of enzyme was defined as the activity necessary to carboxylate one μmol of acetyl CoA to produce malonyl CoA per min.

Acetyl CoA carboxylase activity was, therefore, calculated as follows:

$$\text{Acetyl CoA Carboxylase (munits/mg protein)} = \frac{S}{T \times t} \times \frac{B}{V \times W} \times 1000$$

Where:

S = sample counts (d.p.m.)

T = total counts added per tube (d.p.m.)

t = incubation time

B = bicarbonate concentration per incubation tube in μmol

V = volume of homogenate per incubation tube (ml)

W = protein content of liver homogenate (mg/ml)

HEPATIC ACETYL CoA CARBOXYLASE ASSAY

TIME COURSE

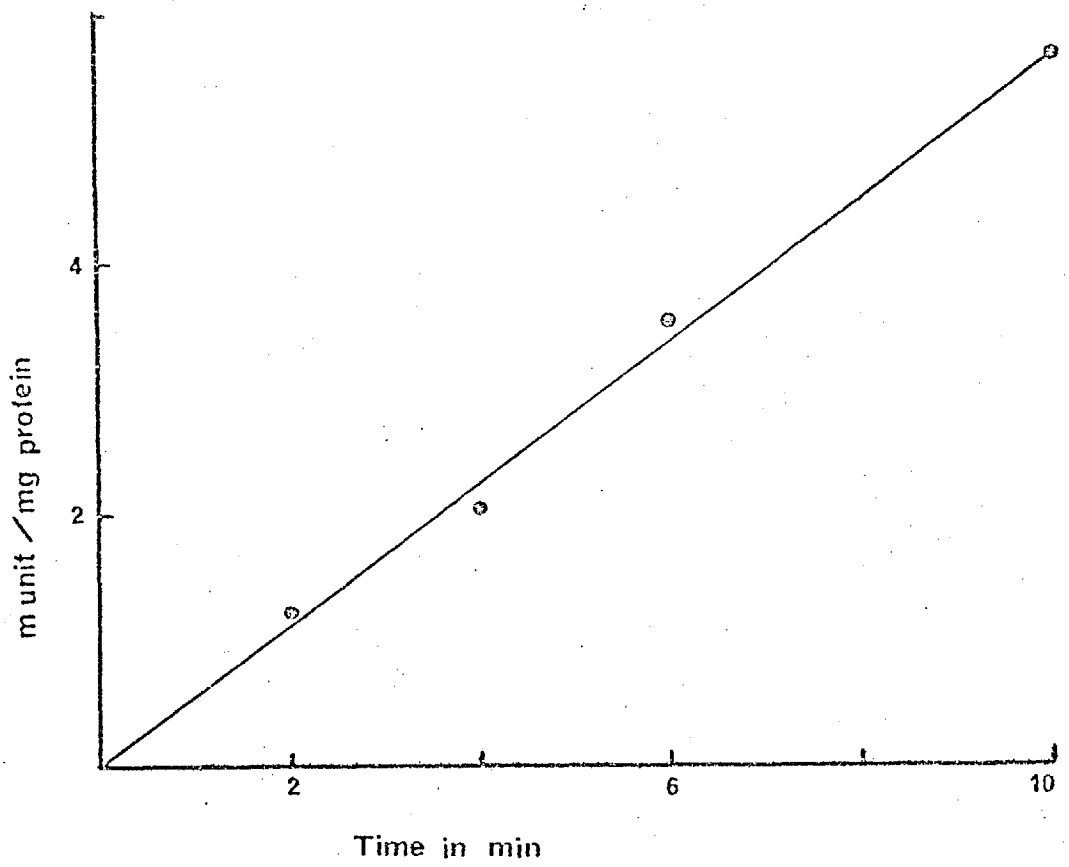


FIGURE (7)

HEPATIC ACETYL CoA CARBOXYLASE ASSAY

PROTEIN CONCENTRATION CURVE

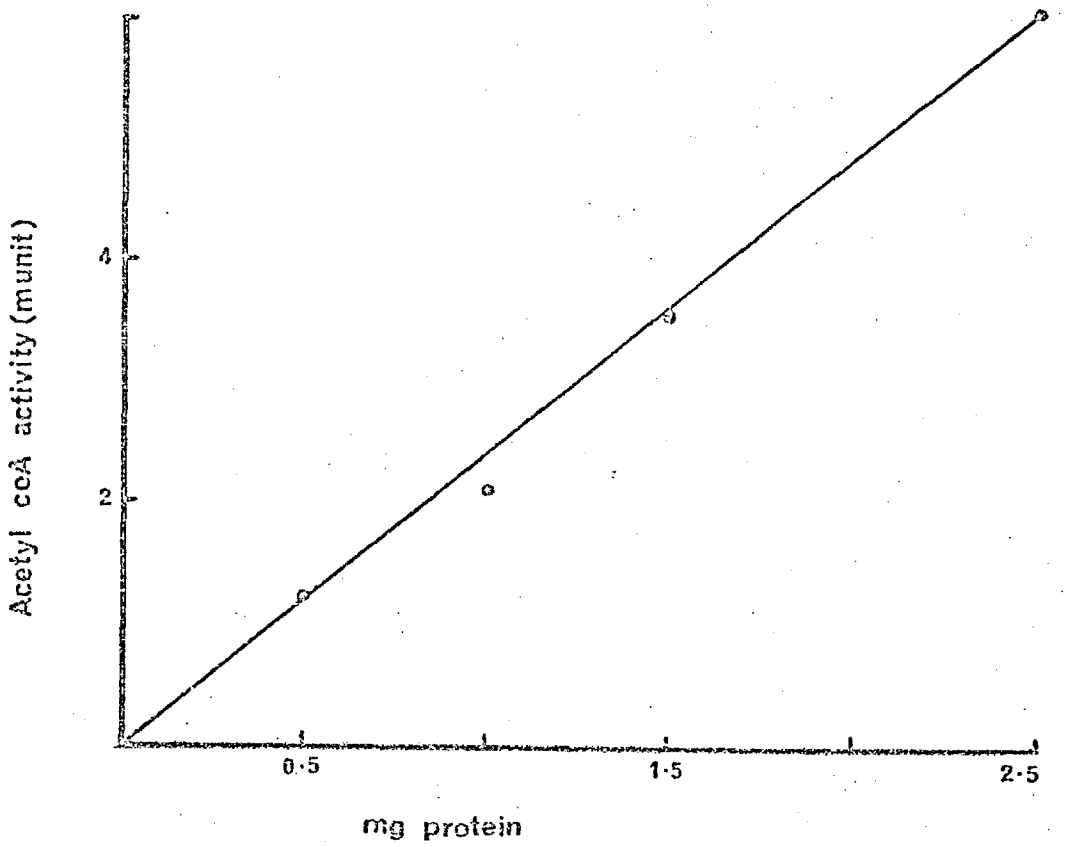


FIGURE (8)

(c) Assay of fatty acid synthetase activity

Fatty acid synthetase activity was measured in the 100,000 x g supernatant of liver homogenates using a modification of the method of Wakil et al (1959). In this procedure the change in extinction coefficient due to oxidation of NADPH as malonyl CoA is converted to long chain fatty acids was determined in a Unicam recording spectrophotometer (model SP800).

Assay Procedure

The complete assay was carried out in silica cuvette maintained at 25°C and with a light path of 1 cm. Each cuvette contained phosphate buffer (pH 7.0; 300 µmol), 2-mercaptoethanol (15 µmol) EDTA (5 µmol) and NADPH (1 µmol). The enzyme preparation (100,000 x g supernatant) was added with 0.3 ml water to bring the total cuvette volume to 2.9 ml. The initial absorbence of this mixture was read against a similarly consistent reagent blank containing no NADPH. When there was no further change in absorbence acetyl CoA (0.2 µmol in 100 µl water) was added and the contents were mixed. The absorbence of the reaction mixture was read at 30 s. intervals over 8 min. Under these conditions the reaction rate was linear with time and protein concentration (Figs. 9 & 10).

Calculation

$$\text{Enzyme activity} = \frac{\Delta E \times Vl}{10^6 \times \Sigma \times d \times V_2} \text{ µmol NADPH/ml enzyme extract/min.}$$

Vl = 3 ml, the total volume of reaction medium

V2 = 0.100 ml, the volume of enzyme extract added

Σ = $6.22 \times 10^6/cm^2/mole$, extinction coefficient at 340 mµ of NADPH

d = 1 cm, light path of the cuvette

HEPATIC FATTY ACID SYNTHETASE ASSAY

TIME COURSE

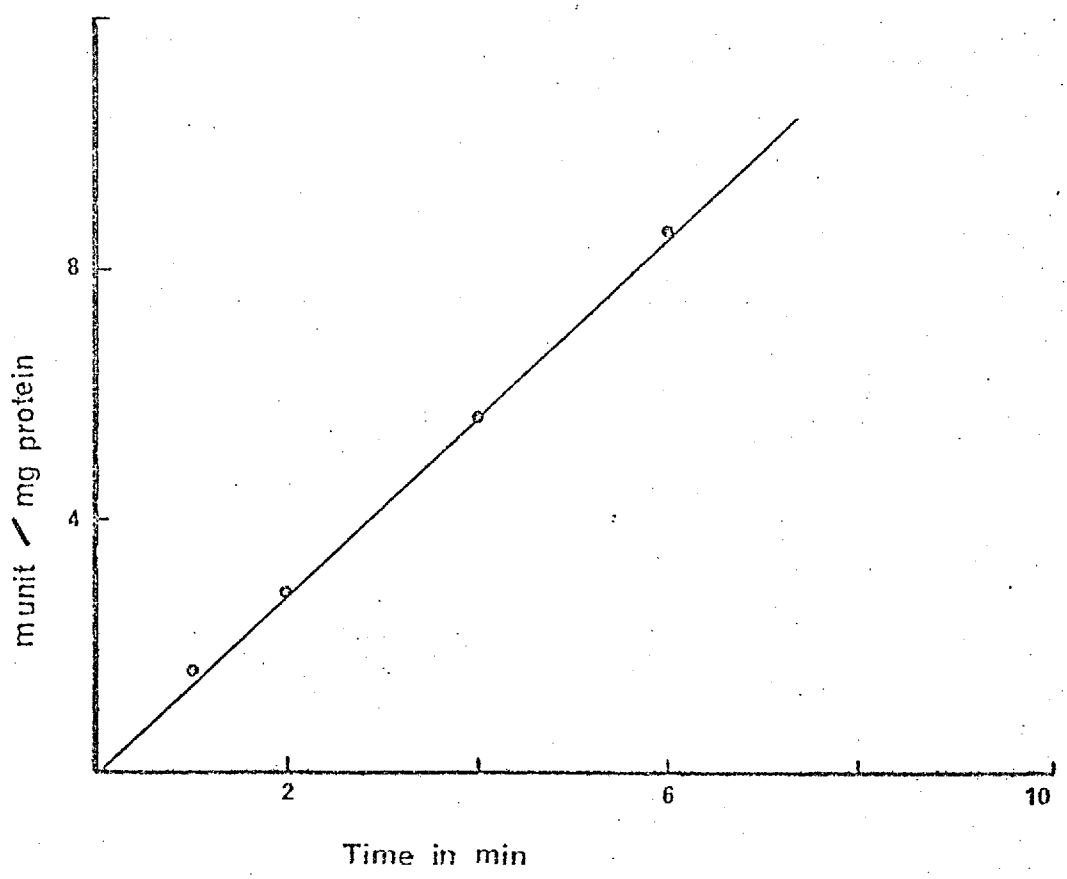


FIGURE (9)

HEPATIC FATTY ACID SYNTHETASE ASSAY

PROTEIN CONCENTRATION CURVE

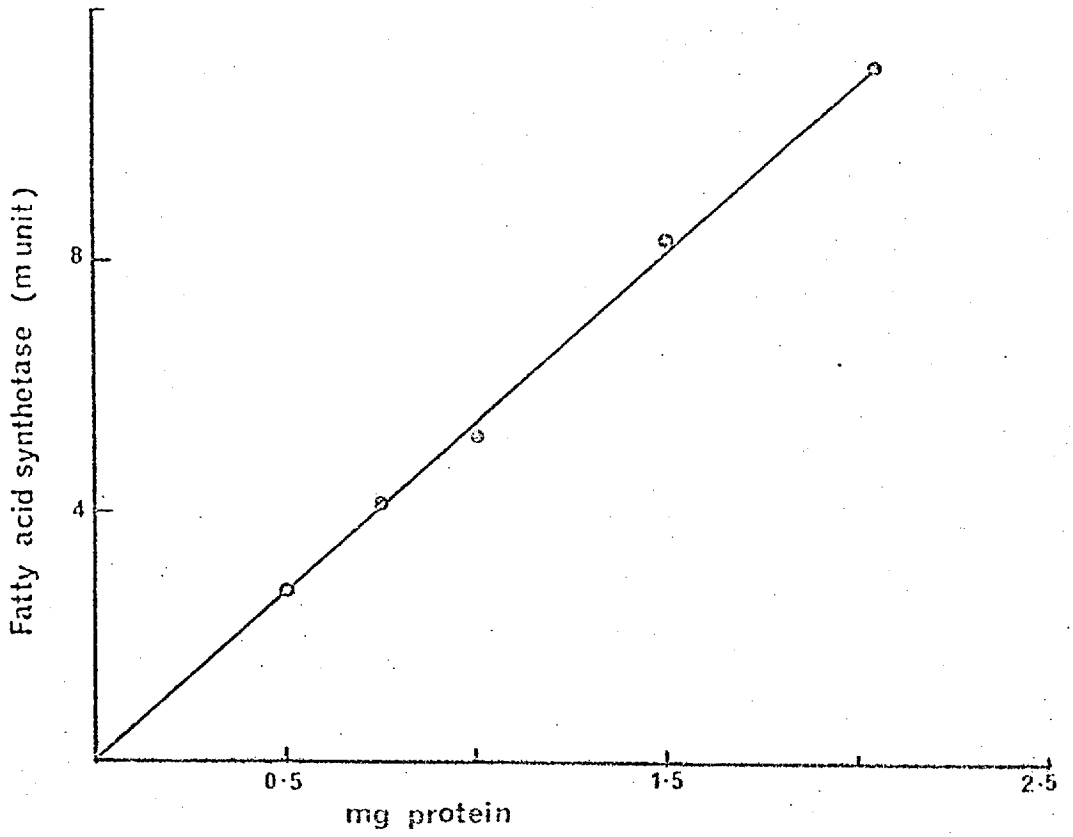


FIGURE (10)

ΔE = change in extinction due to the reaction/min.

Hence the activity $= \frac{\Delta E \times 3}{10^{6} \times 6.22 \times 10^{-6} \times 1 \times 0.1}$ μmoles NADPH/ml of enzyme extract/min.

$$OC = \frac{3000 \times \Delta E}{0.622}$$ nmoles NADPH per ml of enzyme extract per min.

If 1 ml of enzyme preparation (100,000 x g supernatant) contains X mg of protein then enzyme activity equals:

$$\frac{3000 \times \Delta E}{0.622 \times W}$$ nmol NADPH per mg enzyme protein/min.

Assuming that palmitic acid forms the bulk of the fatty acids synthetised by this process then from the overall stoichiometric equation of the reaction:

$$CH_3\ CO.S.CoA + 7HOOC.CH_2.CO.S.CoA + 14\ NADPH + 14\ H^+$$

Acetyl CoA Malonyl CoA

$$= CH_3.(CH_2)\ 8.COOH + 7CO_2 + NADPH + 8\ CoA + 6\ H_2O$$

palmitic acid

the molar ratio of NADPH to malonyl CoA consumed during fatty acid synthesis should be 2 : 1.

Hence enzyme activity equals: $\frac{3000 \times \Delta E}{0.622 \times W} \div 2$

$$= \frac{3000 \times \Delta E}{1.244 \times W}$$ nmol of malonyl CoA/mg of enzyme protein/min. or munits/mg protein.

one unit activity of fatty acid synthetase is defined as the activity required for utilisation of one μmole of malonyl CoA per minute per mg protein.

(2) Measurement of Liver Gluconeogenic Enzymes

(a) Preparation of the Enzyme Extract

The animals were fasted for 36 h. prior to sacrifice

and the liver was removed under pentobarbitone anaesthesia. Portions of liver were removed and homogenised in approximately 10 vol. of ice cold Tris-HCl buffer (0.1 M, pH 7.4) and containing 250 mM sucrose. The homogenate was centrifuged at 25,000 x g for 10 min. at 4°C and the floating fat cake removed. The supernatant was then recentrifuged at 100,000 x g for 20 min. and the supernatant containing the cytoplasmic fraction was separated and immediately frozen at -20°C. No measurable deterioration in the enzyme activity was detected over four weeks of storage.

(b) Assay of Phosphoenolpyruvate Carboxykinase (PEPCK)

PEPCK activity was measured by determining the rate of carboxylation of phosphoenolpyruvate to oxaloacetate using the methods of Chang & Lane (1966) and Sanchez-Medina et al (1971). The oxaloacetate generated from the reaction was then reduced to malate simultaneously with the consumption of proportionate amounts of NADH. In the original technique the change in spectrophotometric extinction of NADH was used to calculate the enzyme activity. In the present study we have modified the original method using a radiometric assay instead. $^{14}CO_2$ provided as NaH $^{14}CO_3$ was therefore used to carboxylate phosphoenolpyruvate to ^{14}C-oxaloacetate, and the latter was converted to acid stable ^{14}C-malate. The acid stable product (malate) was used to calculate PEPCK activity.

The assay tube contained: Tris-HCl buffer (25 µmol, pH 7.4), $MnCl_2$ (7.5 µmol), reduced glutathione (0.5 µmol),

inosine-5-diphosphate (7.5 µmol), NaH $^{14}CO_3$ (12.5 µmol and 2.5 µci), NADH (0.6 µmol), and malate dehydrogenase (12.5 µg/tube). An appropriate amount of liver homogenate was added, and the reaction was initiated by the addition of 7.5 µmol of phosphoenol pyruvate to each incubation tube. The total volume of the assay mixture was 750 µl.

The tubes were covered and incubated at 30°C in a metabolic shaker for 15 min. After incubation the reaction was terminated by the addition of 4 M HCl (250 µl). The acid stable radioactivity was then determined on triplicate aliquots as described previously for the acetyl CoA carboxylase. Under these conditions the reaction was linear with time for 20 min. as shown in Figure (11) and was proportional to the concentration of the homogenate in the assay mixture (Figure (12)).

Enzyme Units and Calculation of the Enzyme Activity

Units of enzyme activity were defined as the activity required to carboxylate one µmol of phosphoenol pyruvate per min., to produce oxaloacetate. PEPCK activity was therefore calculated as follows:

$$\text{PEPCK (munits/mg protein)} = \frac{S}{T \times t} \times \frac{B}{V \times W} \times 1000$$

Where:

S = sample counts (d.p.m.)

T = total counts added/tube (d.p.m.)

t = incubation time

B = bicarbonate concentration in umol/tube

V = volume of homogenate/tube (ml)

W = protein content of liver homogenate (mg/ml)

HEPATIC PHOSPHOENOLPYRUVATE CARBOXYKINASE ASSAY

TIME COURSE

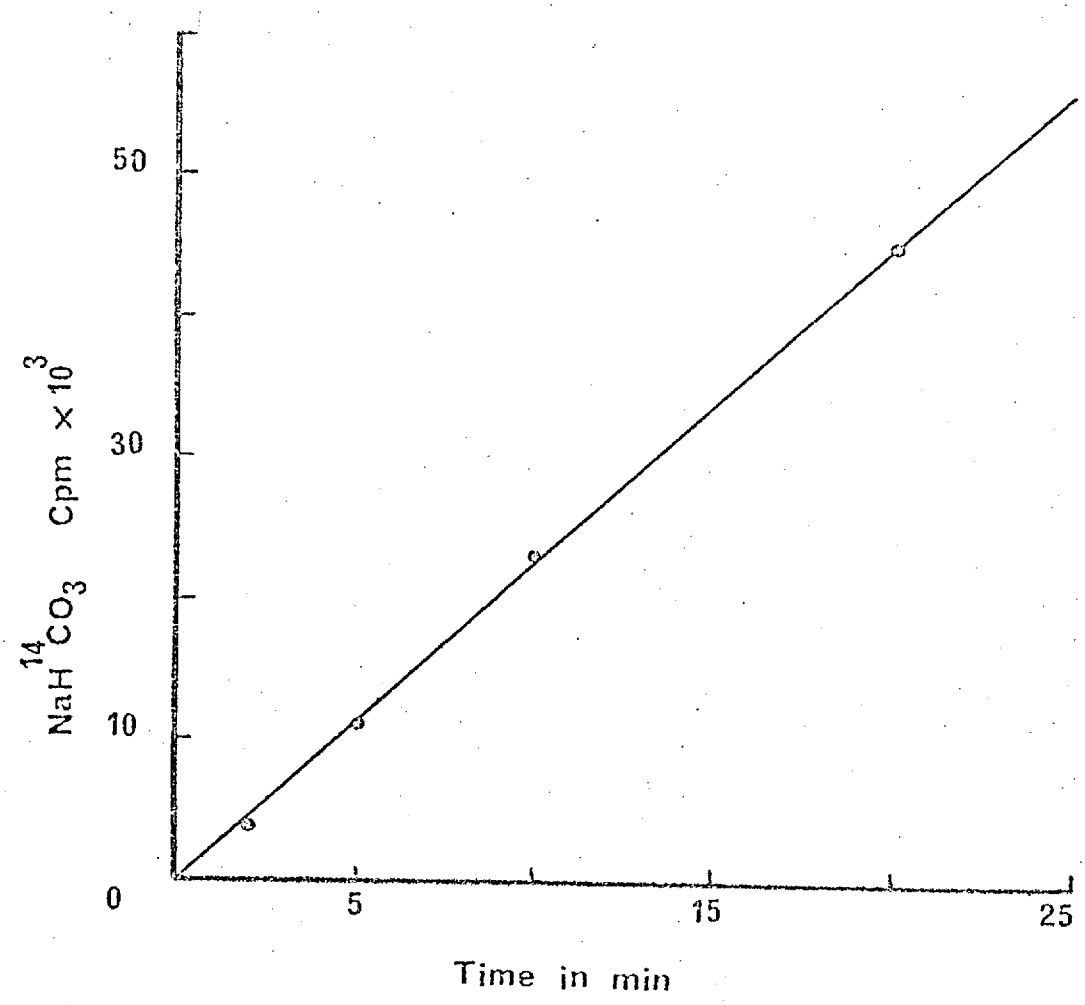


FIGURE (11)

HEPATIC PHOSPHOENOLPYRUVATE CARBOXYKINASE ASSAY

PROTEIN CONCENTRATION CURVE

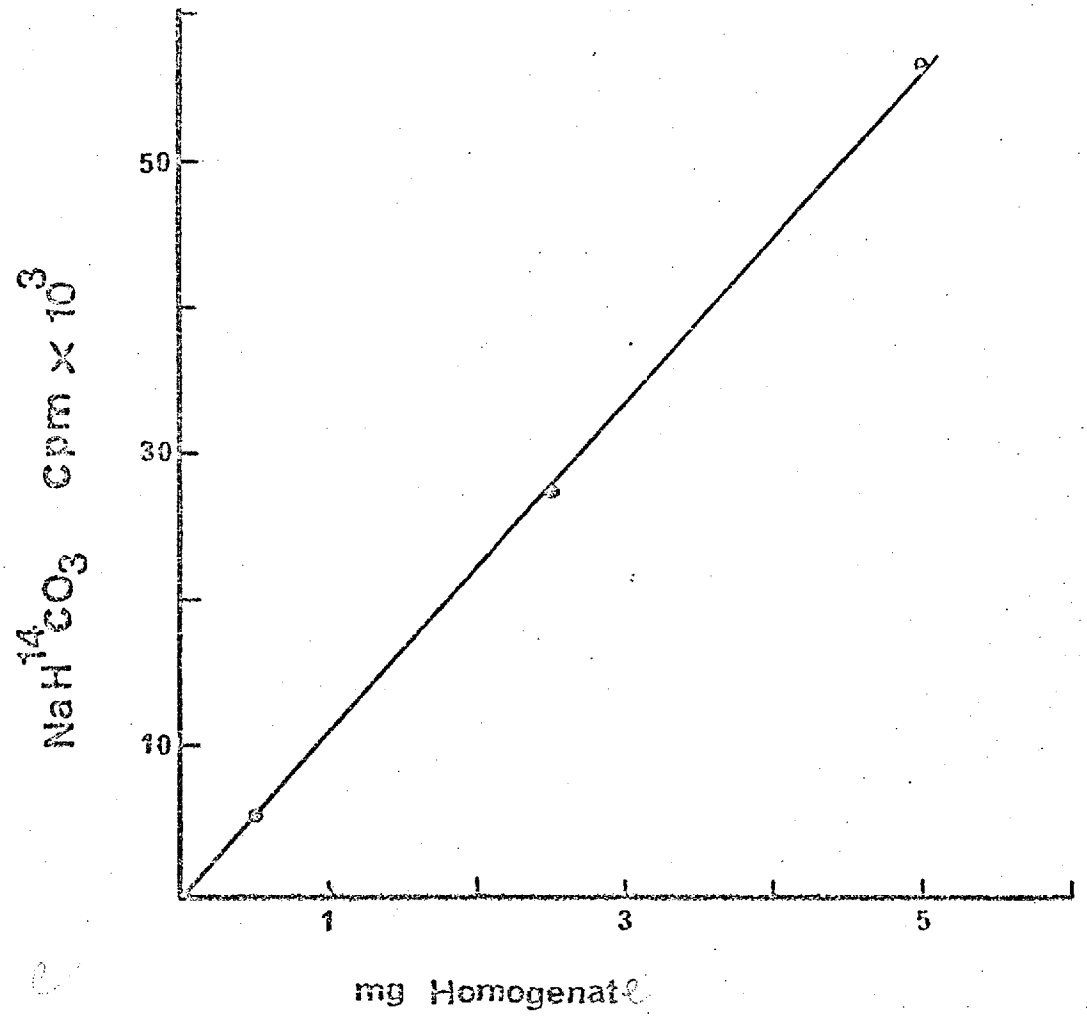


FIGURE (12)

(3) Measurement of Liver Gluconeogenic Intermediates

(a) Preparation of Liver Extracts

Approximately one gram of liver tissue was frozen in liquid N_2. Homogenisation of the frozen tissue was carried out in 6% perchloric acid using an electric homogeniser. Sufficient perchloric acid was added so that the ratio of the final liquid volume to the liver sample was 4 : 1. The water content of the liver was assumed to represent 75% of its weight.

The homogenate was centrifuged at 2000 x g for 20 min. at $4^{o}C$ to precipitate the proteins and the supernatant was separated. This extract was then neutralised using a microburette filled with concentrated solution of $KHCO_3$ (approx. 2 M). The mixture was allowed to stand in cold for 30 min. to allow adequate precipitation of the perchlorate formed during neutralisation. The precipitate was then removed, recentrifuged and the supernatant was used for the assay of the substrates.

(b) Measurement of Pyruvate (pyr), Phosphoenol Pyruvate (PEP) and Glycerate-2-phosphate (2-GP)

These intermediates were measured in liver extracts using a standard enzymatic method (Bergmeyer Methods of Enzymatic Analysis 1974). The procedure is based on the fact that all these substrates could be converted enzymatically to lactate, with the oxidation of stoichiometric amount of NADH. The oxidation of NADH is proportional to the concentration of substrate converted and is measured spectrophotometrically at 340 nm. The reactions involved are as follows:

1. 2-PG $\xrightleftharpoons{\text{Enolase}}$ PEP + H_2O

2. ADP + PEP $\xrightarrow[\text{kinase}]{\text{pyruvate}}$ ATP + pyruvate

3. Pyruvate + NADH + H^+ $\xrightarrow[\text{dehydrogenase}]{\text{lactate}}$ lactate + NAD

Under conditions of the assay the equilibria of reaction (2) and (3) are sufficiently fast in the direction of phosphoenol pyruvate and pyruvate conversion that quantitative reaction was assured, providing that the NADH concentration was more than 0.01 mM.

Procedure

The change in extinction coefficient at 340 nm (1 cm light path) was measured against air at 25°C; the final volume in the cuvette was 2.06-2.22 ml. This assay was also carried out in silica cuvettes.

The neutralised liver extract (0.8 ml) mixed with triethanolamine buffer (1.24 ml) containing triethanolamine hydrochloride (600 mM and pH 7.6), EDTA (6 mM) and NADH (0.2 mM). The contents were mixed in the cuvette and left for 20 min. following which initial absorbence (E_1) was recorded. Lactate dehydrogenase (5 µg powder in 20 µl) was then added and the contents mixed. After 10 minutes the change in extinction was recorded (E_2). A solution (80 µl) containing ADP (2.4 mM), $MgSO_4$ (20 mM) and KCL (74 mM) was added to the cuvette and the contents were mixed. The change in extinction (E_3) was recorded after a further 5 min. Pyruvate kinase (40 µl; 10 µg enzyme protein) was then added and the extinction (E_4) recorded after 5 min. Finally 40 µl containing 10 µg of enolase were added and

absorbence (E_5) was recorded after 5 min.

Calculation

$$\mu\text{mol 3-PG. 2-PG or PEP/ml. deproteinised sample} = \frac{\Delta E \times V}{E \times V_D \times d}$$

Where:

V = volume of the assay mixture

V_D = volume of the deproteinised sample

d = light path of the cuvette (cm)

E = extinction coefficient of NADH (cm/μmol at 340 nm)

ΔE = change in extinction due to the consumption of the substrate

Where:

$$pyr = E_1 - E_2$$

$$pep = E_3 - E_4$$

$$pG = E_4 - E_5$$

From the above equation the concentration of each substrate in μmol/kg wet weight liver could be calculated.

(c) Measurement of Oxaloacetate (oxal), Dihydroxyacetone Phosphate (DAP), D-Fructose-1,6-diphosphate (1,6-F-P_2) and Glyceraldhyde-3-phosphate (GAP)

The enzymatic reactions upon which measurements of these substrates are based could be represented diagramatically as follows:

1. Oxaloacetate + NADH + H^+ $\xleftrightarrow{MDH}$ L - (-) - Malate + NAD^+
2. F - 1, 6 - P_2 $\xleftrightarrow{aldolase}$ DAP + GAP
3. GAP $\xleftrightarrow{TIM}$ DAP
4. DAP + NADH + H^+ $\rightleftharpoons$ L - (-) Glycerol-3-P + NAD^+

In the presence of excess NADH and adequate enzyme concentrations the reaction sequence proceeds rapidly. The decrease in extinction at 340 nm resulting from the oxidation of NADH could then be measured spectrophotometrically.

Assay System

This assay was also carried out in triethanolamine buffer (0.85 ml) contained in a silica cuvette. The triethanolamine buffer contained triethanolamine hydrochloride (400 mM, pH 7.6), EDTA (6 mM) and NADH (17 µM) which were mixed with 1.5 ml of sample extract. The cuvette was left for 10 min. and the extinction at 340 mM was recorded (E1). Malate dehydrogenase (0.3 µg in 5 µl) was added and the contents of the cuvette mixed. The change in extinction (E_2) was recorded. Glycerol-3-phosphate dehyrogenase (10 µl; 3.3 µg/ml) was added and again the change in extinction (E_3) was determined after a further 5 min. Triose-phosphate isomerase (0.165 µg/ml) was added in 10 µl to each cuvette and the extinction (E_4) was recorded after 5 min. Finally the aldolase suspension (10 µl; 5 µg/ml) was added and after 5 min. the change in extinction (E_5) was recorded.

Spectrophotometric measurements were performed at 340 nm in silica cuvette, which had a light path of 1 cm. The total vol. of the assay solution was between 2.265 and 2.295 ml.

Calculation

The change in extinction for each substrate was used to calculate their concentration as follows:

µmol. oxaloacetate. dihydroxyacetone phosphate or D-fructose-1,6-diphosphate/ml deproteinised sample.

$$= \frac{\Delta E \times V}{E \times V_D \times d}$$

Where:

V = volume of the assay mixture

V_D = volume of the deproteinised sample

d = light path of the cuvette (cm)

E = extinction coefficient of NADH (cm^2/µmol at 340 nm)

ΔE = change in extinction due to the consumption of the substrate

Where:

$$\text{oxal.} = E_1 - E_2$$

$$\text{DAP} = E_2 - E_3$$

$$\text{GAP} = E_3 - E_4$$

$$1,6.F.P_2 = E_4 - E_5$$

(d) Measurement of Glucose-6-phosphate

This intermediate was measured by a standard enzymatic method based on the principle that glucose-6-p-dehydrogenase (G6p-DH) catalyses the oxidation of glucose-6-phosphate (G-6-p) by NADP.

Glucose-6-p + NADP $\rightleftharpoons$ 6-phosphogluconolactone + NADPH + H^+

In the presence of suitable excess of NADP the

oxidation of G-6-p was virtually quantitative.

Procedure

The change in optical density due to reduction of NADP was followed spectrophotometrically against air at 25°C and with a light path of 1 cm. The final volume in the cuvette was 1.025 ml. The assay was carried out in silica cuvettes; the assay mixture contained: 0.5 ml neutralised liver extract mixed with 0.5 ml triethanolamine buffer (0.4 M; pH 7.6) 0.01 ml NADP solution (0.2 mM) and 0.01 ml Mg Cl_2 solution (0.5 M). The contents of the cuvette were mixed thoroughly, left to warm up to 25°C and then the optical density was recorded E_1. Using a small glass spatula 0.005 ml of G-6-p-DH solution (0.25 mg protein/ml) was mixed. After completion of the reaction (3-5 min. after the addition of the enzyme) the optical density was recorded E_2.

Calculation

$$\mu\text{moles G-6-p/ml deproteinised sample} = \frac{E \times V}{E \times V_D \times d}$$

Where:

E = difference in extinction $E_1 - E_2$

V = volume of the assay mixture

V_D = volume of the deproteinised sample

d = light path of the cuvette (cm)

E = extinction coefficient when d = 1

4. Measurement of Hepatic c-AMP

Estimation of c-AMP content of liver tissue extracts was performed using a specific protein binding assay. The theoretical basis of this method has been described in detail by Gill and Garren (1970), Gilman (1970) and Brown & Ekins (1971). The modifications introduced by Kissebah et al (1974) and employed in this thesis include the partial purification of the binding protein from bovine adrenal cortices and the extraction of c-AMP from liver tissue in 80% ethanol.

(a) Extraction of c-AMP from Liver Tissue

Following sacrifice of rats, liver tissue was immediately frozen in liquid nitrogen. The tissue was then homogenised in four volumes of 80% alcohol using an electrically driven homogeniser. The water content of liver was assumed to represent 75% of its weight. The homogenate was centrifuged at 3000 x g for 10 min. at $4^{o}C$ to sediment the precipitated proteins. The clear supernatant was then removed and evaporated to dryness under a stream of air. The residue containing the c-AMP was then dissolved in an appropriate volume of the assay buffer and stored frozen at $-20^{o}C$.

(b) Reagents

(i) Preparation of the Binding Protein

Fresh bovine adrenals were obtained from the slaughter house on ice and immediately dissected to separate the cortices. The tissue was then homogenised

in two volumes of buffer containing Tris-HCl (50 mM; pH 7.4), sucrose (250 mM), KCl (25 mM) and $MgCl_2$ (5 mM). Homogenisation was performed at 4°C using an electrically driven homogeniser. The homogenate was first centrifuged at 2000 x g for 20 min. to separate the floating fat cake. The extract was recentrifuged at 5000 x g for another 20 min. to sediment cell debris. The clear supernatant was then recentrifuged at 16,000 x g for 20 min. at 4°C.

The c-AMP binding protein was precipitated from the supernatant using solid ammonium sulphate (40 grams%) which was added while stirring at 4°C. The precipitate was isolated by centrifugation at 16,000 x g for 20 min., solubilised in appropriate volume of buffer and dialysed against 20 volumes of the same buffer. This partially purified preparation was subdivided into aliquots and stored at -20°C.

With each new batch of binding protein a dilution curve was constructed and the dilution which produced a binding capacity between 30 and 40% of labelled c-AMP in the absence of added c-AMP standard was considered as the most appropriate way for this assay.

(ii) c-AMP Tracer

(8-^{3}H) adenosine 3', 5'-cyclic phosphate, ammonium salt was obtained from the Radiochemical Centre, Amersham. The specific activity of this material was 20,000-30,000 mci/mmol. The contents of the vial were diluted with 50% ethanol in water so that 50 ul of the final solution

contained approximately 4000 cpm (< 0.5 picomoles of c-AMP). The solution was then stored at -20°C.

(iii) c-AMP Standard

First stock of c-AMP solution was prepared by dissolving 277 µg of 3', 5'-c-AMP in 10 mls of 50% ethanol in water. A second standard containing 40 pmol/50 µl was then prepared by diluting the first stock 100 times in the working buffer. This solution was aliquoted and stored at -20°C. On the day of the experiment an aliquot was thawed and serial dilutions were prepared to contain 0.5, 1, 2.5, 5, 10, 20 and 40 pmoles per 50 µl.

(iv) Assay Buffer

The working buffer contained Tris-HCl (50 mM), theophylline (8 mM) and mercaptoethanol (6 mM; pH 7.4). This buffer was used for dilution of samples, tracer, standards, binding protein and the assay mixture.

(c) Assay Mixture

The c-AMP assay was carried out in plastic tubes. Duplicate estimations were performed. Each tube contained 100 µl; working buffer, 50 µl; ^{3}H-c-AMP, 50µl; of either the standard or the unknown sample and 100 µl of the diluted binding protein. Non-specific binding was determined in tubes from which the binding protein was omitted. The tubes were adequately mixed and incubated at 4°C for 1.5 hours.

c-AMP PROTEIN BINDING ASSAY

STANDARD CURVE

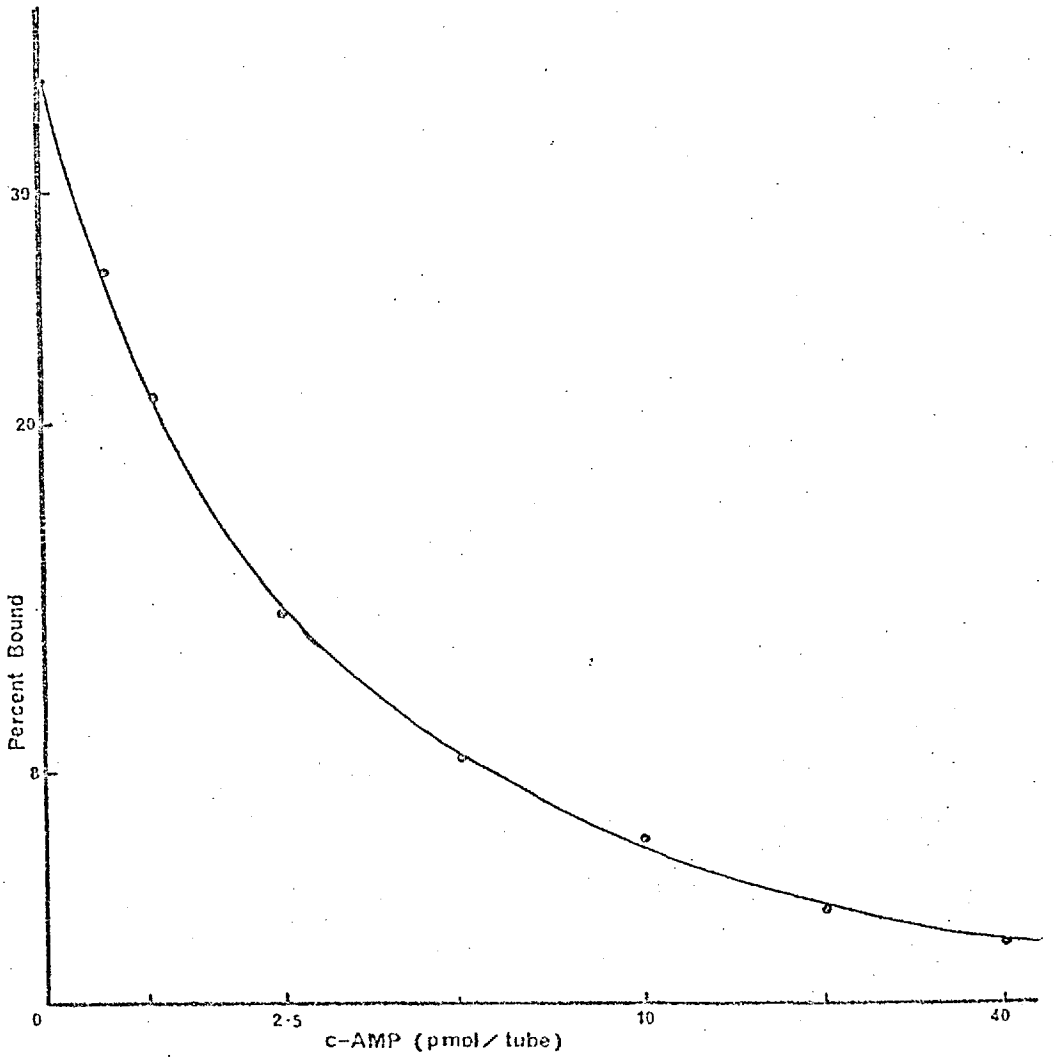


FIGURE (13)

Following incubation, free c-AMP was separated from bound ligand using albumin coated charcoal (12 grams of Norite A charcoal and 2 grams of albumin were dissolved in 100 mls of working buffer). To each assay tube 100 µl of the charcoal mixture was added and mixed with the tube contents. The charcoal pellet was then sedimented at $4^{o}C$ by centrifugation at 2000 g for 30 min. Hundred µl of the supernatant containing protein bound c-AMP were taken from each tube and counted in 10 mls of Kinnard Scintillation Fluid (Naphthalene; 160 g, xylene; 760 ml, dioxan; 760 ml, ethanol; 460 ml, PPO; 10 g, ∝-NPO; 100 mg). Each sample was counted twice for 20 min. in a refrigerated scintillation spectrometer. Figure (13) shows a typical standard curve using this procedure. Tissue extracts were diluted in the working buffer to produce binding ratios falling within the sensitive part of the assay curve.

5. Protein Determination

Total protein content of liver tissue extracts was determined by the method of Lowry et al (1951) using bovine albumin as a standard.

(a) Reagents

Reagent A: contained 2% sodium carbonate in 0.1 N sodium hydroxide.

Reagent B: was prepared as 1% copper sulphate in an aqueous solution of 2% sodium potassium tartrate.

Reagent C: was freshly prepared by mixing 2 mls of reagent B with 100 ml of reagent A.

Folin-Ciocalteu phenol reagent was purchased from BDH Chemical Company and diluted with distilled water to a concentration of 1 M.

Crystalline bovine serum albumin was used to prepare a stock solution containing 500 µg/ml. From the stock, serial dilutions were made to containing 250, 100 and 50 µg/ml.

(b) Procedure

In clean glass tubes 200 ml of either sample or albumin standards were added, followed by the addition of another 200 µl of distilled water. Two mls of freshly prepared solution C were added to each tube and the mixture allowed to stand for 10 min. While the tube was being vigorously mixed, 0.2 ml of diluted Folin reagent was added. The reaction was then continued for 30 min. and read against a blank in Unicam spectrophotometer at wavelength 750nm. From the standard curve readings, the protein concentration in the samples was determined.

CHAPTER III

EXPERIMENTAL PROCEDURES, RESULTS AND DISCUSSION

SECTION 1

CHANGES IN LIVER LIPOGENIC AND GLUCONEOGENIC ENZYMES INDUCED BY FEMALE GONADAL STEROIDS; ROLE OF INSULIN AND GLUCAGON

I N T R O D U C T I O N

From the data reviewed in Chapter I it appears that oestrogenic preparations could lead to a rise in plasma triglyceride concentration predominantly due to enhancement of hepatic lipogenesis and stimulation of triglyceride synthesis. Oestrogens also reduce the fasting plasma glucose concentration probably as a result of the inhibition of hepatic gluconeogenesis. Progestogens, on the other hand, produce either opposing action to oestrogens or no effect.

Oestrogens and progestogens appear to influence the level of circulating insulin and glucagon and it may be that some of the effects of these steroids are mediated by changes either in the secretion or in the action of these peptide hormones.

In this section we have investigated the effects of 17-β oestradiol and progesterone, the naturally occurring female gonadal steroids, on the rate limiting enzymes regulating hepatic lipogenesis and gluconeogenesis. We have also examined the effects of these steroids on insulin and glucagon secretion as well as the responsiveness of liver cells to the pancreatic hormones. We were then able to evaluate the contribution of changes in insulin and glucagon secretion and/or action towards the alterations in hepatic lipogenic and gluconeogenic activity observed during oestrogen or progesterone therapy.

EXPERIMENTAL PROCEDURES AND METHODS

Female Wistar rats (120-140 gms body weight) were ovariectomised and divided into four main groups, (A, B, C and D).

Group 'A' served as controls for the oestrogen treated rats. Each animal was given a daily subcutaneous (s.c.) injection of 0.2 ml corn oil Since the oestrogen was found to induce anorexia in rats and impair their normal gain of weight, the control group was pair fed to achieve similar nutritional status and body weight.

Group 'B' received a daily s.c. injection of 0.2 ml corn oil containing oestradiol for 8 weeks. Animals in this group were subdivided into three subgroups, B_1, B_2 and B_3, each receiving a different dose of oestradiol 1, 10 and 100 µg/kg/day/rat respectively.

Group 'C' served as control for the progesterone treated group and were given daily s.c. injections of corn oil. Since the small dose of progesterone used in this study did not alter the feeding habits of rats and did not influence their growth pattern, animals in this group and in group 'D' were fed ad libitum.

Group 'D' was given daily s.c. injections of progesterone for 8 weeks. Animals in this group were also subdivided into three subgroups, D_1, D_2 and D_3, each receiving different doses of progesterone 0.05, 0.5 and 5 mg/kg/day/rat.

In each experiment conducted the last dose of steroid was administered 24 h. before the animals were sacrificed. For measurements of fasting plasma hormones (insulin and glucagon) and metabolites (triglycerides and glucose), animals were fasted overnight and anaesthetised with pentobarbitone. The main portal vein and the abdominal aorta were exposed and blood was withdrawn. The plasma separated from portal vein blood was assayed for basal immunoreactive insulin and glucagon. Fasting plasma levels of glucose and triglycerides were measured in samples obtained from abdominal aorta.

To determine the secretory capacity of the alpha and beta cells of the pancreas we have also measured the hormonal levels achieved during the intravenous infusion of alanine or glucose. L-alanine was dissolved in distilled water and neutralised with Na oH to pH between 7 and 7.5. The alanine solution was prepared so that each animal was given 1mmol of alanine/kg. The total volume infused per rat was less than 0.2 ml. One fourth of the alanine dose was given as a priming injection followed by continuous infusion of the remaining solution. Animals were anaesthetised with pentobarbitone and the main femoral vein was exposed. T-shaped fine catheter was introduced through the femoral vein and connected with a syringe mounted on an automatically driven pump. Towards the end of the infusion period the portal vein was exposed to obtain blood from the main portal vein for measurement of glucagon to determine the secretory capacity of alpha cells. Basal levels of these hormones

were measured in similarly treated rats that were infused simultaneously with saline instead of alanine.

Animals from each group were also infused with glucose (5.5 mmol/kg) to examine the secretory function of beta cells of the pancreas. One fourth of the glucose dose was given as a pulse injection and the remaining solution was administered by continuous infusion over a period of 15 min. Basal hormone levels were also measured during a saline infusion. The total volume infused per rat was less than 0.2 ml. Plasma samples were obtained from the portal vein for insulin measurement.

For the assay of liver lipogenic enzymes (acetyl CoA carboxylase and fatty acid synthetase) animals were subjected to periods of high carbohydrate feeding and fasting as described in the methodology section. The rats were anaesthetised by pentobarbitone and the liver was removed through an abdominal incision. Homogenates were immediately prepared for the assay of liver enzyme activity. Measurements of phosphoenolpyruvate carboxykinase activity and gluconeogenic intermediates were performed on rat livers obtained from rats after 36 h. of fasting. For the assay of PEPCK the liver was homogenised to prepare the cytoplasmic fraction as described in the methodology section. Liver samples for the analysis of gluconeogenic intermediates were prepared by decapitation of unanaesthetised rats, rapid excision of the liver and immediate freezing in liquid nitrogen. The frozen tissue was then deproteinised and extracted in perchloric acid. The neutralised

extract was then used for the determination of pyruvate, oxaloacetate, phosphoenol pyruvate, 2-phosphoglycerate, dihydroxyacetone phosphate, fructose-1,6-diphosphate, glucose-6-phosphate and glucose.

To assess the responsiveness of liver cells to glucagon, frozen liver extracts were used to determine the tissue c-AMP level before and after the administration of exogenous glucagon. Animals were anaesthetised by pentobarbitone, the liver and the portal vein were exposed through an abdominal incision. Glucagon was given intraportally as a pulse injection and the liver was removed, immediately frozen and c-AMP extracted. Unstimulated values were determined in sham-operated animals subjected to similar surgical manipulation and given a pulse injection of saline instead.

R E S U L T S

I. 17-β-Oestradiol

A. Effect of Oestradiol on Immunoreactive Insulin and Glucagon Concentrations in Portal Vein Blood

(1) Basal Levels: Table (1) shows the mean fasting plasma insulin and glucagon levels in portal vein blood of female ovariectomised rats treated with increasing concentrations of 17-β-oestradiol for periods of 6-8 weeks.

The mean fasting plasma insulin in portal vein blood of control animals was $3.4 \pm 0.25 \times 10^{-10}$M which corresponds to 52 ± 4 μU/ml. Administration of 17-β-oestradiol 1 μg/kg body weight/day in corn oil did not produce any significant changes in the plasma insulin level. On the other hand, treatment with 17-β-oestradiol in doses of 10 and 100 μg/kg/day lead to a small but significant reduction in insulin levels (Table 1 & Figure 14a).

Fasting plasma glucagon in portal vein blood of the control animals averaged $1.7 \pm 0.26 \times 10^{-10}$M (600 ± 91 pg/ml). As with insulin daily subcutaneous injections of small doses of 17-β-oestradiol (1μg/kg/day) did not affect the basal glucagon levels. Oestradiol in doses of 10 and 100 μg/kg/day on the other hand, produced marked and significant reductions in glucagon levels. The concentrations of this hormone in portal vein blood averaged 0.32 ± 0.08 and $0.25 \pm 0.06 \times 10^{-10}$M with the 10 and 100 μg doses of oestradiol respectively (Table 1 and Figure 14b).

The results shown in Table (1) indicate that in overnight fasted control rats the ratio between insulin and glucagon levels in portal vein blood, each expressed in its molar concentration, (I/G), averaged 2.2 ± 0.25 (Mean ± SEM) and was not significantly altered by small doses (1 µg/kg/day) of 17-β-oestradiol treatment. As shown in Figure 14c, the administration of oestrogen in doses of 10 and 100 µg/kg/day resulted in a significant increase in the portal vein insulin/glucagon molar ratio (I/G) with mean values 3-4 times higher than that of control animals. The increase in molar ratio was observed despite some decrease in the absolute insulin concentration since the reduction in the absolute glucagon level was very marked.

(2) Response to Alanine: Figure (15) shows a time course of the effect of infusing alanine (1 mmol/kg) to control rats. Within two minutes from the start of infusion the portal vein glucagon concentration was raised 3-4 times above the basal value found in rats receiving saline infusion. The elevation in plasma glucagon was then maintained for the subsequent twelve minutes of the infusion period. Table (2) indicates that the steady state glucagon concentration in response to alanine was dependent on the amino acid dose infused and that alanine 1 mmol/kg was sufficient to produce maximal stimulation of alpha cell secretion.

Table (3) demonstrates the effects of oestradiol therapy upon the pancreatic alpha cell response to maximal stimulation with alanine (1 mmol/kg). It is

evident that treatment of animals with oestradiol 10 or 100 µg/kg/day markedly suppressed the capacity of alpha cells to secrete glucagon. Both the absolute concentration and the increment above basal were significantly lower in the oestrogen treated rats. No effect, however, was found in animals treated with small oestradiol doses of 1 µg/kg/day (Figure 17a).

(3) Response to Glucose: As depicted in Figure (16) the infusion of glucose 5.5 mmol/kg via the femoral vein to control rats caused a 200-300% rise above the level of immunoreactive insulin measured in portal vein blood of control rats. The effect was demonstrable within two minutes and was maintained for the remaining period of infusion. The dose of glucose infused was sufficient to produce maximal stimulation of insulin secretion and no further increase was observed with higher glucose concentration (Table 4).

Table (3) shows that in animals treated with 1 µg/kg/day of oestradiol plasma insulin concentrations during glucose infusion were not significantly different from that of controls rats. On the other hand, the administration of oestradiol in doses of 10 or 100 µg/kg caused a significant reduction in the absolute levels of insulin achieved during glucose infusion. Moreover the percentage increment above basal was also decreased in the oestradiol treated rats. These results suggest that moderate and large doses of oestradiol (10 & 100 µg/kg) suppress the

secretory capacity of the beta cells to secrete insulin in response to glucose challenge in vivo (Figure 17b).

B. Effect of Oestradiol on Liver Lipogenic Enzymes

Table (5) and Figure (18) show that the mean acetyl CoA carboxylase activity in liver homogenates of control rats was 2.4 ± 0.4 munit/mg protein. Administration of oestradiol in doses between 10 and 100 µg/kg/day for 8 weeks produced a significant enhancement of the activity of this enzyme ($P < 0.001$). The mean acetyl CoA carboxylase activity in livers of animals given the 10 µg dose was 6.2 ± 0.6 munit/mg protein while the activity in the animals treated with the 100 µg doses averaged 5.8 ± 0.5 munit/mg protein. These values were 2-3 fold that of the controls. Daily subcutaneous injections of 1 µg/kg/day of oestradiol, on the other hand, did not produce any significant stimulation of acetyl CoA carboxylase activity.

Figure (19) shows the activity of acetyl CoA carboxylase in liver homogenates of control rats and animals treated with oestradiol (10 µg/kg/day) assayed in the presence of increasing concentrations of acetyl CoA. The concentration of acetyl CoA in the assay mixture was varied from 0.05 to 0.5 mmol/litre. It is evident that at all levels of substrate concentrations the activity of the enzyme was much greater in the oestrogen treated rats. When the concentration of acetyl CoA exceeded 0.2 mM the activity in the oestradiol treated rats was

approximately three times the value in liver homogenates of control rats. These results indicate that the changes in acetyl CoA carboxylase seen during oestradiol therapy were due to an increase in the maximal activity of the enzyme and could represent the induction of this enzyme.

The mean activity of fatty acid synthetase in liver homogenates of control rats was 4.1 ± 1.2 munit/mg protein and was increased significantly to 8.9 ± 2 by the administration of 10 µg of oestradiol/kg body weight/day. A further increase to a mean of 10.1 ± 2.5 was observed when the daily oestrogen dose injected was 100 µg/kg/day. No significant changes were observed in the activity of this enzyme in rats given 1 µg 17-β-oestradiol/day (Table 5).

As depicted in Figure (18) the increase in acetyl CoA carboxylase and fatty acid synthetase activities observed in animals treated with moderate or large doses of oestradiol was associated with 2-3 fold increase in fasting plasma triglyceride levels. On the other hand, the small dose of oestrogen (1 µg/kg/day) which did not alter hepatic enzyme activities had no significant effects on plasma triglyceride concentrations.

C. Effect of Oestradiol on Liver Gluconeogenic Enzymes and Intermediates

Table (6) shows that the activity of phosphoenol-pyruvate carboxykinase (PEPCK) in liver homogenates of control rats averaged 49.1 ± 2.9 mU/mg protein. Administration of 17-β-oestradiol 10 µg/kg/day produced a slight

but significant reduction in the activity of this enzyme (Mean ± SEM = 33.5 ± 1.6). A more marked reduction in PEPCK activity was observed in the animals given larger doses of the steroid (100 µg/kg/day). In this latter situation the mean activity was 22.5 ± 2.3 mU/mg protein). On the other hand, no significant changes in the activity of this enzyme were observed during the period of low dose (1 µg/kg/day) of oestradiol therapy (Figure 20).

As presented in Figure (21) it appears that the decrease in PEPCK activity in livers obtained from oestradiol treated rats was demonstrable at all concentrations of phosphoenol pyruvate. These results suggest that the inhibition of PEPCK by oestrogen is probably due to the reduction in the maximal activity or the amount of enzyme available in the liver.

To explore the in vivo significance of the decreased PEPCK in livers of oestrogen treated rats, hepatic gluconeogenic intermediates were also measured in control and oestrogen treated rats. These results are shown in Table (6) and are presented in a sequential form as described by Exton and Park (1966) in Figure (22). It is evident that the administration of oestradiol (100 µg/kg/day) produced a significant increase in the hepatic concentration of pyruvate and oxaloacetate with a fall in phosphoenol pyruvate as compared to the levels of these precursors in control animals. These results indicate suppression of the pathway separating pyruvate and phosphoenol pyruvate

and are consistent with the finding of reduced phosphoenolpyruvate carboxykinase activity. The crossover pattern of gluconeogenic intermediates thus shown in Figure (22) provides an in vivo evidence to indicate the inhibition of gluconeogenesis in livers of animals treated with oestradiol at PEPCK step. Indeed the fasting plasma glucose measured in blood samples obtained from the abdominal aorta was significantly reduced by the administration of oestradiol to these animals and was parallel to changes in PEPCK (Figures 20 & 22).

D. Effect of Oestradiol on Liver c-AMP Concentration and the Hepatic Response to Exogenous Glucagon

In liver extracts obtained from control rats the basal c-AMP concentration averaged 0.44 ± 0.03 pmol/mg wet wt. As shown in Figure (23) the administration of glucagon 1 µg/kg into the portal vein produced an instantaneous rise in liver c-AMP content with peak values occurring 15 sec. following the injection of glucagon. The nucleotide concentration remained elevated for another 30-45 sec. before returning towards basal value thereafter. The intraportal administration of glucagon resulted in 4-6 times increase in liver c-AMP assayed in liver extracts obtained from control rats 30 sec. after the injection of glucagon.

In animals receiving oestradiol the basal c-AMP concentration was significantly lower than in control rats ($P < 0.01$).

Furthermore, the nucleotide concentration 30 sec. following the intraportal administration of glucagon was significantly lower than that observed in pair fed control rats (Table 7 & Figure 24).

Table 1: Effect of Oestradiol Administration on the Concentrations of Insulin and Glucagon in Portal Vein Blood of Female Ovariectomised Rats

Treatment Groups	Plasma Concentration x 10^{-10}M		Molar Ratio Insulin/Glucagon
	Insulin	Glucagon	
Pair-fed Control (n = 18)	3.4 ± 0.25	1.7 ± 0.26	2.2 ± 0.25
Oestradiol			
1 µg (n = 12)	3.6 ± 0.38	1.68 ± 0.10	2.14 ± 0.25
P	(NS)	(NS)	(NS)
10 µg (n = 20)	**2.8 ± 0.4	**0.32 ± 0.08	*8.8 ± 1.2
P	<0.05	<0.001	<0.001
100 µg (n = 8)	**2.4 ± 0.2	**0.25 ± 0.06	*9.6 ± 1.4
P	<0.002	<0.001	<0.001

(n) = number of animals in each group.

Values are Means ± SEM.

P = probability values for significance of differences from means of controls using student "t" test.

Non-significant differences (NS) means $P > 0.05$.

The duration of steroid therapy was 8 weeks of daily subcutaneous injections.

The doses of steroids shown represent the injected dose/kg body weight/day solubilised in 0.2 ml of corn oil. The control group received daily subcutaneous injections of the solvent corn oil.

** = significant decrease from pair-fed control.

* = significant increase from pair-fed control.

EFFECT OF OESTRADIOL ON

PORTAL VEIN PLASMA INSULIN LEVEL

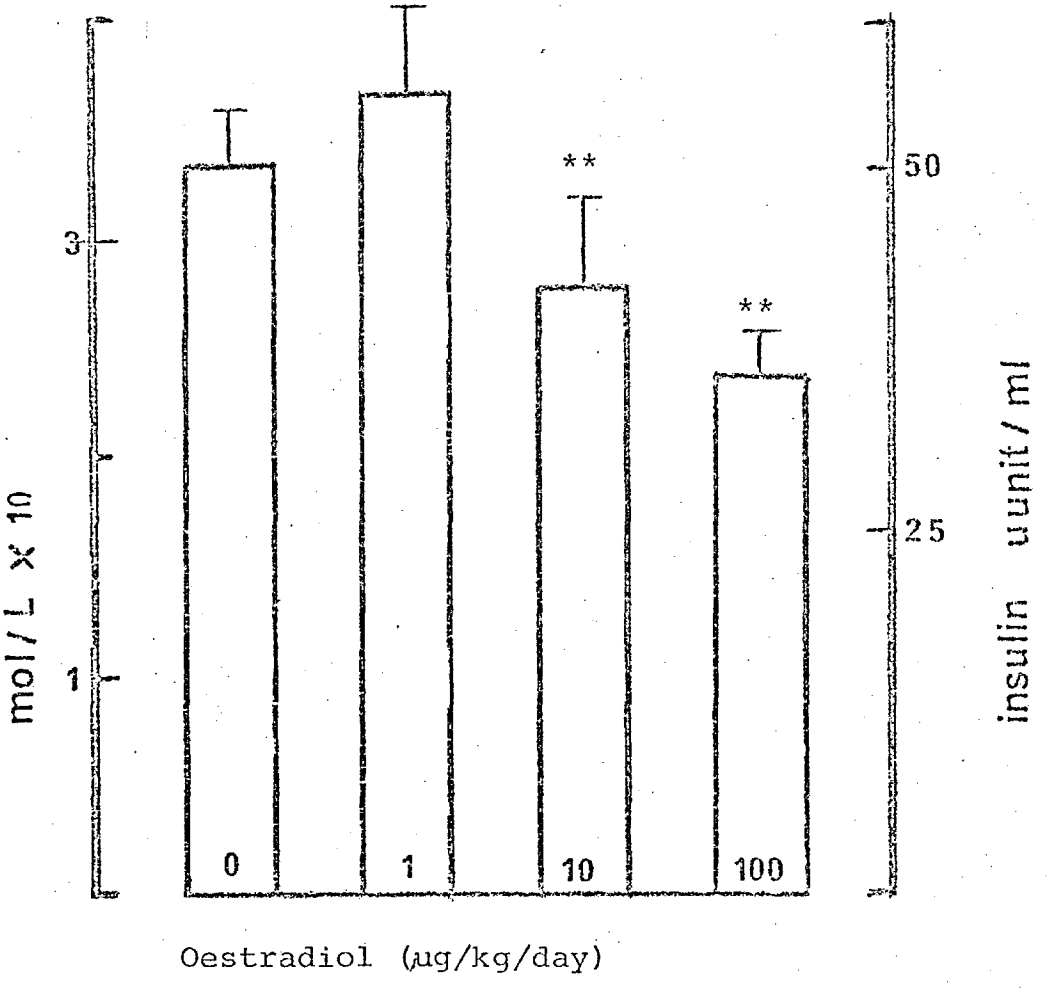


FIGURE (14a)

The dose of oestradiol in each group was given for 8 weeks by daily s.c. injection.

The number of animals used is shown in Table 1.

Bars represent Means and SEM.

** = Significant decrease as compared to pair-fed controls ($P < 0.05$).

EFFECT OF OESTRADIOL ON

PORTAL VEIN PLASMA GLUCAGON LEVEL

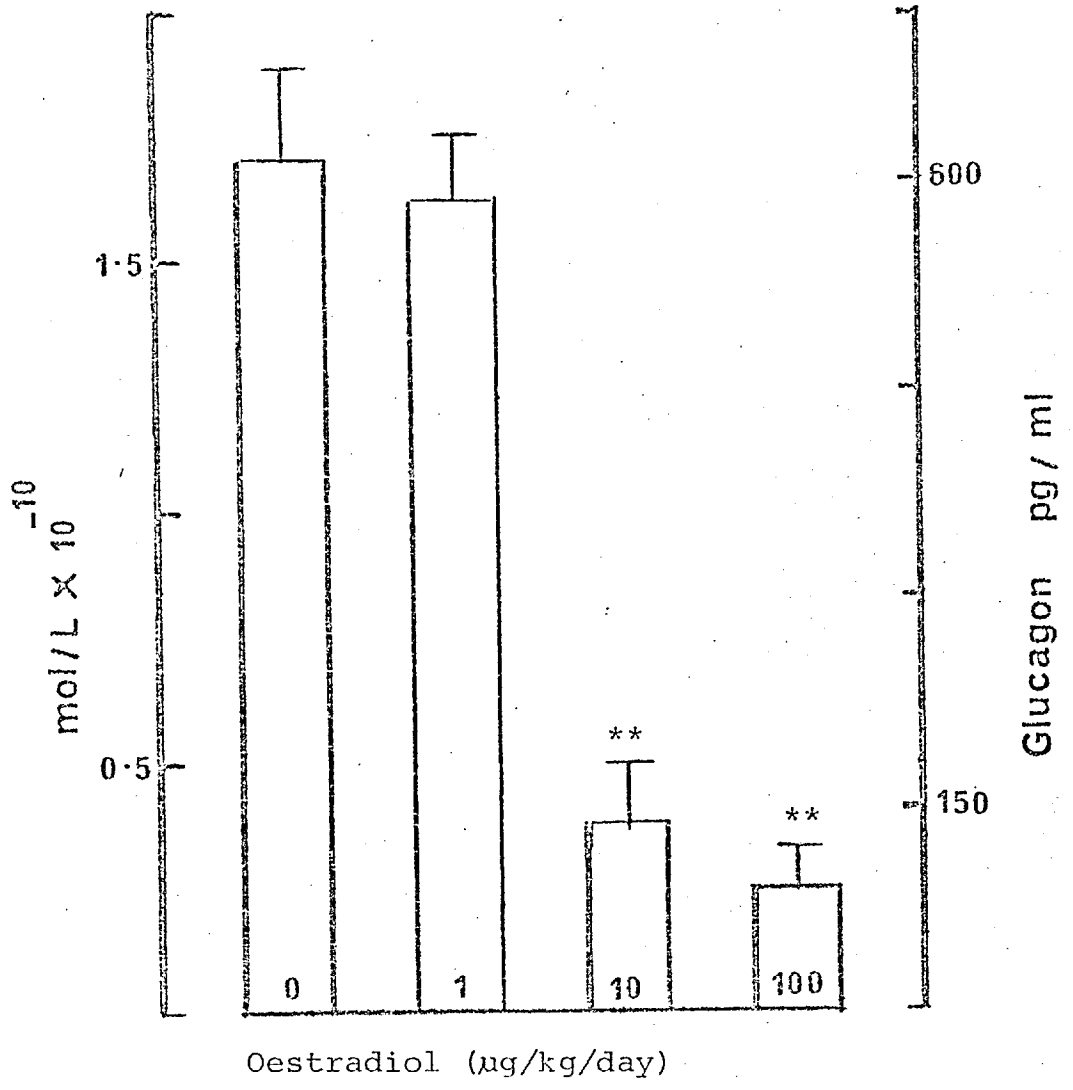


FIGURE (14b)

Oestradiol was given to each group of rats for 8 weeks.

The number of animals in each group is shown in Table 1.

Bars represent Means and SEM.

** = significant decrease as compared to pair-fed controls ($P < 0.001$).

EFFECT OF OESTRADIOL ON PORTAL VEIN PLASMA INSULIN : GLUCAGON MOLAR CONCENTRATION RATIO (I/G)

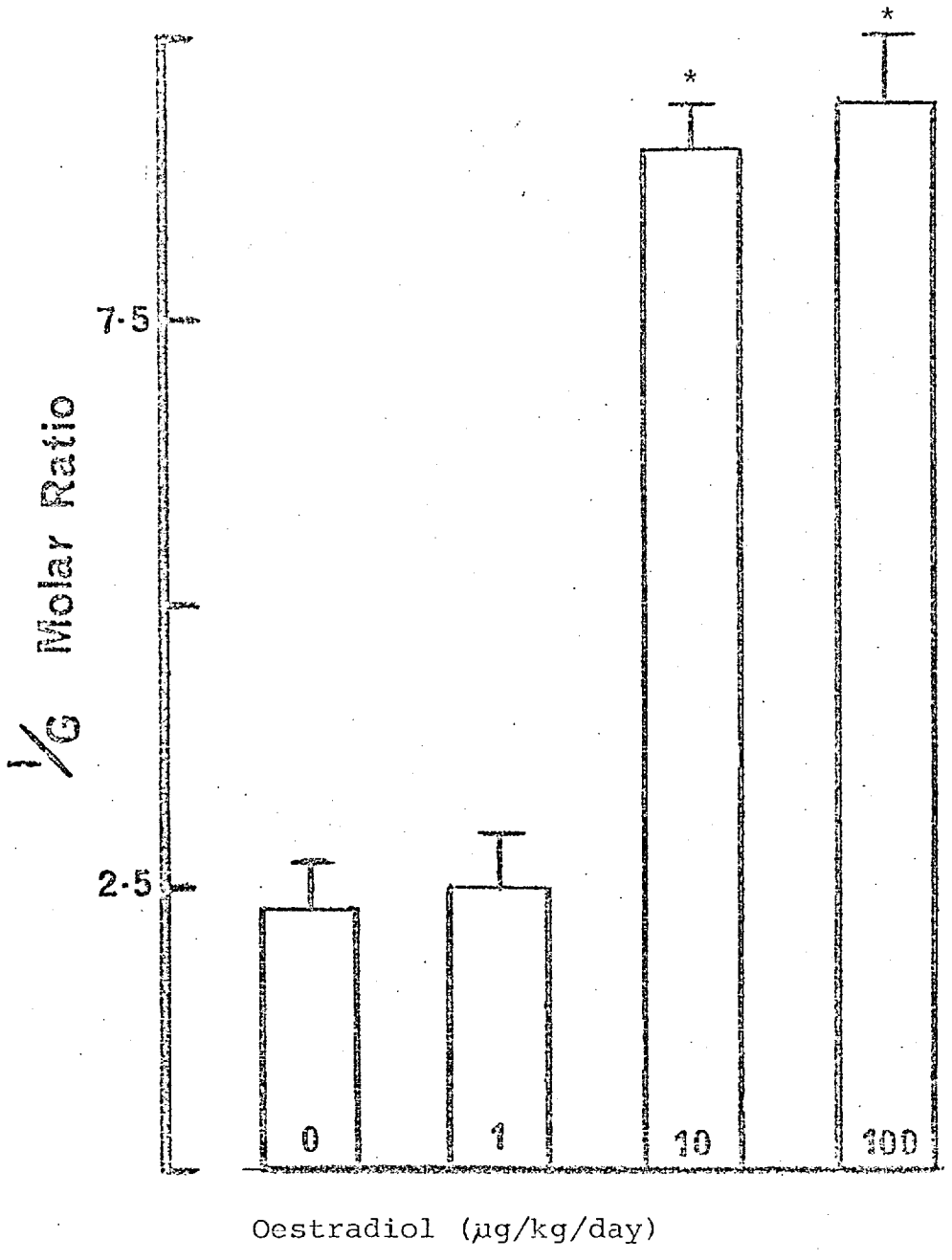


FIGURE (14c)

Oestradiol was given daily by s.c. injection for 8 weeks.

The number of rats used in shown in Table 1.

I/G ratio is calculated from portal vein insulin and glucagon, each expressed in its molar concentration.

Bars represent Means and SEM.

* = Significant increase as compared to pair-fed controls ($P < 0.001$).

EFFECT OF ALANINE INFUSION ON PORTAL VEIN PLASMA GLUCAGON IN CONTROL RATS

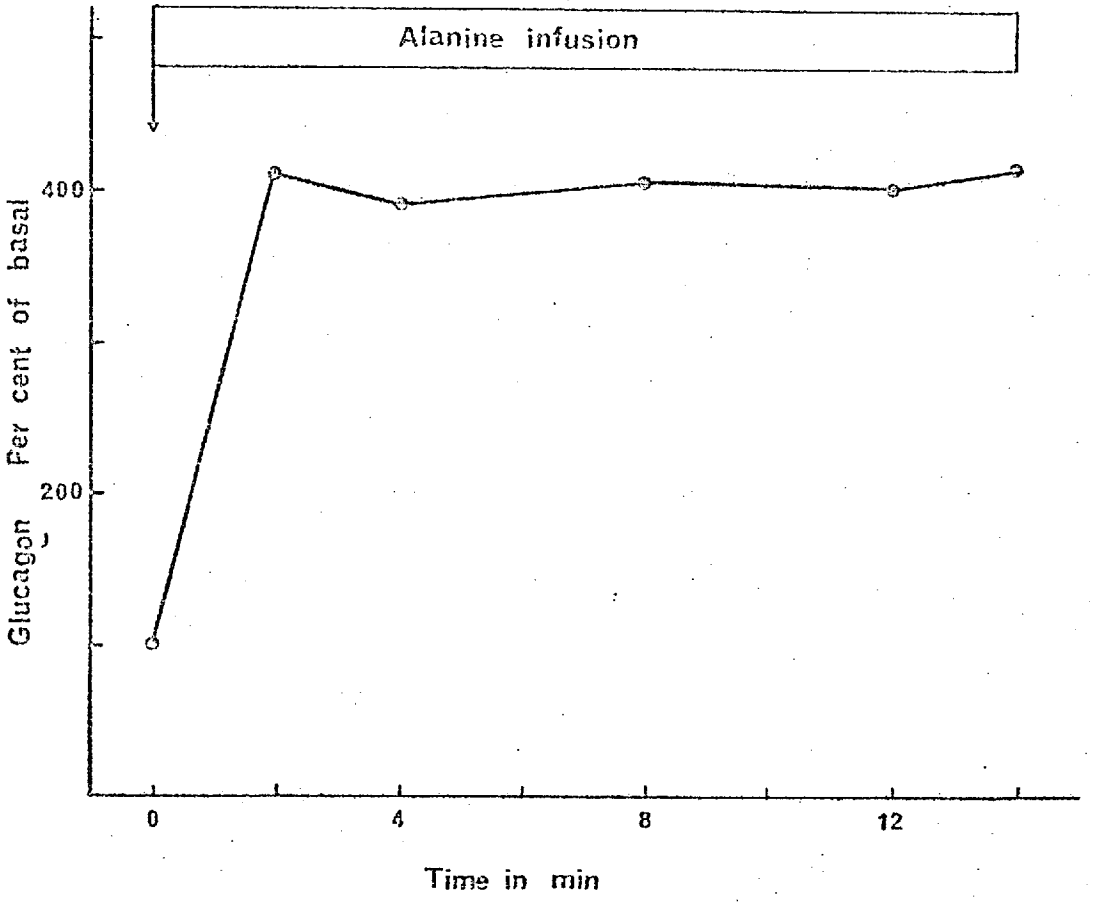


FIGURE (15)

0.2 ml of alanine solution was infused through a cannula inserted in the femoral vein of anesthetised rats. The total dose of alanine administered was 1 mmol/kg. One fourth of the dose was given as a pulse injection followed by a continuous infusion of the remaining solution over a period of 15 min. Animals were sacrificed at appropriate intervals for determination of portal vein glucagon. Unstimulated values (basal) were determined in rats treated with a similar procedure but infused with saline instead of alanine. Each point represents the mean of results obtained in three rats.

Table (2): Effects of Alanine Infusion on Portal Vein Plasma Glucagon Levels

Infusion Medium	Portal Vein Glucagon Concentration $\times 10^{-10}$ M
Saline	1.42 ± 0.11
Alanine (mmol/kg)	
0.20	3.2 ± 0.18
0.40	6.4 ± 0.19
0.60	9.6 ± 0.28
1.0	9.8 ± 0.34

Results are Means ± SEM of values obtained in four rats in each group.

EFFECT OF GLUCOSE INFUSION ON PORTAL VEIN PLASMA INSULIN CONCENTRATION IN CONTROL RATS

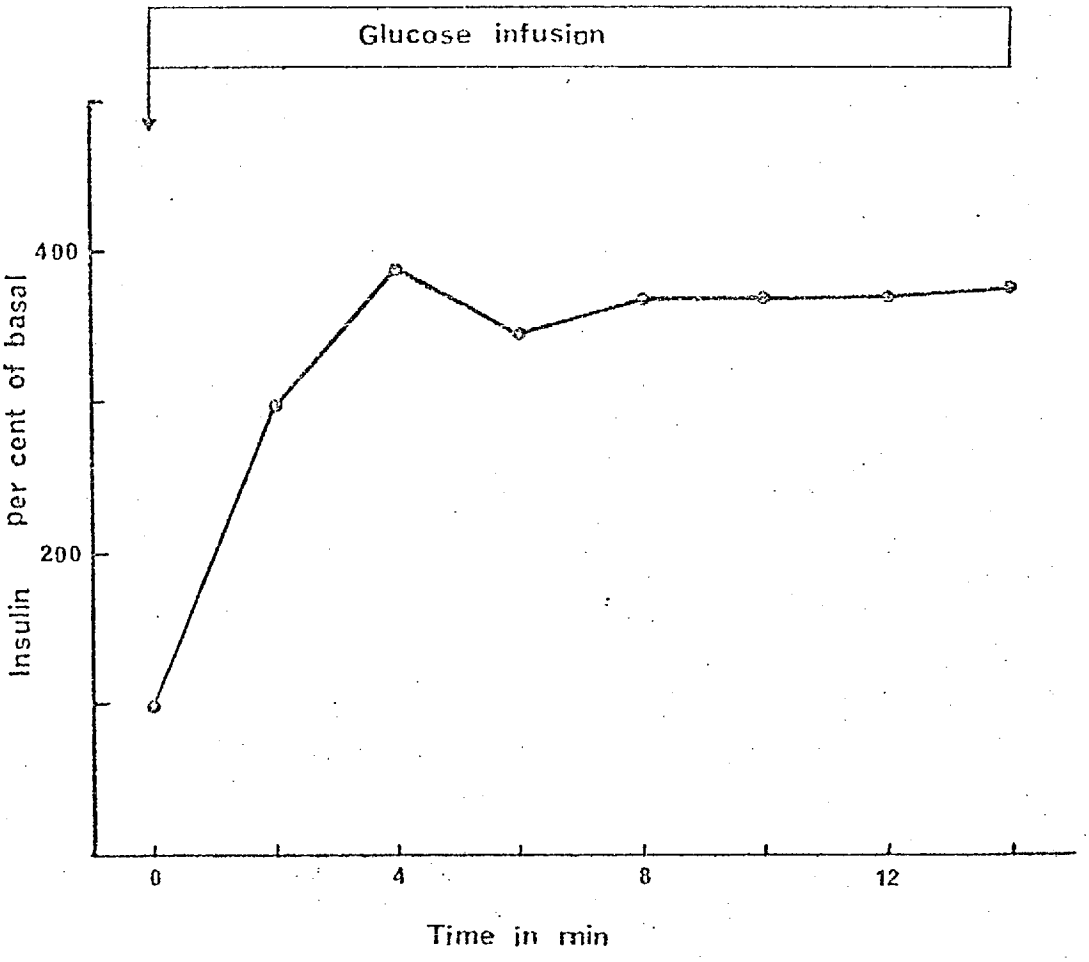


FIGURE (16)

0.2 ml of glucose solution was infused through a cannula inserted in the femoral vein of anesthetised rats. The total dose of glucose administered in 15 min. was 5.5 mmol/kg. One fourth of the dose was given as a pulse injection followed by a continuous infusion of the remaining solution. Animals were sacrificed at appropriate intervals for determination of portal vein insulin. Unstimulated values (basal) were determined in rats treated with a similar procedure but infused with saline. Each point represents the mean of results obtained in three rats.

Table 3: Effect of Oestradiol on the Pancreatic Islets Response to Glucose and Alanine Infusion

Experimental Conditions	Insulin Concentration x 10^{-10}M		Glucagon Concentration x 10^{-10}M	
	Saline	Glucose	Saline	Alanine
Pair-fed Control	3.2 ± 0.16	12.6 ± 0.82 (394%)	1.68 ± 0.14	9.6 ± 0.42 (571%)
Oestradiol (µg/kg/day)				
1	3.4 ± 0.12	12.9 ± 0.76 (379%)	1.72 ± 0.11	9.2 ± 0.38 (535%)
10	**2.3 ± 0.11	**7.2 ± 0.42 (313%)	**0.46 ± 0.06	**1.26 ± 0.08 (274%)
100	**2.0 ± 0.09	**6.8 ± 0.36 (340%)	**0.39 ± 0.04	**0.86 ± 0.04 (221%)

Values are Means ± SEM obtained in 6-8 rats in each group.

Values in parentheses represent percentage increment above basal values.

** Significantly lower than that of pair-fed control rats ($P < 0.005$).

Table (4): Effects of Glucose Infusion on Portal Vein Plasma Insulin Concentration

Infusion Medium	Portal Vein Insulin Concentration x 10^{-10} M
Saline	2.9 ± 0.12
Glucose (mmol/kg)	
1.0	3.8 ± 0.11
2.5	6.3 ± 0.24
4.0	18.4 ± 0.46
5.5	19.9 ± 0.68

Results are Means ± SEM of values obtained in four rats in each group.

EFFECT OF OESTRADIOL ON PORTAL VEIN GLUCAGON INCREMENT IN RESPONSE TO ALANINE

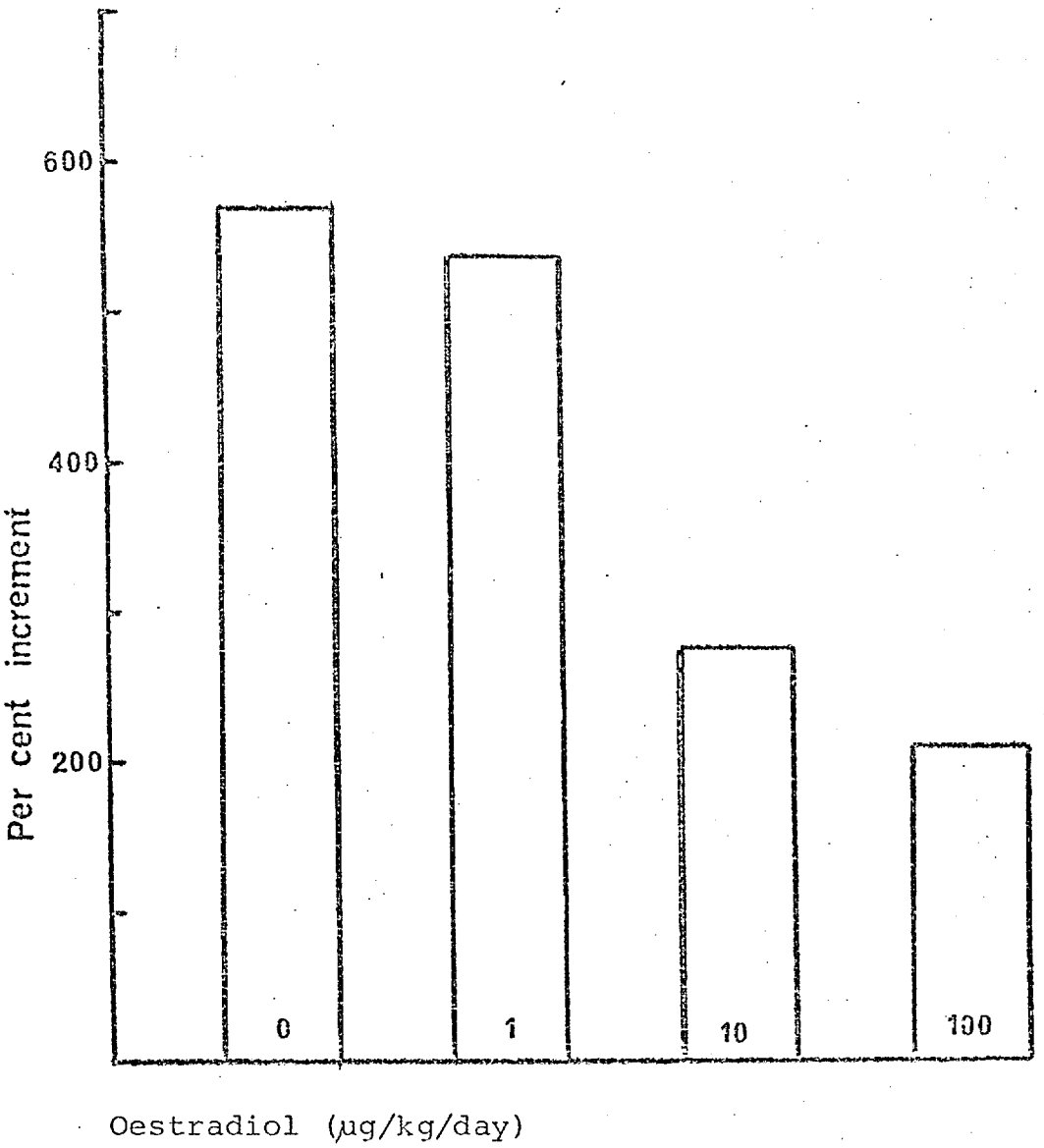


FIGURE (17a)

Animals receiving increasing doses of oestradiol were examined under basal conditions and following stimulation with alanine (see legend to figure 15).

Results were expressed as percent increment above basal.

EFFECT OF OESTRADIOL ON PORTAL VEIN INSULIN INCREMENT IN RESPONSE TO GLUCOSE

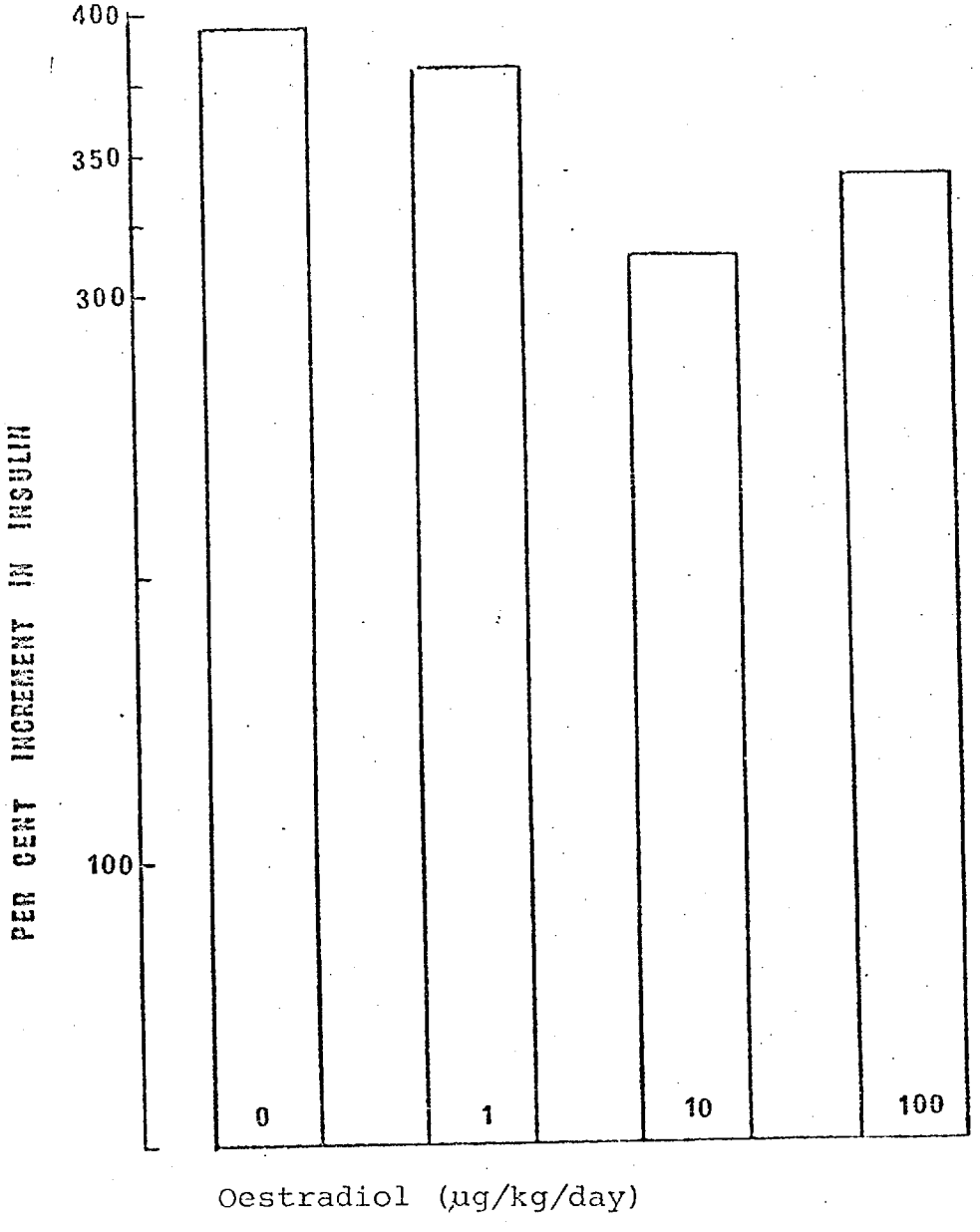


FIGURE (17b)

Animals receiving increasing doses of oestradiol were examined under basal conditions and following stimulation with glucose (see legend to Figure 16).

Results were expressed as per cent increment above basal.

Table 5: Effect of Oestradiol Administration on Hepatic Acetyl CoA Carboxylase, Fatty Acid Synthetase Activities and Fasting Plasma Triglyceride Concentration in Ovariectomised Rats

Experimental Conditions	Hepatic Enzyme Activity (munit/mg protein)		Triglycerides (mM/L)
	Acetyl CoA Carboxylase	Fatty Acid Synthetase	
Control	2.4 ± 0.4	4.1 ± 1.2	0.74 ± 0.08
Oestradiol (µg/kg/day)			
1	2.4 ± 0.4	4.4 ± 1.2	0.68 ± 0.1
10	*6.2 ± 0.6	*8.9 ± 2	*1.76 ± 0.18
100	*5.8 ± 0.5	*10.1 ± 2.5	*1.84 ± 0.2

Oestradiol was given as daily subcutaneous injections in corn oil for 8 weeks.

Values are Means ± SEM obtained in 6-8 rats in each group.

* Denotes significant increase as compared to control values ($P < 0.001$).

EFFECT OF OESTRADIOL ON FASTING PLASMA TRIGLYCERIDES, HEPATIC ACETYL CoA CARBOXYLASE AND FATTY ACID SYNTHETASE ACTIVITIES

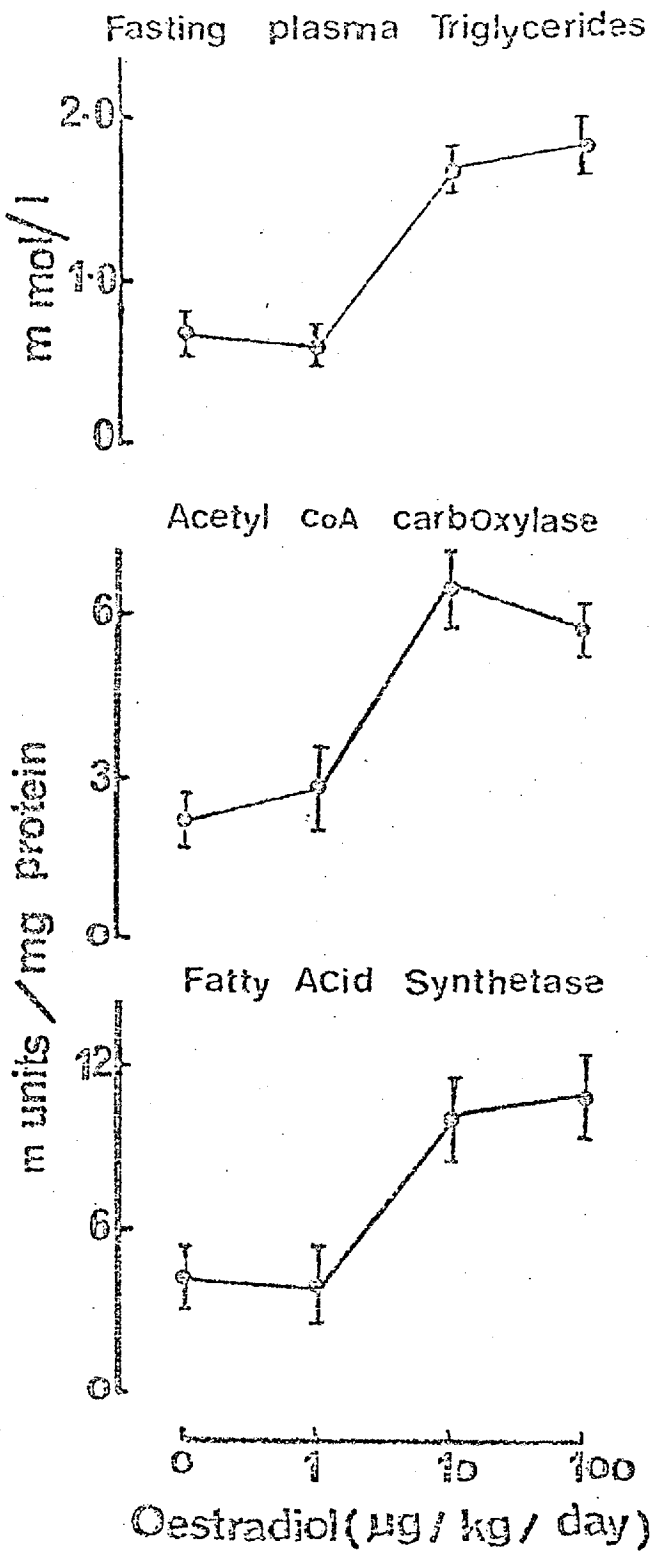
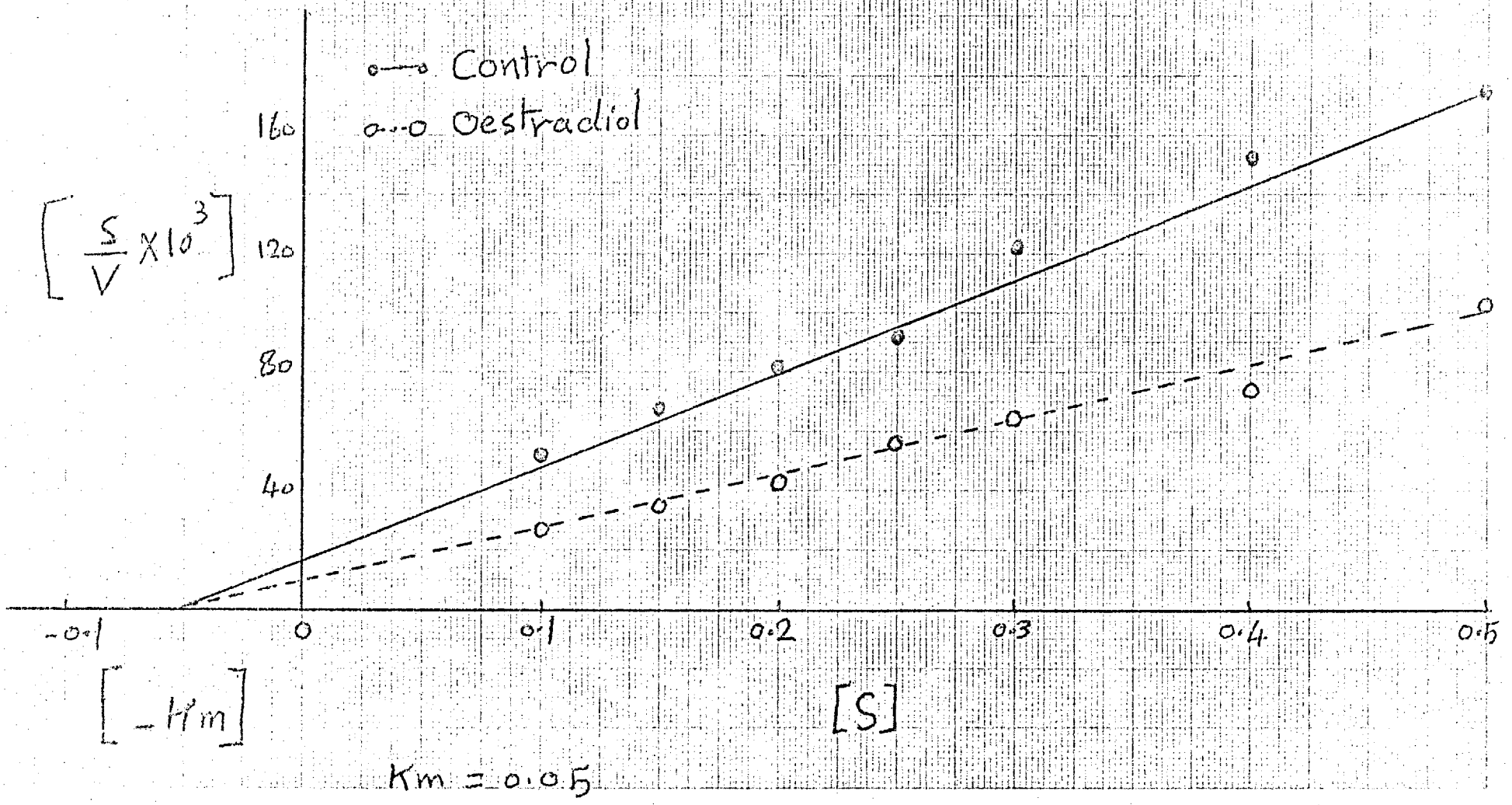


FIGURE (18)

Fasting plasma triglyceride concentration was measured in blood obtained from aorta of overnight fasted rats. Lipogenic enzymes were assayed under in vitro optimal conditions using liver homogenates of rats subjected to periods of fasting and refeeding to reveal maximal activity (see section on methods). Results are Means ± SEM obtained in 6-8 rats. Oestradiol was given daily for 8 weeks.

Linear plot, Reaction Kinetics of Acetyl CoA Carboxylase

(S = acetyl CoA concentration mmol/L, V = acetyl CoA carboxylase activity mU/mg)



EFFECT OF ACETYL CoA CONCENTRATION ON ACETYL CoA CARBOXYLASE IN LIVER HOMOGENATES OF CONTROL AND OESTRADIOL TREATED RATS

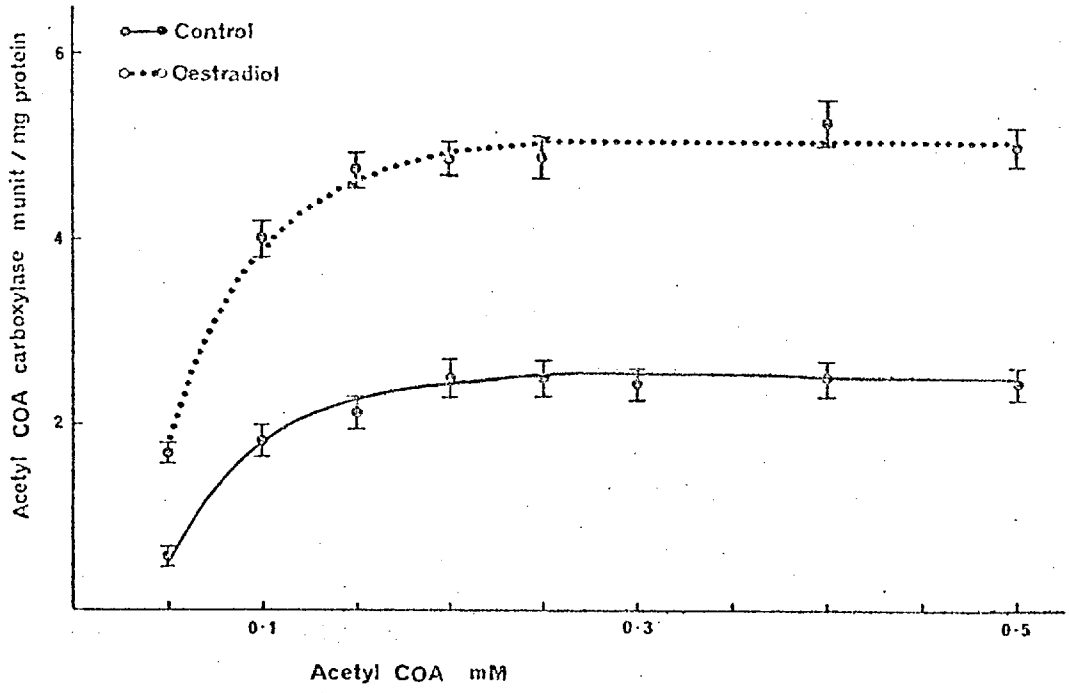


FIGURE (19)

Acetyl CoA carboxylase activity was assayed in the presence of increasing concentrations of acetyl CoA. The activity was measured in liver homogenates from pair-fed control rats and from animals treated with 100 µg of oestradiol/kg/day. Each point represents the Mean ± SEM of duplicate estimate in 4 rats.

Table 6: Effect of Oestradiol Therapy on Liver PEPCK and Gluconeogenic Intermediates

Experimental Conditions	PEPCK Activity mU/mg protein	Gluconeogenic Intermediates (μmoles/kg wet weight liver)							
		Pyr	Oxal	PEP	2-PG	DAP	FDP	G-6-P	Glu
Pair-fed Control	49.1 ± 2.9	43	6.25	60	36	25.8	24.3	256.5	8200
Oestradiol (ug/kg/day)									
1	48.6 ± 2.3	42 ± 2	6.4 ± 0.8	58 ± 1.2	32 ± 1.2	27 ± 4.2	26 ± 2.4	248 ± 2.4	7800 ± 460
10	**33.5 ± 1.6	*62 ± 2	*12.1 ± 0.6	*42 ± 1.6	39 ± 4	28.2 ± 4.6	*39 ± 2.3	259 ± 2.8	8100 ± 680
100	**22.5 ± 2.3	*77 ± 3	*16.2 ± 0.8	*34 ± 3	49 ± 6	29.5 ± 1.8	42.5 ± 0.6	365 ± 2.3	7380 ± 1176

? = increase ?

Oestradiol was given as daily subcutaneous injections in corn oil for 8 weeks. The control animals received daily injections of corn oil.

Values are Means ± SEM of estimations in 6-8 rats.

* denotes significant increase ($P < 0.01$) when compared to control.
** indicates significant decrease ($P < 0.005$).

Abbreviations: Pyr, pyruvate; oxal, oxaloacetate; PEP, phosphoenol pyruvate; 2-PG, 2-phosphoglycerate; DAP, dihydroxyacetone phosphate; FDP, fructose, 1-6, diphosphate; G-6-P, glucose-6-phosphate; Glu, glucose.

EFFECT OF OESTRADIOL ON FASTING PLASMA GLUCOSE, LIVER PHOSPHOENOLPYRUVATE CARBOXYKINASE (PEPCK) AND PORTAL VEIN I/G RATIO

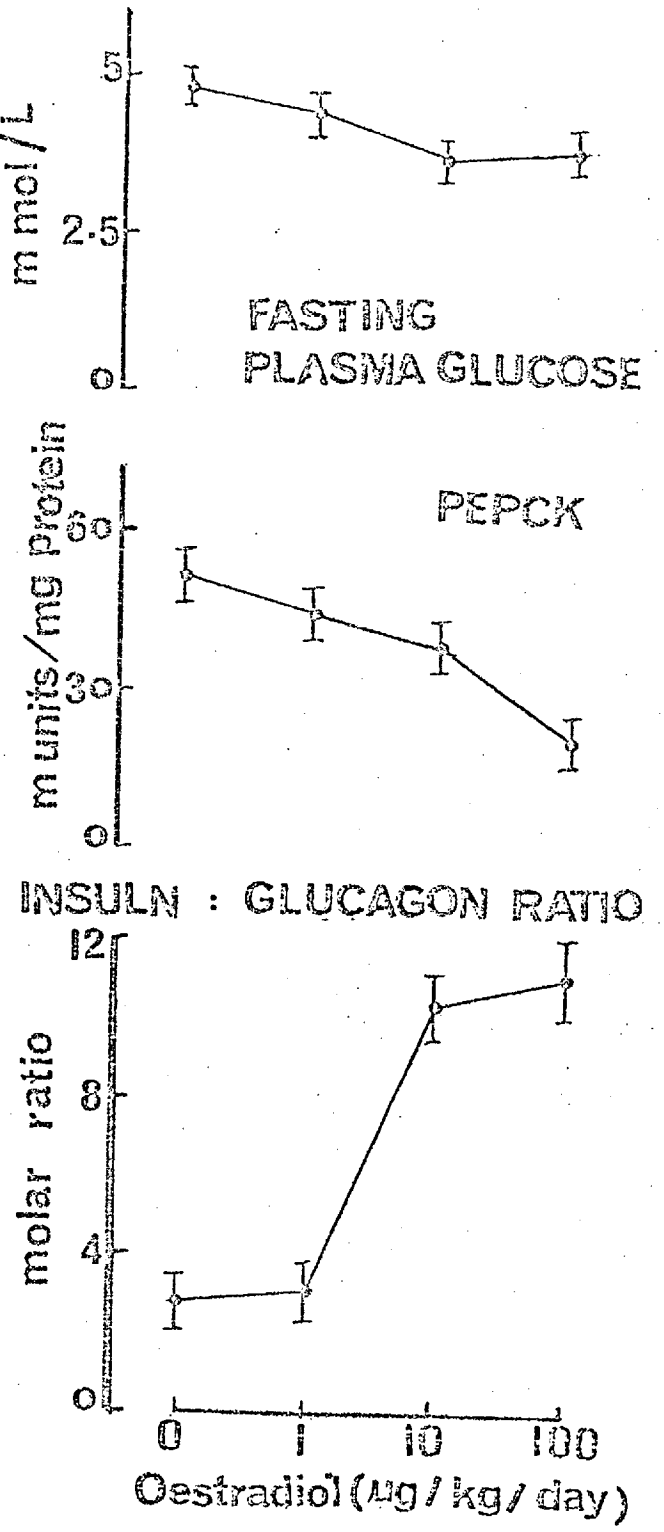


FIGURE (20)

Plasma glucose was measured in blood obtained from abdominal aorta, while insulin and glucagon were measured in samples from portal vein. Rats were fasted overnight prior to blood withdrawal. PEPCK was assayed under in vitro optimal conditions in liver homogenates of 36 h. fasted rats. Values are Means ± SEM of results obtained in 6-8 rats.

EFFECT OF PHOSPHOENOL PYRUVATE CONCENTRATION ON LIVER PHOSPHOENOLPYRUVATE CARBOXYKINASE (PEPCK) OF CONTROL AND OESTRADIOL TREATED RATS

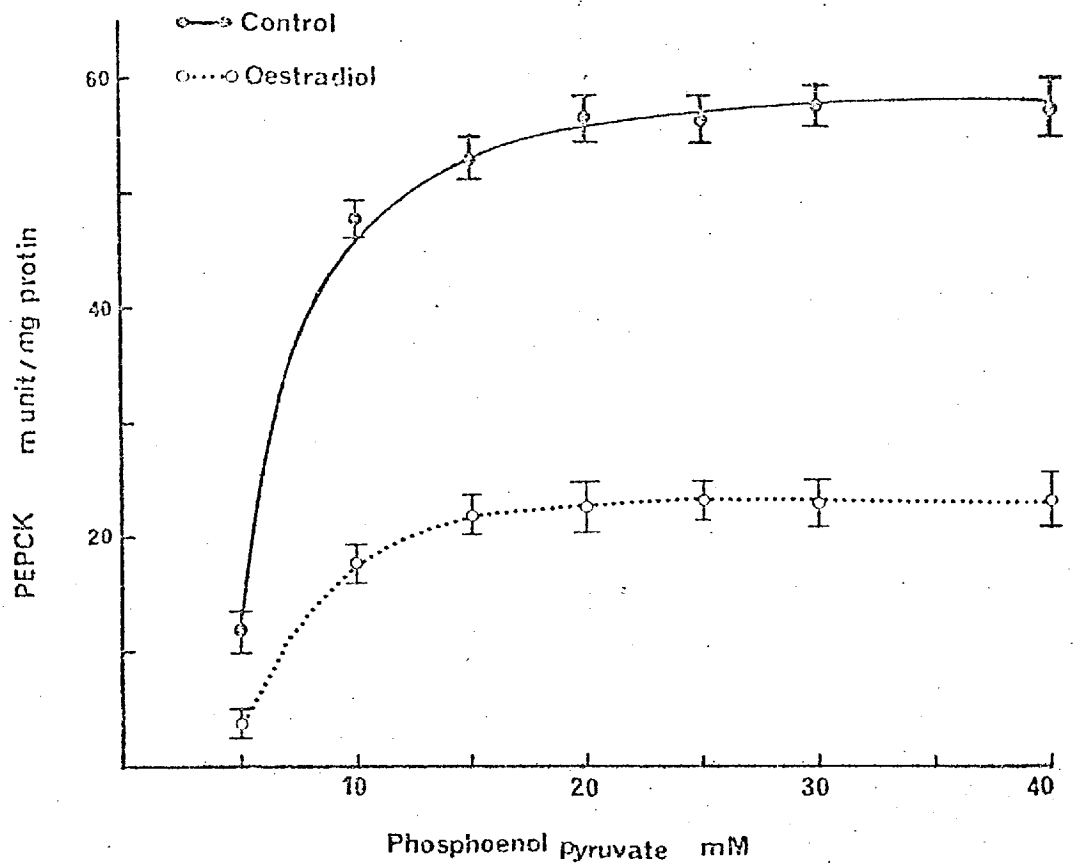


FIGURE (21)

PEPCK activity was assayed with increasing concentrations of phsophoenol pyruvate. Each point represents the mean of duplicate estimates obtained in 3-4 rats. Oestradiol was given in a dose of 100 µg/kg/day for 8 weeks.

The small increase in glucose-6-phosphate may be due to the presence of a side pool in which glucose-6-phosphate is formed from glycerol via the activity of the enzyme glycerokinase (Fugine 3).

It is unlikely, however, that this pathway will be a major source of glyconeogenesis. The ∝ -glycerophosphate formed will be predominantly utilised in the estrification of fatty acids resulting from the concordant stimulation of lipogenesis during oestrogen treatment.

EFFECT OF OESTRADIOL ON LIVER GLUCONEOGENIC INTERMEDIATES AND FASTING PLASMA GLUCOSE

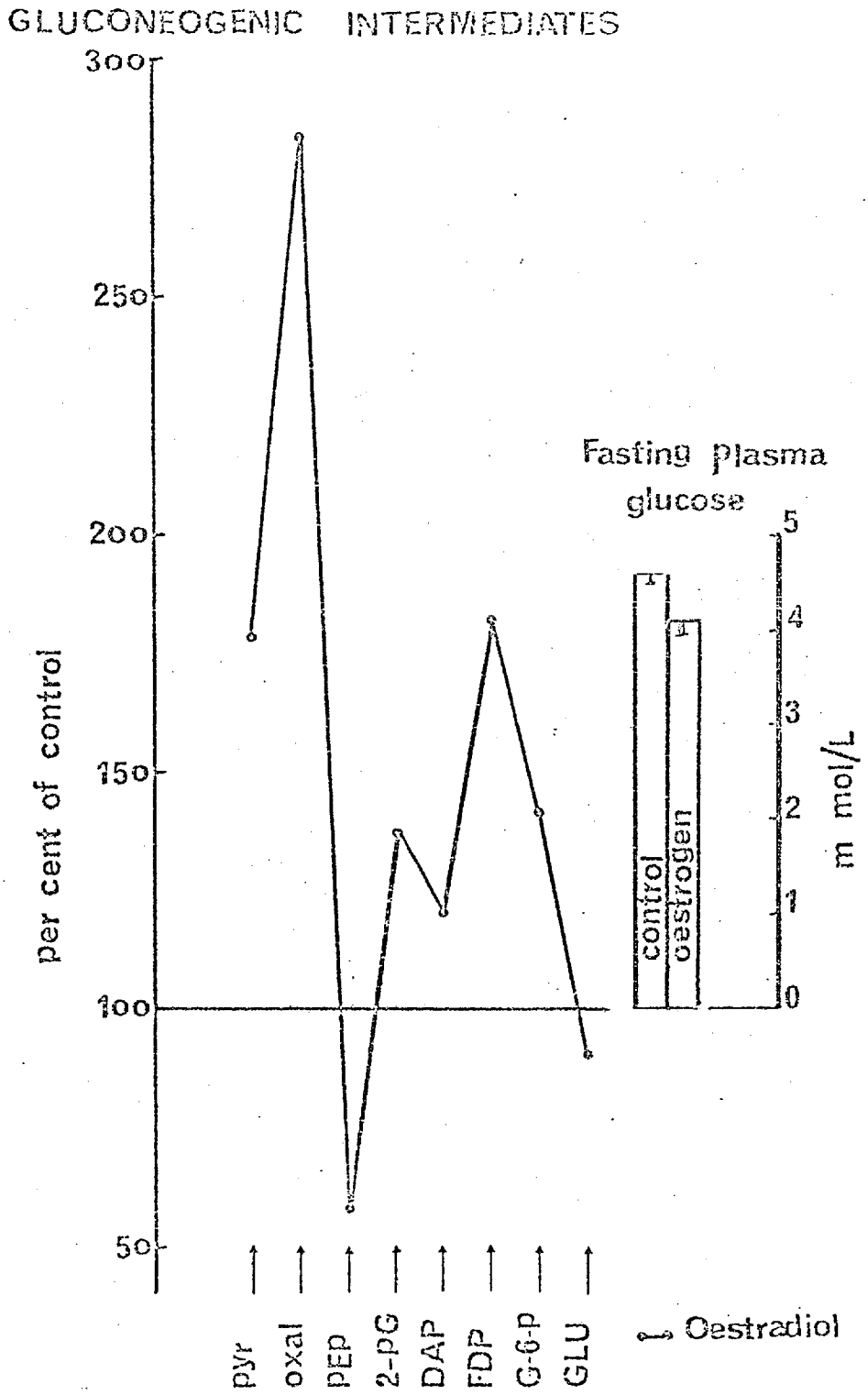


FIGURE (22)

Animals were treated with oestradiol (100 µg/kg/day) for 8 weeks. The liver was extracted and assayed for measurement of intermediates. Results are expressed as percentage of similar measurements obtained in pair-fed control animals. Each point represents the mean of values obtained in 6 rats. For abbreviation (see legend to Table 6).

EFFECT OF THE INTRAPORTAL ADMINISTRATION OF GLUCAGON UPON HEPATIC c-AMP CONTENT

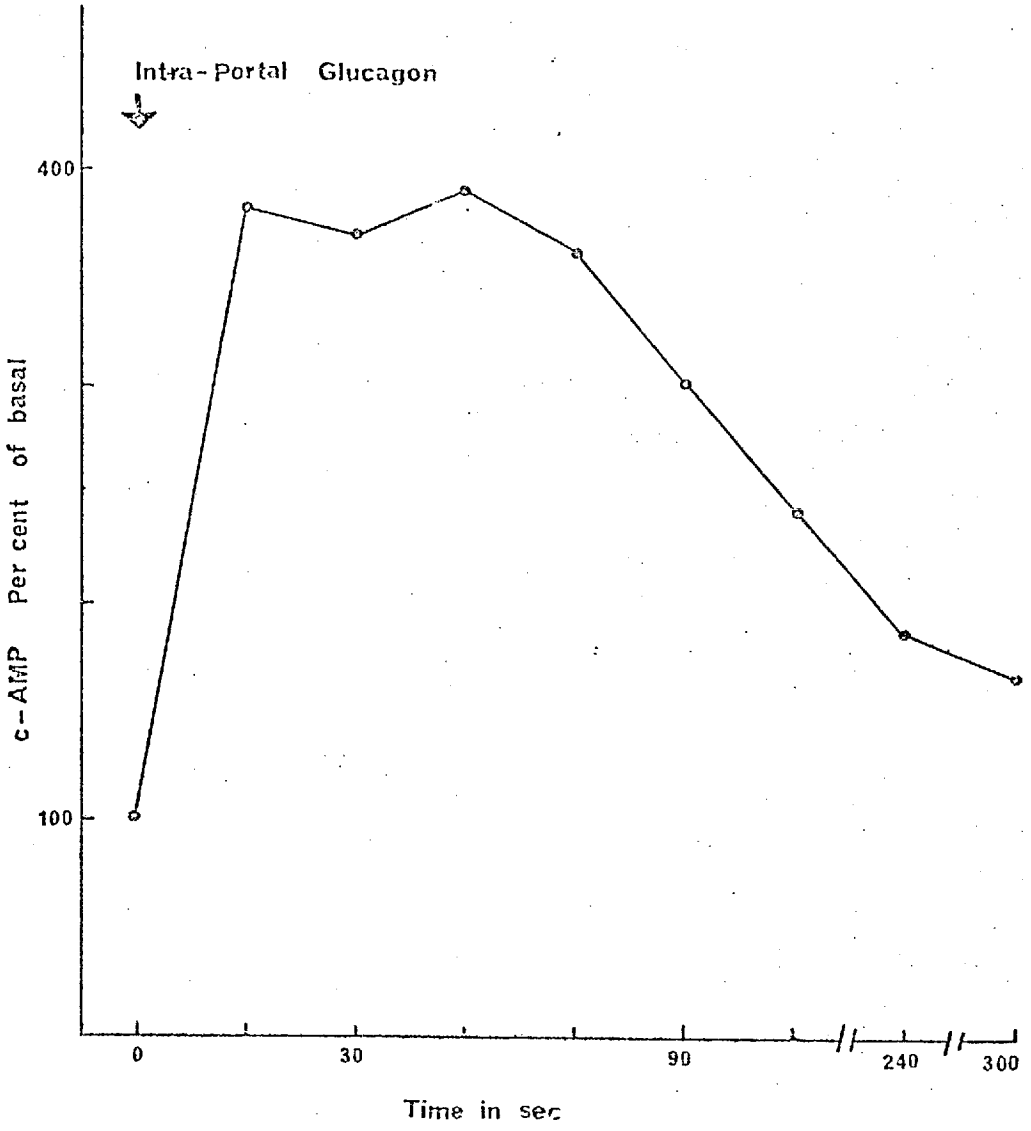


FIGURE (23)

Anaesthetised animals were injected through the main portal vein with glucagon (1 µg/kg/). Animals were sacrificed at appropriate intervals and liver extracted for c-AMP measurement. Basal values were determined in rats injected with a similar volume of saline instead. Each point represents the mean of duplicate estimates in samples obtained from 4 rats.

Table (7): Effects of Oestradiol on Basal and Glucagon Stimulated Hepatic c-AMP Content

Experimental Conditions	c-AMP Content (pmol/mg wet weight liver)	
	Basal	Glucagon Stimulated
Pair-fed Controls	0.44 ± 0.04	1.82 ± 0.08
Oestradiol (µg/kg/day)		
10	**0.28 ± 0.02	**0.85 ± 0.06
100	**0.25 ± 0.03	**0.9 ± 0.12

Basal c-AMP Content was determined in liver extracts of overnight fasted rats.

Stimulated levels of c-AMP were estimated in tissue samples obtained 30 sec. following the intraportal injection of 1 µg of glucagon/kg body weight.

Values are Means ± SEM.

The probability value for significance from the control was determined using the student "t" test.

** = significantly decreased from control ($P < 0.01$).

EFFECT OF OESTRADIOL ON BASAL AND GLUCAGON STIMULATED HEPATIC c-AMP CONTENT

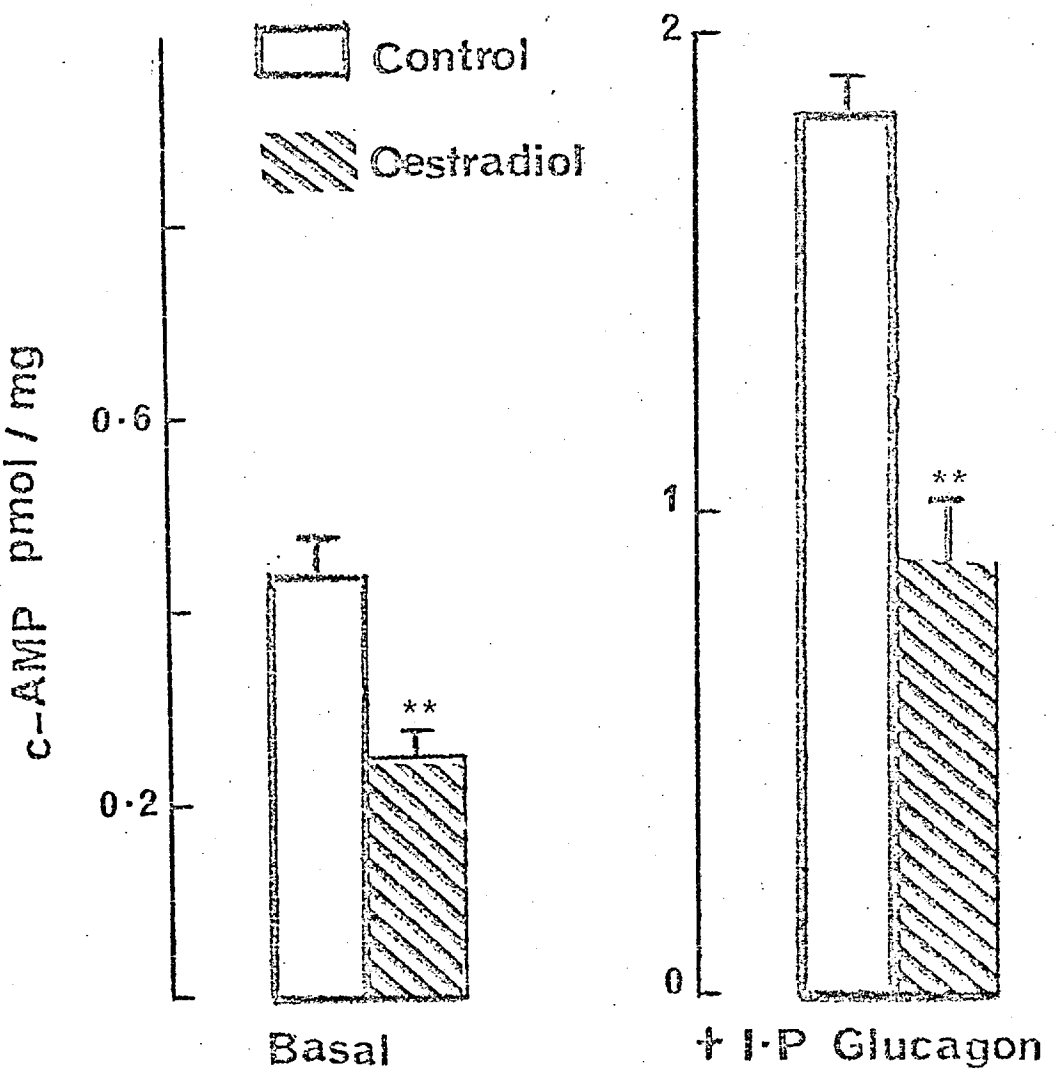


FIGURE (24)

Liver c-AMP concentration was measured under basal conditions and following glucagon stimulation as described in Figure (23). Bars represent Means and SEM of results obtained in 6-8 rats.
** = Significant decrease compared to pair-fed controls ($P < 0.01$)

II. Progesterone

A. Effect of Progesterone on Immunoreactive Insulin and Glucagon Concentrations in Portal Vein Blood

(1) Basal Levels: Table (8) shows the effect of progesterone administration on portal vein insulin levels. The mean portal vein insulin concentration in control rats was $3.9 \pm 0.32 \times 10^{-10}$M ($56 \pm 6$ μU/ml). The daily s.c. injection of small doses of progesterone (0.05 mg/kg) did not produce any significant changes in insulin concentration. On the other hand, the administration of progesterone in a dose of 0.5 of 5 mg/kg/day to ovariectomised rats produced a significant increase in basal plasma insulin concentration (Mean ± SEM = 4.98 ± 0.6 and $4.8 \pm 0.3 \times 10^{-10}$M respectively).

The mean basal plasma glucagon of control rats in this group was $1.82 \pm 0.19 \times 10^{-10}$M. (650 ± 100 pg/ml) and small doses of progesterone (0.05 mg/kg/day) did not significantly alter its concentration. As with insulin, however, large doses of progesterone (0.5 or 5 mg/kg/day) produced a significant increase in the portal vein glucagon level (Mean ± SEM = 2.65 ± 0.19 and $2.4 \pm 0.16 \times 10^{-10}$M respectively).

Table (8) and Figure (25 a,b,c) indicate that the levels of insulin and glucagon in portal vein blood were equally increased in rats by the administration of progesterone so that the ratio between the two hormones remained similar to that of controls.

These results suggest that these doses of progesterone stimulate the pancreatic beta and alpha cells to the same

degree and hence produce proportionate increases in the concentration of both insulin and glucagon.

(2) Response to Alanine: Table (9) shows that treatment of rats with progesterone enhanced the alpha cell response to alanine. The mean glucagon level in portal vein blood of control rats was increased from a basal of $1.76 \pm 0.08 \times 10^{-10}$M to reach a peak value of $8.4 \pm 0.36 \times 10^{-10}$M 15 min. after the infusion of alanine. In rats treated with progesterone 5 mg/kg/day, on the other hand, the basal glucagon concentration averaged $3.1 \pm 0.18 \times 10^{-10}$M and was increased to a mean of $16.4 \pm 0.82 \times 10^{-10}$M which was significantly higher than that of controls. Also a significant increase in glucagon response was observed in rats treated with 0.5 mg of progesterone.

(3) Response to Glucose: Administration of progesterone to rats also enhanced the insulin response to glucose. In control rats the basal insulin concentration in portal vein blood averaged $4.2 \pm 0.12 \times 10^{-10}$M and was raised to $14.1 \pm 1.6 \times 10^{-10}$M after the infusion of glucose. In rats receiving daily s.c. injections of progesterone 5 mg/kg/day the basal insulin averaged $6.2 \pm 0.82 \times 10^{-10}$M and was increased to $29 \pm 3.2 \times 10^{-10}$M during the period of glucose infusion. Similarly a significant increase in the insulin response to glucose was observed in rats treated with progesterone 0.5 mg/kg/day. Smaller doses of progesterone on the other hand had no significant effects (Table (9)).

B. Effect of Progesterone on Liver Lipogenic Enzymes

The data depicted in Table (10) and in Figure (26) indicate that the acetyl CoA carboxylase and fatty acid synthetase activities in liver homogenates obtained from control rats averaged 1.65 ± 0.24 and 4.2 ± 0.6 munits/mg protein respectively. Daily s.c. injections of progesterone 0.05, 0.5 or 5 mg/kg to rats did not significantly affect the activities of these enzymes. The lack of effect on the activities of lipogenic enzymes was demonstrable at a wide range of substrate concentrations (Figure (27)) and was not associated with significant changes in plasma triglyceride concentrations. (Fig.26).

C. Effect of Progesterone on Liver Gluconeogenic Enzymes and Intermediates

Liver homogenates obtained from fasted control rats showed that the mean phosphoenolpyruvate carboxykinase activity was 42 ± 1.6 mU/mg protein (Tab.11and Fig.28). The daily s.c. injection of progesterone 0.05, 0.5 or 5 mg/kg/day did not significantly influence the activity of this enzyme in liver homogenate. This pattern was demonstrable at all concentrations of phosphoenol pyruvate as shown in Figure (29).

Progesterone also did not alter the relative concentrations of gluconeogenic intermediates of rat liver as compared to that of control rats (Tab.11). The crossover pattern shown in Figure (30) indicates that the

administration of this steroid had no significant influence on the gluconeogenic pathway nor did it produce a significant change in fasting plasma glucose concentration (Fig.28& Fig.30).

D. Effect of Progesterone on Liver c-AMP Concentration and the Hepatic Response to Exogenous Glucagon

Figure (31) shows that the mean basal c-AMP content of liver extracts obtained from control rats was 0.49 $\pm$ 0.025 pmol/mg wet wt. The administration of progesterone in a dose of 5 mg/kg/day did not significantly alter the basal hepatic c-AMP content. Similarly, the increment in hepatic c-AMP in response to the intraportal administration of glucagon was not different in progesterone treated rats as compared to control animals (Table 12 and Figure 31).

Table 8: Effects of Progesterone Administration on the Concentration of Insulin and Glucagon in Portal Vein Blood of Ovariectomised Rats

Treatment Groups	Plasma Concentration x 10^{-10} M		Molar Ratio Insulin/Glucagon
	Insulin	Glucagon	
Control (n = 12)	3.9 ± 0.32	1.82 ± 0.19	2.08 ± 0.19
Progesterone (µg/kg/day)			
0.05 (n = 8)	3.8 ± 0.26	1.85 ± 0.2	2.05 ± 0.2
P	(NS)	(NS)	(NS)
0.5 (n = 7)	4.98 ± 0.6	2.65 ± 0.19	2.01 ± 0.3
P	<0.01	<0.005	(NS)
5 (n = 11)	4.80 ± 0.3	2.4 ± 0.16	2.0 ± 0.26
P	<0.02	<0.005	(NS)

(n) = number of animals in each group.

Values are Means ± SEM.

P = probability values for significance of differences from means of controls using student "t" test.

Non-significant differences (NS) means $P < 0.05$.

The duration of steroid therapy was 6-8 weeks of daily subcutaneous injections.

The doses of steroids shown represent the injected dose/kg body weight/day solubilised in 0.2 ml of corn oil. The control group received daily subcutaneous injections of the solvent corn oil.

EFFECT OF PROGESTERONE ON

PORTAL VEIN PLASMA INSULIN LEVEL

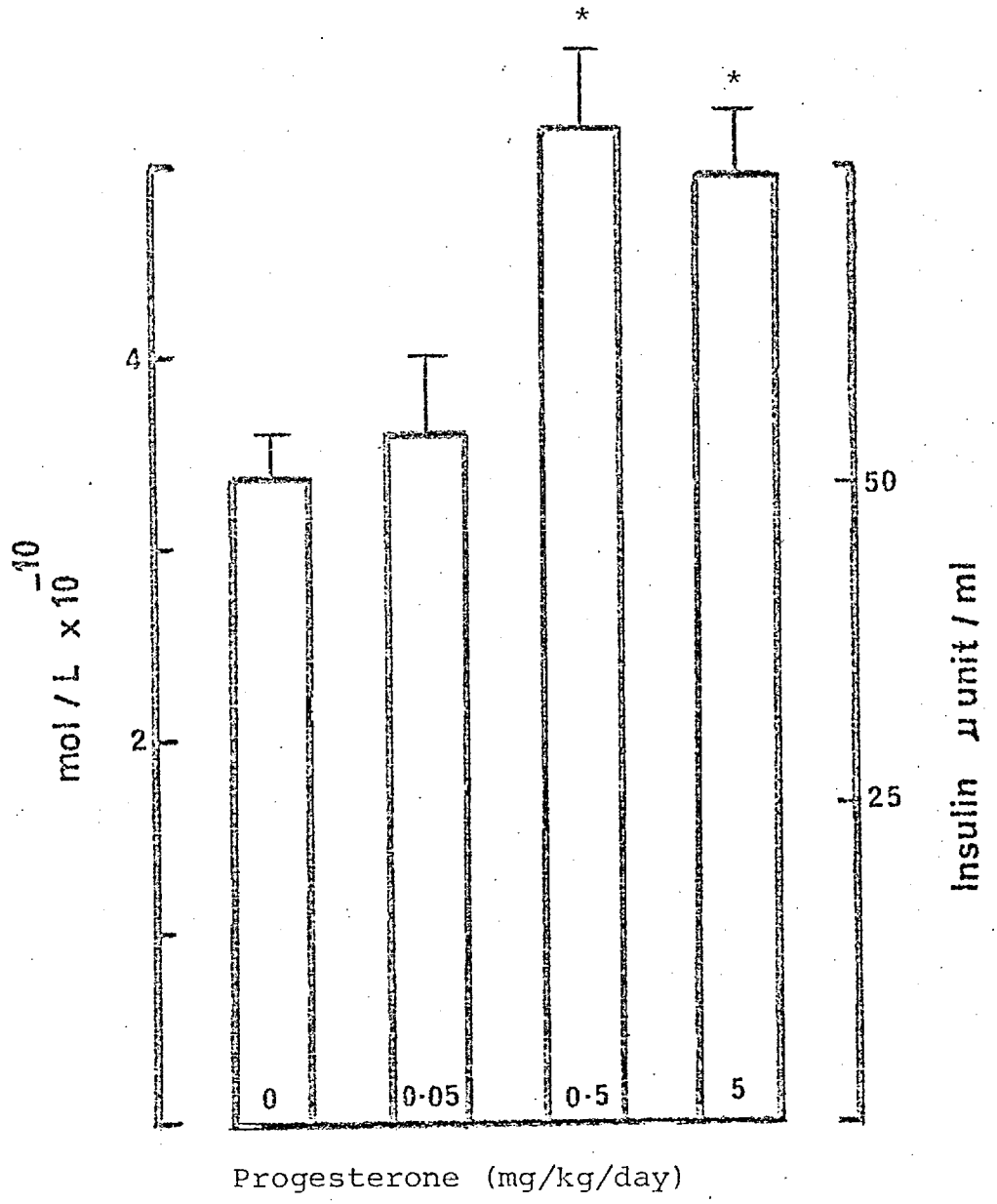


FIGURE (25a)

Progesterone was given daily by s.c. injection for 8 weeks. Number of rats in each group is shown in Table 8. Bars are Means and SEM.

* = Significant increase ($P < 0.02$).

EFFECT OF PROGESTERONE ON

PORTAL VEIN PLASMA GLUCAGON LEVEL

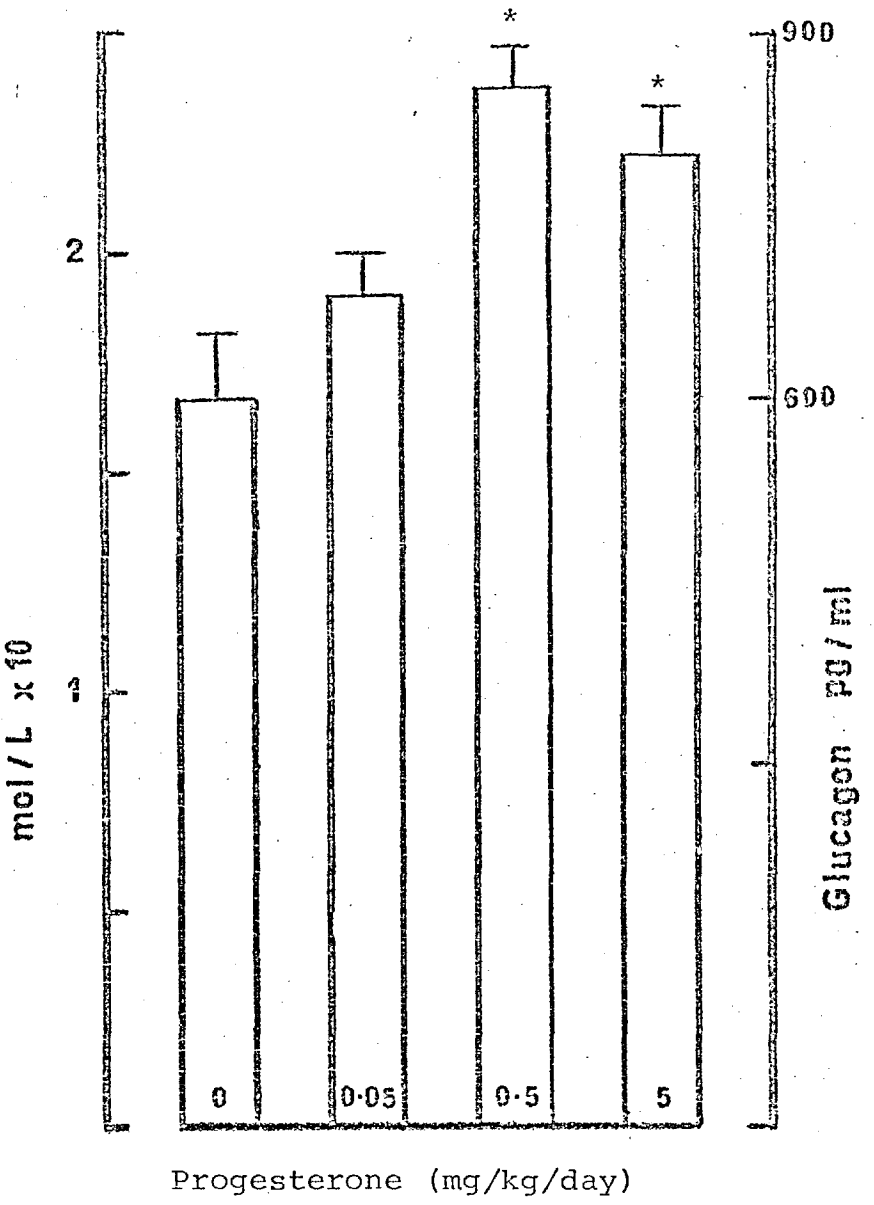


FIGURE (25b)

Progesterone was given daily by s.c. injection for 8 weeks. Number of rats in each group is shown in Table 8. Bars are Means and SEM.

* = Significant increase ($P < 0.005$).

EFFECT OF PROGESTERONE ON PORTAL VEIN PLASMA INSULIN : GLUCAGON MOLAR CONCENTRATION RATIO (I/G)

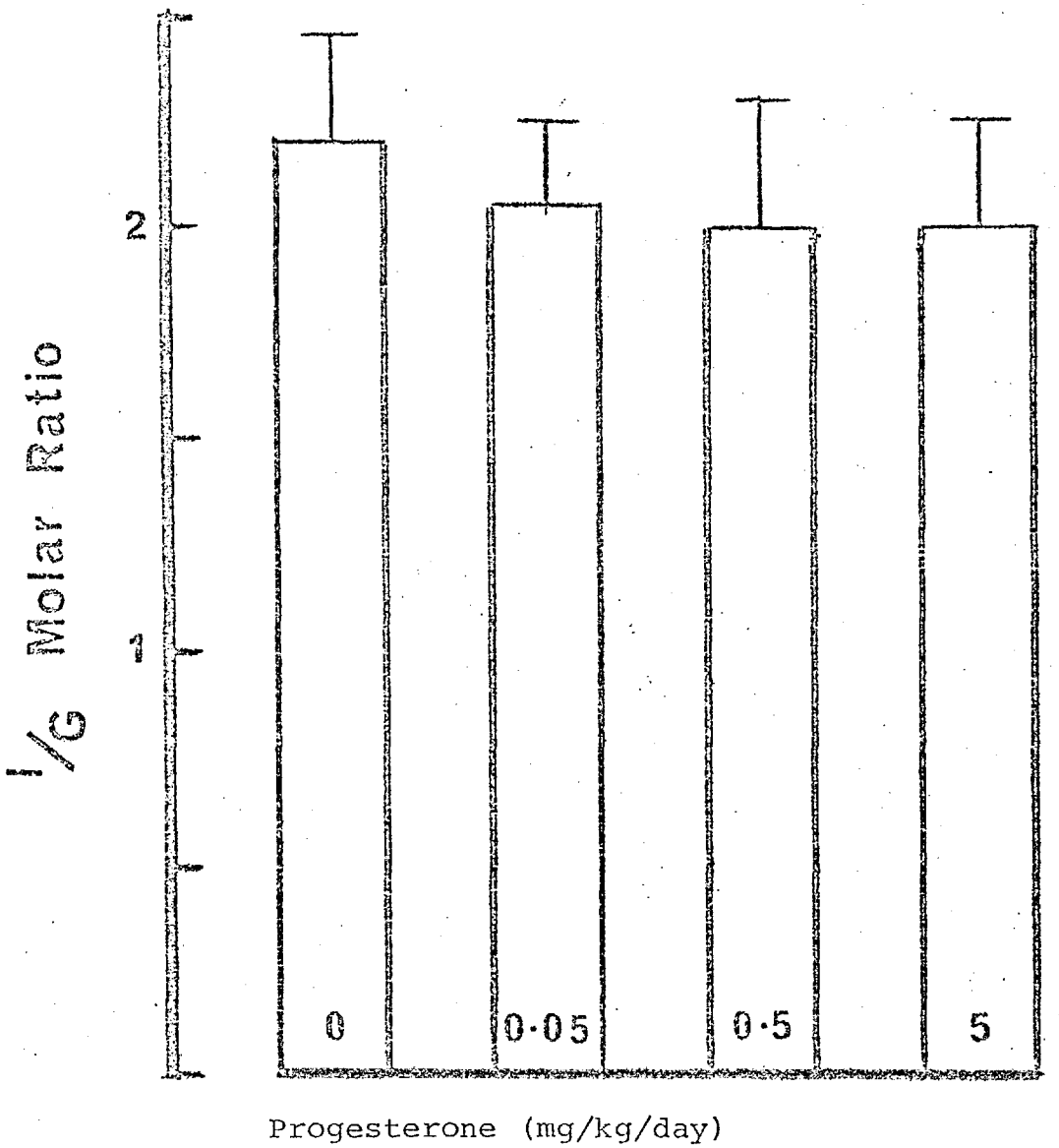


FIGURE (25c)

Bars represent the ration of insulin : glucagon in portal vein, each expressed in its molar concentration.

For further details see Table 8.

Table 9: Effect of Progesterone on the Pancreatic Islet Response to Glucose and Alanine Infusion

Treatment Groups	Insulin Concentration x 10^{-10}M		Glucagon Concentration x 10^{-10}M	
	Saline	Glucose	Saline	Alanine
Controls	4.2 ± 0.12	14.1 ± 1.6 (336%)	1.76 ± 0.08	8.4 ± 0.36 (477%)
Progesterone (µg/kg/day)				
0.05	3.9 ± 0.11	13.8 ± 1.8 (354%)	1.82 ± 0.12	9.41 ± 0.8 (517%)
0.5	5.6 ± 0.68	*26 ± 2.6 (464%)	2.86 ± 0.22	*14.2 ± 0.76 (497%)
5	6.2 ± 0.82	*29 ± 3.2 (468%)	3.10 ± 0.18	*16.4 ± 0.82 (529%)

Values are Means ± SEM obtained in 6-8 rats in each group.

Values in parentheses represent increment above basal values.

* indicates significant increase ($P < 0.01$).

Table 10: Effect of Progesterone Therapy on Hepatic Acetyl CoA Carboxylase, Fatty Acid Synthetase Activities and Plasma Triglyceride Levels in Female Ovariectomised Rats

Experimental Conditions	Hepatic Enzyme Activity (munit/mg protein)		Triglycerides (mmol/L)
	Acetyl CoA Carboxylase	Fatty Acid Synthetase	
Control	1.65 ± 0.24	4.2 ± 0.6	0.78 ± 0.1
Progesterone (ug/kg/day)			
0.05	1.82 ± 0.30	4.1 ± 0.8	0.92 ± 0.15
P	(NS)	(NS)	(NS)
0.5	2.4 ± 0.38	4.6 ± 0.6	0.76 ± 0.09
P	(NS)	(NS)	(NS)
5	2.2 ± 0.36	4.2 ± 0.7	0.74 ± 0.08
P	(NS)	(NS)	(NS)

Progesterone was given as daily subcutaneous injections in corn oil for 6-8 weeks.

Values are Means ± SEM obtained in 6-8 rats in each group.

NS = non-significant difference from control ($P > 0.05$).

EFFECT OF PROGESTERONE ON FASTING PLASMA TRIGLYCERIDES, HEPATIC ACETYL CoA CARBOXYLASE AND FATTY ACID SYNTHETASE ACTIVITIES

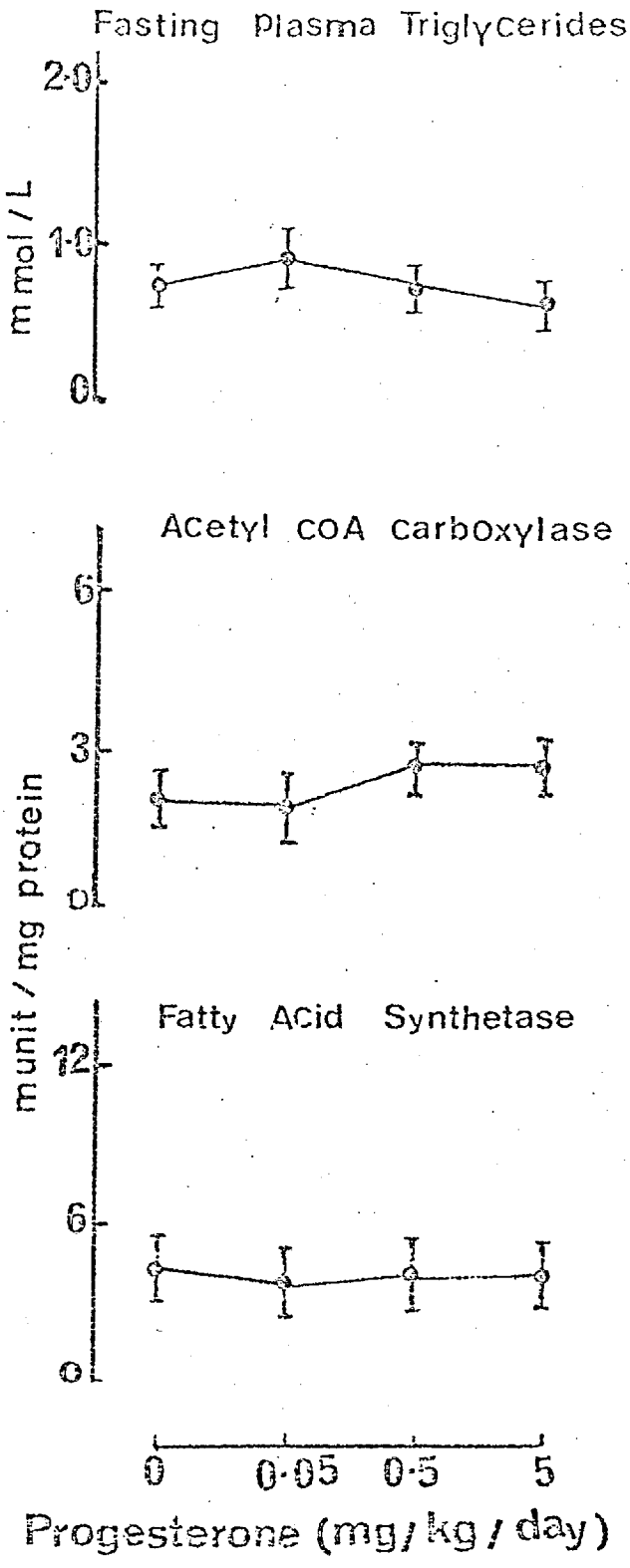


FIGURE (26)

Blood obtained from the abdominal aorta of overnight fasted rats was assayed for triglycerides. Lipogenic enzymes were measured under optimal conditions in liver homogenates. Results are Means ± SEM obtained in 6-8 rats. Progesterone was given daily for 8 weeks.

EFFECT OF ACETYL CoA CONCENTRATION ON ACETYL CoA CARBOXYLASE ACTIVITY OF LIVER HOMOGENATES

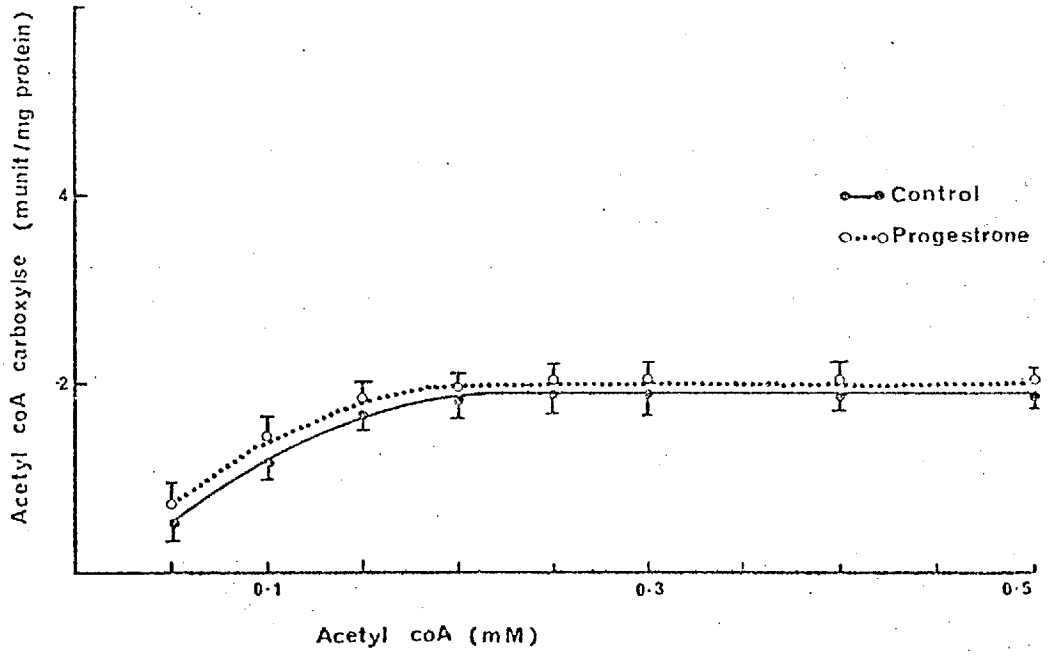


FIGURE (27)

Rat liver acetyl CoA carboxylase was assayed with increasing concentrations of acetyl CoA. Progesterone treated rats were given s.c. injections 5 mg/kg/day for 8 weeks. Each point represents Mean and SEM of duplicates estimates on samples obtained from four rats.

Table 11: Effect of Progesterone Therapy on Liver PEPCK and Gluconeogenic Intermediates

Experimental Conditions	PEPCK Activity mU/mg protein	Gluconeogenic Intermediates (umoles/kg wet weight liver)							
		Pyr	Oxal	PEP	2-PG	DAP	FDP	G-6-P	Glu
Control	42 ± 1.6	46 ± 3	6.8 ± 0.6	56 ± 2	39 ± 1.6	28 ± 6	28.6 ± 1.8	248 ± 2.6	7900 ± 360
Progesterone Treated (ug/kg/day)									
0.05	38 ± 2.6	42 ± 4	6.7 ± 0.4	62 ± 1.8	26 ± 2	27.2 ± 1.6	24 ± 2.1	236 ± 1.8	8200 ± 250
P	(NS)	(NS)	(NS)	(NS)	(NS)	(NS)	(NS)	(NS)	(NS)
0.5	41 1.8	39 ± 8	6.2 ± 0.3	58 ± 1.6	29 ± 3	24 ± 2	29 ± 1.6	242 ± 4.2	7800 ± 480
P	(NS)	(NS)	(NS)	(NS)	(NS)	(NS)	(NS)	(NS)	(NS)
5.0	46 1.9	45 ± 4	6.5 ± 1.5	54 ± 5	38 ± 5	24.5 ± 3	24.5 ± 2	276 ± 2.5	8250 ± 1000
P	(NS)	(NS)	(NS)	(NS)	(NS)	(NS)	(NS)	(NS)	(NS)

Progesterone was given as daily subcutaneous injections in corn oil for 6-8 weeks. The control animals received daily injections of corn oil. Values are Means ± SEM of results in 6-7 rats.

Non-significant differences (NS) means $P > 0.05$.

Abbreviations: Pyr, pyruvate; oxal, oxaloacetate; PEP, phosphoenol pyruvate; 2-PG, 2-phosphoglycerate; DAP, dihydroxyacetone phosphate; FDP, fructose, 1-6, diphosphate; G-6-P, glucose-6-phosphate; Glu, glucose.

EFFECT OF PROGESTERONE, ON FASTING PLASMA GLUCOSE, LIVER PHOSPHOENOLPYRUVATE CARBOXYKINASE (PEPCK) AND PORTAL VEIN I/G RATIO

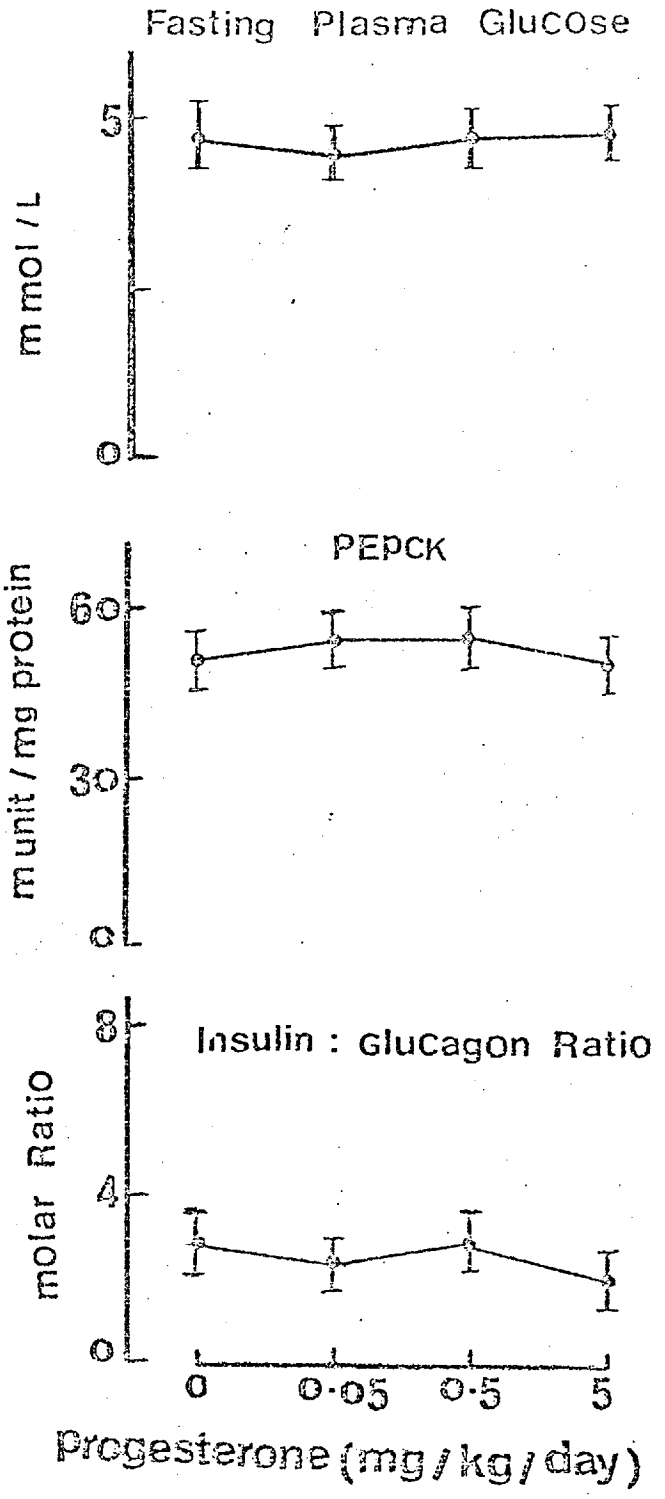


FIGURE (28)

Fasting plasma glucose was measured in blood obtained from aorta, while insulin and glucagon were measured in samples from the portal vein. Rats were fasted overnight prior to blood withdrawal. PEPCK was assayed under *in vitro* optimal conditions in liver homogenates of 36 h. fasted rats. Values are Means ± SEM of results obtained in 6-8 rats.

EFFECT OF PHOSPHOENOL PYRUVATE CONCENTRATION ON PHOSPHOENOLPYRUVATE CARBOXYKINASE (PEPCK) ACTIVITY OF LIVER

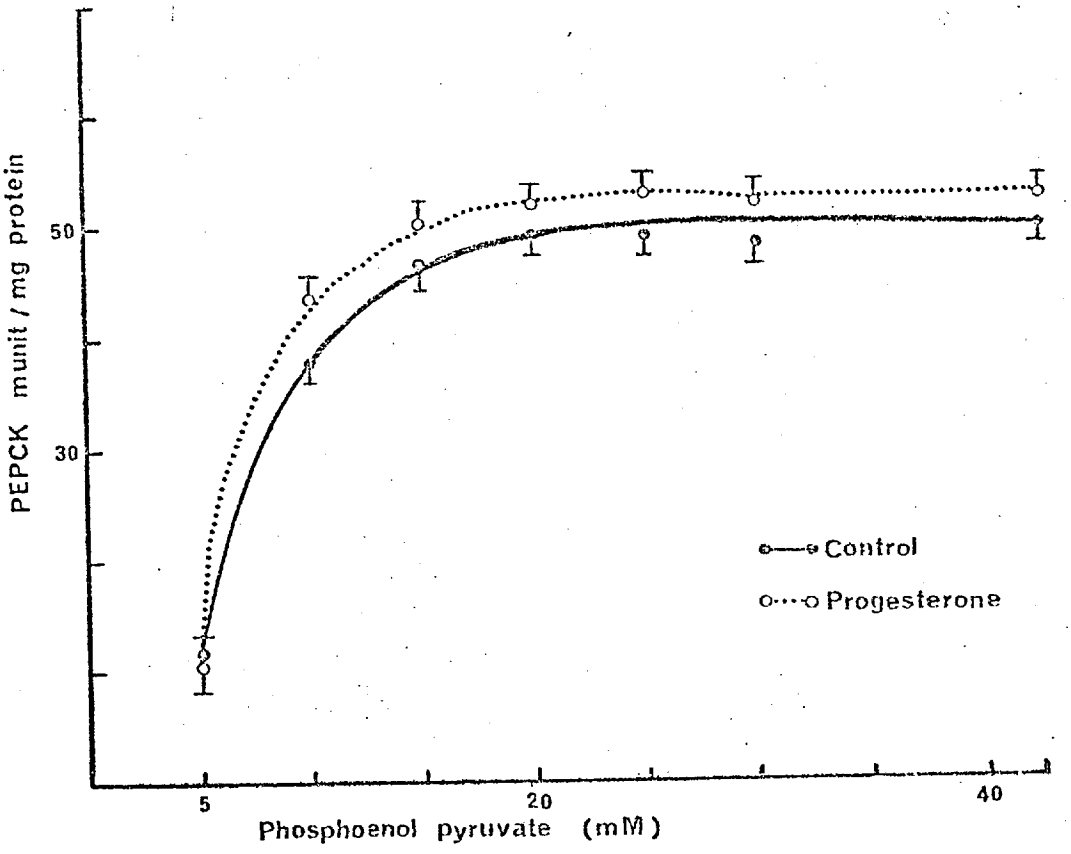


FIGURE (29)

Liver PEPCK was assayed with increasing concentrations of phosphoenol pyruvate. Progesterone treated rats were given s.c. injection 5 mg/kg/day for 8 weeks. Each point represents the Mean and SEM of duplicates estimates of samples obtained from four rats.

EFFECT OF PROGESTERONE ON LIVER GLUCONEOGENIC INTERMEDIATES AND FASTING PLASMA GLUCOSE

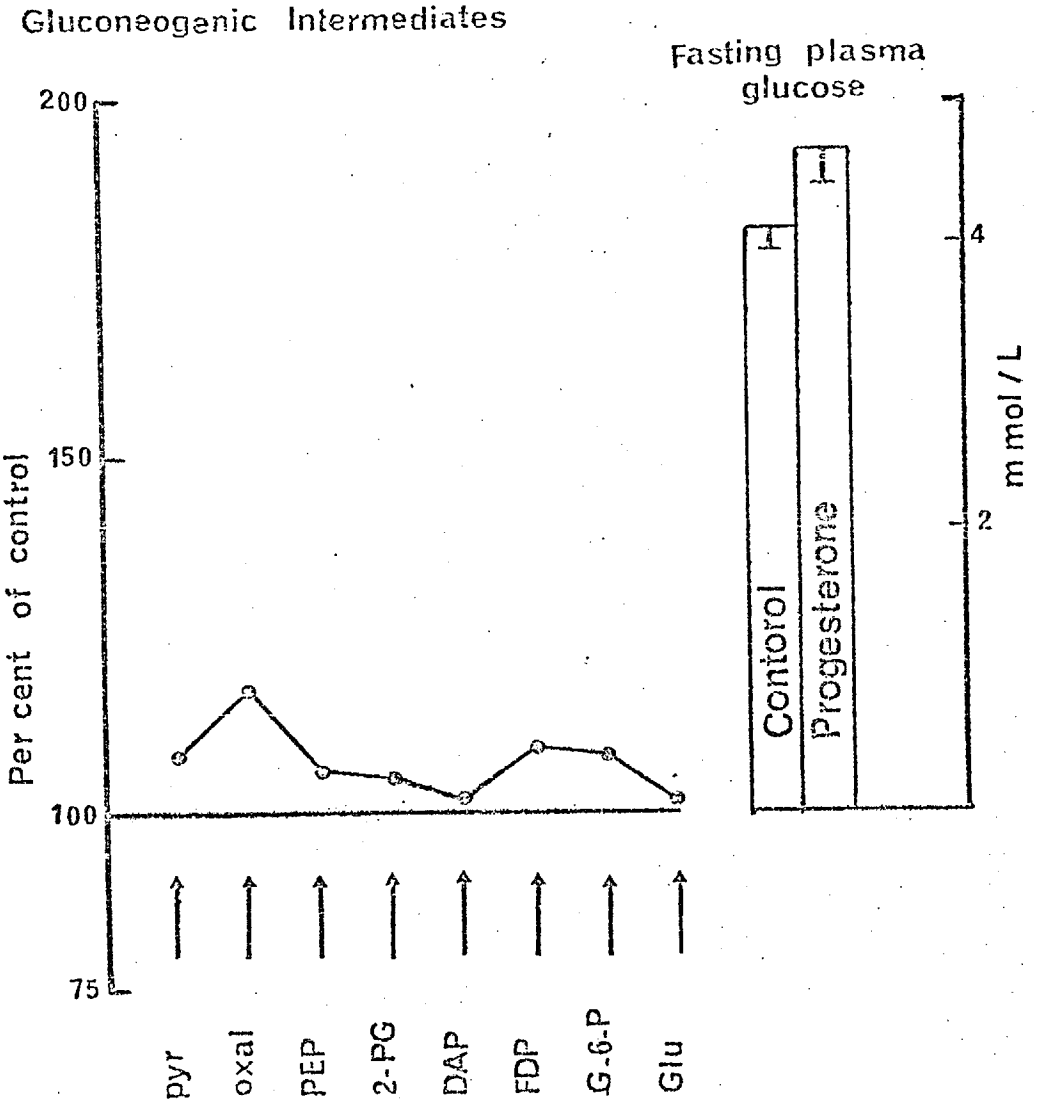


FIGURE (30)

Animals treated with progesterone (5 mg/kg/day) for 8 weeks. The liver was then extracted and assayed for the intermediates. Results are expressed as percentage of similar estimates obtained in pair-fed control animals. Each point represents the mean of values obtained in 6 rats.

(For abbreviations see legend to Table 11.)

Table (12): Effects of Progesterone on Basal and Glucagon Stimulated Hepatic c-AMP Content

Experimental Conditions	c-AMP Content (pmol/mg/wet weight liver)	
	Basal	Glucagon Stimulated
Controls	0.49 ± 0.04	2.1 ± 0.11
Progesterone (mg/kg/day)		
0.5	0.48 ± 0.04	2.16 ± 0.09
5.0	0.46 ± 0.03	2.22 ± 0.06

Basal c-AMP content was determined in liver extracts of overnight fasted rats.

Stimulated levels of c-AMP were estimated in tissue samples obtained 30 sec. following the intraportal injection of 1 µg of glucagon/kg body weight.

Values are Means ± SEM.

EFFECT OF PROGESTERONE ON BASAL AND GLUCAGON-STIMULATED HEPATIC c-AMP CONTENT

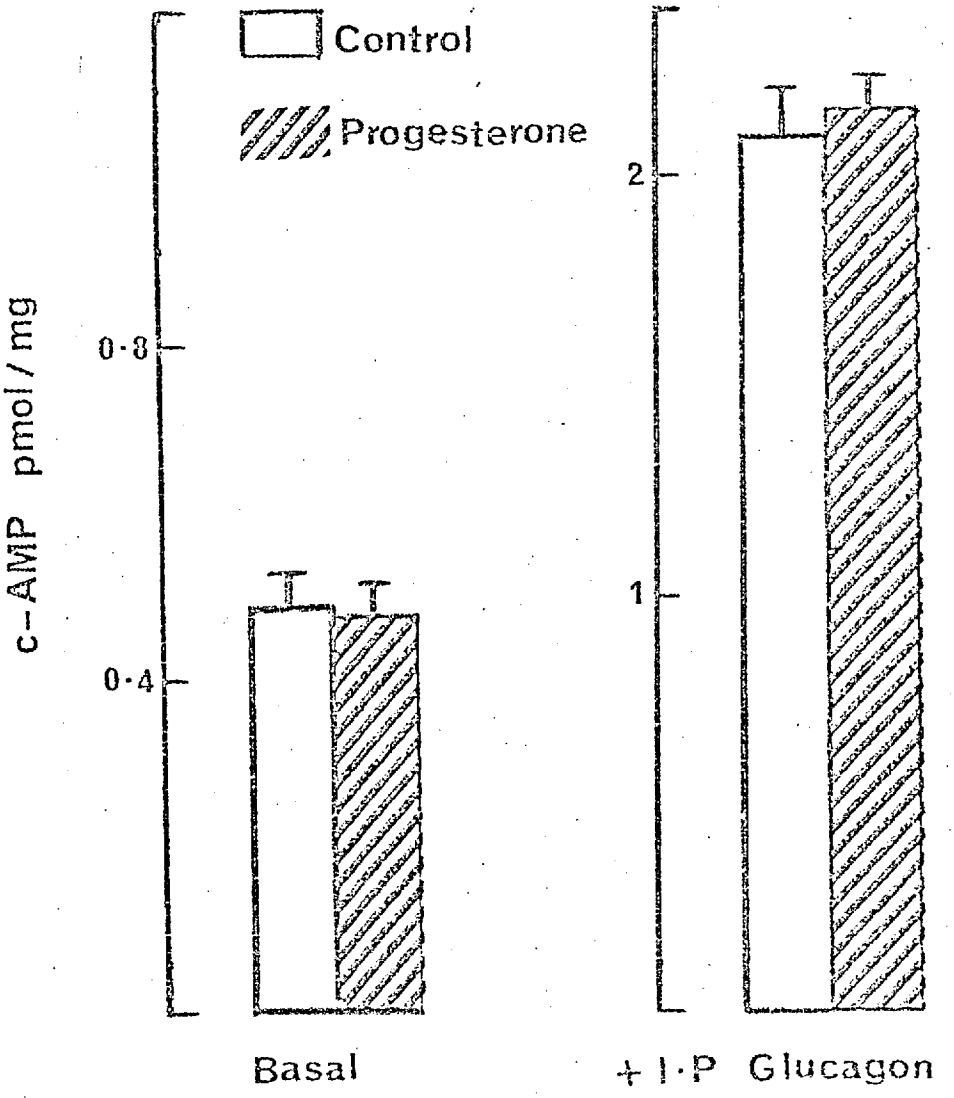


FIGURE (31)

Liver c-AMP content was measured under basal conditions and following glucagon stimulation as desribed in Figure 23. Bars represent Means and SEM of results obtained in 6-8 rats.

D I S C U S S I O N

The results of the present investigation indicate that the administration of oestrogen to ovariectomised rats produces a dose dependent rise in plasma triglyceride concentration and a fall in fasting plasma glucose. Progesterone, on the other hand, had no significant effects. These findings suggest that the effects of oestrogens and progesterone in humans could be reproduced experimentally in rats and hence this animal model could be used to study the metabolic effects of these steroids.

Afolabi (1974) has examined the effect of therapeutic doses of oestradiol and of progesterone administered to gonadectomised rats. In these animals the intake of oestrogenic steroids enhanced the hepatic release of triglycerides into the plasma resulting in hypertriglyceridaemia. The administration of oestrogens to rats stimulated the synthesis and secretion of plasma VLDL apoprotein peptides from liver slices incubated in vitro. The rates of hepatic lipogenesis from glucose and/or acetate were also increased. The results shown in the present study indicate that the enhanced lipogenesis observed with oestrogens could be due to the increased activity of hepatic acetyl CoA carboxylase and fatty acid synthetase enzymes. The increases in activity of these enzymes during oestrogen therapy were dose dependent and were parallel to the magnitude of rises in plasma triglyceride concentration.

The activities of acetyl CoA carboxylase and fatty acid synthetase enzymes represent the key or the rate-limiting steps in the control of hepatic lipogenesis (Numa et al 1965, and Vagelos et al 1966). An increase in the activity of these enzymes as in obese mice leads to enhancement of hepatic lipogenesis (Chang et al 1967, and Winand et al 1968). On the other hand, a decreare in the activity of these enzymes as in diabetic livers is associated with a proportionate decrease in the rate of fatty acid synthesis (Wieland et al 1963). Indeed, Windmueller & Spath (1967) have implied that the rate of hepatic triglyceride secretion might be determined by the activity of these enzymes since a close correlation was demonstrable between the fatty acid synthesising capacity and the rate of triglyceride release from rat liver.

The increased activity of the fatty acid synthesising enzymes during oestradiol therapy could be due to either an increase in the catalytic efficiency of an unchanged mass of the enzyme or an increase in the quantity of enzyme protein. The observations made in this study are in keeping with the possibility that oestradiol enhances the activities of liver acetyl CoA carboxylase and fatty acid synthetase by increasing the quantities of these enzymes per unit mass of liver tissue, since the enhancement was observed in assay conditions containing optimal concentrations of substrate and cofactors. Further, the differences in protein content of liver between control and gonadal steroid treated rats were not significant,

and the activities of these enzymes expressed per mg of liver extract protein were markedly increased in the oestradiol treated rats, suggesting that the effect is specific and not simply due to a generalised increase in liver protein synthesis.

Another important finding in the present investigation is the demonstration of decreased activity of the enzyme phosphoenolpyruvate carboxykinase in liver homogenates obtained from oestradiol treated rats suggesting that the steroid could inhibit gluconeogenesis at this step and hence lower the fasting plasma glucose. Oliver (1973), using the same animal model, has also demonstrated a decrease in fasting plasma glucose and a rise in plasma pyruvate. It was, therefore, concluded that oestrogens impair the utilisation of small molecules such as pyruvate and its conversion to glucose. Matute & Kalkhoff (1973) have also demonstrated that the conversion of alanine or pyruvate to glucose was impaired in livers of oestrogen treated rats. Indeed, our crossover pattern of gluconeogenic intermediates in liver extracts of oestrogen treated rats showing elevated levels of pyruvate and oxaloacetate and reduced levels of phosphoenol pyruvate confirms that oestrogens inhibit the in vivo rate of gluconeogenesis at the PEPCK step.

Exton et al (1970) have reviewed the evidence suggesting an important role of PEPCK step in the hormonal control of gluconeogenesis in mammalian liver. The enzyme undergoes adaptive changes in response to many hormones

including insulin and glucagon which correlates with their effects on hepatic gluconeogenesis. This conclusion has been confirmed by other investigators (Yeung & Oliver 1968; Philppichin & Ballerd 1970 and Exton et al 1971b).

The effects of oestrogen on liver lipogenic and gluconeogenic enzymes could be mediated via three main mechanisms:

Firstly, it is possible that the steroid might exert a direct effect modulating the synthesis and/or degradation of liver enzymes.

Secondly, oestrogen might influence the secretion of some other hormones which in turn could influence the hepatic activity.

Thirdly, oestrogens might modify the responsiveness of the liver cells to the circulating hormonal or humeral agents to alter their biological effectiveness on liver cells.

It is now well recognised that the mammalian liver cell cytoplasm contains a specific receptor protein which binds the oestrogen molecules. The complex so formed is translocated into the nucleus to bind in a specific manner the nuclear chromatin (Steggles et al 1971) which in turn could influence the synthesis of messenger RNA and protein synthesis. Indeed there is sufficient evidence that hepatic enzyme induction may represent a physiological function of some gonadal steroids. Thus, the administration of oestradiol benzoate to ovariectomised

rat causes significant induction of the enzyme aminotransferase and tryptophan oxygenase (Braidman & Rose 1971). In fact Martin & Martin (1976) have demonstrated that the intake of oestrogen containing preparations increases the enzyme γ-glutamyl-transpeptidase (G.G.T) released into the plasma and the magnitude of this effect is proportional to the degree of elevation in serum triglycerides. It was, therefore, proposed that the enhanced lipogenic activity during oestrogen therapy might represent a direct effect of oestrogen on liver enzymes.

On the other hand, there is evidence to suggest that the effects of oestrogens might be secondary to the associated changes in pancreatic hormone levels which in turn could profoundly influence the hepatic enzyme activity.

Alterations in peripheral blood levels of insulin in response to female gonadal steroid therapy have been widely documented in humans and animals. Thus several reports have been published to confirm the concensus that circulating insulin levels are increased in subjects taking these steroids. Wynn & Doar (1969) have found an increase in the mean plasma-insulin response to glucose in women receiving the combined oral contraceptives, though the mean fasting level was unchanged. Spellacy et al (1969) and Beck (1969) were able to demonstrate a rise in both basal and glucose stimulated insulin levels in women on sequential therapy. The prospective study

carried out by Yen & Vela (1968) on non-diabetic women has also demonstrated basal and post glucose challenge hyperinsulinaemia after three months of conventional oral contraceptive regimes. Further, the parenteral administration of natural oestrogens in eleven different investigations that have been recently reviewed by Kalkhoff (1975) resulted in improvement of carbohydrate tolerance and produced hyperinsulinaemia in the majority of cases.

Evidence from other sources seems to contradict the above findings. Thus Javier et al (1968) have reported decrease in insulin levels in women receiving long term treatment with these preparations. While Spellacy et al (1972a) have found no change in insulin levels after six months use of a variety of oestrogenic preparations.

The inconsistencies cited above indicate that the effects of oestrogen and progesterone on plasma insulin are variable and may depend on the type of steroid (natural or synthetic), the route of administration (oral or parenteral), the dosage schedule (conventional or sequential) as well as the pre-existing status of carbohydrate tolerance and endocrine pancreatic reserve. Furthermore, measurements of insulin levels in peripheral blood, unfortunately, do not provide precise information either of the effects of these steroids on pancreatic beta cell function, nor as to the consequent effects of changes in the level of this hormone on liver metabolism. Since the entire pancreatic production of insulin has to

traverse the liver before reaching the peripheral blood, changes in the rate of hepatic removal of this hormone could markedly influence its peripheral concentration. Indeed oestrogen containing preparations have been reported to alter the metabolic clearance rate of insulin in human subjects (Srivastava et al 1975).

The results shown in this study indicate that the administration of oestradiol to ovariectomised rats reduces the basal level of insulin in portal vein blood. Moreover, the reserve capacity of beta cells to secrete insulin in response to glucose infusion was impaired. Progesterone, on the other hand, increased both the basal and glucose-stimulated insulin levels in the portal vein blood. These data suggest that these steroids modulate the activity of the pancreatic beta cells to alter the release of insulin and that the effect of oestrogen and progesterone are antagonistic.

The changes in insulin secretion during oestrogen and progesterone therapy do not necessarily mean that insulin activity in the liver is altered in the same direction, since the steroid might also influence the secretion of antagonistic hormones. In fact, oestrogen and progesterone appear to influence the pancreatic alpha cell function to modulate the secretion of glucagon. Thus oestradiol administered to ovariectomised rats reduced the basal and the alanine stimulated glucagon levels measured in samples of portal vein blood, while progesterone increased these levels. An effect of

oestrogen-progestogen containing preparations on plasma glucagon has also been demonstrated recently in women receiving these preparations (Beck et al 1975). In the latter study the authors have demonstrated a decrease in the arginine stimulated glucagon levels following the administration of oestrogen containing oral contraceptive preparations.

Oestradiol had a more suppressive effect on the pancreatic alpha cells than on beta cells resulting in a greater reduction of glucagon than insulin levels. In the oestradiol treated rats, therefore, the I/G molar concentration ratio in portal vein blood was increased to a level 2-3 times higher than that of control animals. Progesterone, on the other hand, equally increased the activity of both alpha and beta cells stimulating the secretion of insulin and glucagon to the same magnitude and hence the I/G ratio remained unaltered.

For many years the magnitude of insulin and glucagon effects on liver metabolism has been considered to be directly proportionate to the absolute concentration of the hormone reaching the liver (Jefferson et al 1968, and Friedmann et al 1967). Recent studies, however, have suggested that the control of liver metabolism is dependent on the relative molar ratio between insulin and glucagon rather than the absolute levels of either of the two hormones. Parrilla et al (1974) have reported that a 100-1000 fold change in insulin and glucagon concentrations at a constant I/G molar concentration ratio did not alter the

rates of gluconeogenesis, ketogenesis, proteolysis and fatty acid oxidation in rat perfused liver. On the other hand, a shift in the ratio between insulin and glucagon irrespective of their absolute concentrations produced concordant changes in hepatic activity. This bi-hormonal control of liver carbohydrate and lipid metabolism has now been confirmed in a variety of experimental and clinical studies. The data supporting this concept has been reviewed in detail in the Introduction Section which suggest that the insulin : glucagon molar ratio might be the important signal in vivo determining the anabolic or catabolic state of the organism. An increase in the I/G molar ratio in portal vein blood as seen with oestrogen therapy would favour the stimulation of hepatic lipogenesis and inhibition of gluconeogenesis. On the other hand, a proportionate increase in the absolute levels of both hormones with a constant I/G ratio as in progesterone treated rats would not affect liver metabolism.

Another mechanism by which gonadal steroids could influence the liver lipogenic and gluconeogenic activity could be related to the influence of the steroid upon sensitivity of the liver cells to either insulin or glucagon. Thus, in adipose tissue the sensitivity to insulin is enhanced by the administration of oestrogen to rats (Gilmour & McKerns 1966 and Salans 1971). Oestrogen also enhances the responsiveness of skeletal muscle to insulin and promotes lean tissue anabolism (McKerns et al 1958, and Knowlton et al 1942). On the

other hand, large doses of oestradiol were found to promote resistance to the glucagon induced glycogenolysis (Thomas, 1963).

Many investigations have been recently introduced to suggest that the actions of insulin and glucagon on liver lipogenic and gluconeogenic activities are mediated by the effects of these hormones on hepatic c-AMP concentrations. Glucagon provokes an immediate increase in hepatic c-AMP whereas insulin decreases the nucleotide level and counteracts the response of the second messenger to glucagon. The changes in the c-AMP level could then influence the activity of rate-limiting enzymes which control hepatic metabolism (for review see the Introduction Section). The concentration of hepatic c-AMP in vivo would then be an adequate index of alterations either in the secretions and/or the sensitivity of liver cells to either of these hormones.

In the present study the administration of oestrogen to female ovariectomised rat decreased basal hepatic c-AMP content suggesting that the insulin activity in these livers is increased relative to glucagon. This insulin "excess" pattern is consistent with the finding of an increase in portal vein I/G molar concentration ratio. In liver extracts of progesterone treated rats, on the other hand, the c-AMP concentration was similar to that of control rats since the I/G ratio was not significantly altered by the administration of this steroid. Further, in oestradiol treated rats the hepatic nucleotide

response to exogenously administered glucagon was markedly impaired. These results suggest that oestrogens decrease the sensitivity of liver cells to glucagon which might have also enhanced the biological effectiveness of circulating insulin.

In conclusion, the results of this investigation indicate that the administration of pharmacological doses of oestradiol to ovariectomised rats reduces the activity of both alpha and beta cells of the pancreas, and increases the relative I/G molar concentration ratio in portal vein blood. Oestrogens also decrease the responsiveness of liver cells to glucagon. The altered bi-hormonal ratio together with the changes in glucagon action through their effects on the liver c-AMP could be responsible for the changes in rate-limiting enzymes of hepatic liopogenesis and gluconeogenesis resulting in the abnormalities in lipid and carbohydrate metabolism seen during therapy with the steroid. Oestrogens might also have a direct effect causing the induction of liver lipogenic enzymes. Progesterone, on the other hand, produced a proportionate increase in insulin and glucagon levels, did not alter the molar I/G ratio, and did not affect hepatic c-AMP concentration. Consequently progesterone neither affected the activity of the liver enzymes, nor induced any significant changes in plasma glucose and triglyceride concentrations.

The mechanisms by which gonadal steroids could alter

the pancreatic alpha and beta cell activities and the role of the adrenal cortex in this process will be discussed (see General Discussion and Conclusion Section).

SECTION 2

EFFECTS OF GLUCOCORTICOIDS ON LIVER LIPOGENIC AND GLUCONEOGENIC ENZYMES; ROLE OF INSULIN AND GLUCAGON

I N T R O D U C T I O N

The data reviewed in the Introduction Section of this thesis indicate that glucocorticoid therapy leads to increased plasma triglyceride concentration and to impairment in glucose tolerance in patients on long term treatment. While in some studies the effects of glucocorticoids were related to decreased peripheral utilisation of triglyceride and glucose, recent evidence in man and in animals has shown that the steroid has more profound effects on liver metabolism and that increased hepatic production of these molecules might be the predominant abnormality. The biochemical events leading to the glucocorticoid action in liver and the relationship to these changes remain uncertain.

Adrenocorticoid hormones are known to exert direct influence upon several hepatic enzyme systems and it is conceivable that some of their effects on lipid and carbohydrate metabolism are mediated via a similar process. On the other hand, glucocorticoids appear to alter the secretion of insulin and glucagon and may also modulate the liver cell responsiveness to these hormones. Since insulin and glucagon are important regulators of hepatic glucose and triglyceride release, changes in circulatory levels and/or actions of the pancreatic hormones might provide an additive mechanism whereby

glucocorticoids could produce their metabolic effects.

In the present study the effects of adrenalectomy and glucocorticoids on liver lipogenic and gluconeogenic enzyme activities were examined. Concurrently, the portal vein blood levels of insulin and glucagon and the responsiveness of hepatocytes to these hormones were assessed. The changes in hepatic enzyme activity were then related to alterations in the secretion and/or actions of pancreatic hormones.

EXPERIMENTAL PROCEDURE

The effects of adrenalectomy and corticosterone therapy were investigated in female Wistar rats of 120-140 g body weight. Because adrenocortical hormones are known to influence the metabolism of gonadal steroids which in turn could induce changes in liver metabolism, animals were ovariectomised ten days prior to the present study. Rats were divided at random into two main groups, 'A', and 'B'.

Animals in group 'A' were used to study the metabolic and hormonal effects of adrenalectomy and corticosterone replacement. For this purpose, rats in this group were subdivided into:

Subgroup A_1; animals in this subgroup served as sham-operated controls. Under pentobarbitone anaesthesia,

abdominal incisions were made and both adrenals were exposed, manipulated, but not excised. Each animal was given a daily intramuscular injection of 0.2 ml corn oil during the whole period of the study.

Subgroup A_2; animals were ovariectomised, both adrenal glands were exposed and excised under direct vision. Following the surgical procedure, rats were allowed free access to 0.9% saline with the drinking water to prevent intravascular volume depletion resulting from loss of salt due to hypoadrenocorticism. An intramusclular injection of 0.2 ml corn oil was given to each animal daily.

Subgroup A_3; rats were adrenalectomised as in subgroup A_2 but were given daily intramuscular injections of replacement doses of corticosterone (5 mg/kg/day) solubilised in 0.2 ml of corn oil.

Animals in group 'B' were ovariectomised non-adrenalectomised female rats which received increasing doses of corticosterone injections. The subgroups B_2, B_3, B_4, B_5, B_6 and B_7 were treated with 5, 10, 15, 20, 25 and 50 mg doses of corticosterone/kg/day respectively. The dose required per animal per day was solubilised in 0.2 ml of corn oil. Since the administration of corticosterone caused anorexia in rats, the control animals in subgroup B_1 were pair-fed and given daily intramuscular injections of corn oil.

The experimental conditions and methods for collection

of samples employed in the present study were similar to those previously described in Chapter III.

R E S U L T S

I. Effects of Adrenalectomy and Corticosterone Replacement

A. Effects of Adrenalectomy on Immuno-reactive Insulin and Glucagon Concentrations in Portal Vein Blood

1. Basal Levels

Table (13) shows that the mean basal insulin level in portal vein blood of sham-operated overnight fasted control rats was $3.6 \pm 0.32 \times 10^{-10}$M (55 ± 4.8 μU/ml). The mean glucagon level was $1.8 \pm 0.2 \times 10^{-10}$M (636 ± 71 pg/ml) and the insulin to glucagon molar concentration ratio averaged 2.0 ± 0.28.

Bilateral adrenalectomy resulted in a significant decrease in portal vein basal insulin while glucagon levels remained unchanged. In the adrenalectomised rats the mean basal insulin level was $1.8 \pm 0.15 \times 10^{-10}$M while the glucagon level was $2.1 \pm 0.025 \times 10^{-10}$M. The insulin : glucagon molar ratio in portal vein blood of the adrenalectomised rats was, therefore, 0.86 ± 0.2 which was significantly lower than that found in the sham-operated controls (Table 13 and Figure 32 a, b & c).

As shown in Talbe (13) the administration of replacement doses of corticosterone 5 mg/kg/day to adrenalectomised

animals increased the portal level of insulin to a mean value of $3.8 \pm 0.36 \times 10^{-10}$M. The I/G ratio in the treated rats was 2.05 ± 0.026 which is similar to the control value (Figure 32c).

These results suggest that bilateral adrenalectomy reduced the basal insulin secretion in rats resulting in a decrease in the insulin : glucagon ratio in portal vein blood, an effect which was abolished by the administration of corticosterone in replacement doses.

2. Response to Alanine

Animals from each subgroup (A_1, A_2 and A_3) were infused with alanine (1 mmol/kg) for 15 min. and the capacity of the alpha cells to secrete glucagon was assessed. The unstimulated glucagon levels in rats from each group were measured during a similar period of saline infusion. In sham-operated animals the portal vein glucagon level was raised during the alanine infusion to 320% above the unstimulated value. As shown in Table (14) the glucagon increment following the alanine infusion to adrenalectomised rats was 366% above basal and was not significantly different from that observed in sham-operated rats. Furthermore, replacement doses of corticosterone had no significant effects upon the glucagon response to alanine.

The results, therefore, indicate that adrenalectomy does not influence the secretory capacity of the alpha cells to release glucagon in response to stimulation with the amino acid alanine.

3. Response to Glucose

During glucose infusion (5.5 mmol/kg for 15 min.) to sham-operated control rats, the peak portal vein insulin level was 14.7 ± 0.8 x 10^{-10}M which corresponds to an increment of 250% above the basal values observed in saline infused rats. Bilateral adrenalectomy markedly suppressed the insulin response to glucose. The increment in portal vein insulin in these rats did not exceed 150% above the unstimulated value. On the other hand, the administration of corticosterone in replacement doses to adrenalectomised rats restored the insulin response to normal. The peak insulin concentration in this latter subgroup was 13.1 ± 0.62 x 10^{-10}M representing 264% increment above basal value (Table 14).

B. Effect of Adrenalectomy on Liver Lipogenic Enzymes

Figure (33) shows that adrenalectomy leads to a small but significant reduction in plasma triglyceride concentration. The mean plasma level in the overnight fasted sham-operated control rats was 0.64 ± 0.08 mmol/L, while in the adrenalectomised rats it was 0.40 ± 0.06 mmol/L. Treatment of adrenalectomised rats with corticosterone 5 mg/kg/day for 8 weeks raised the plasma triglyceride concentration to a mean of 0.70 ± 0.10 mmol/L which is not significantly different from that of sham-operated controls (Table 15).

Bilateral adrenalectomy also resulted in a significant

decrease in acetyl CoA carboxylase and fatty acid synthetase activities assayed in liver homogenates under optimal conditions meant to express the maximum catalytic activities of these enzymes (see Methodology Section). The mean acetyl CoA carboxylase in sham-operated control rats was 2.6 ± munit/mg protein and was reduced to 1.75 ± 0.2 munit/mg protein following corticosterone replacement therapy. The mean fatty acid synthetase in sham-operated rats was 5.6 ± 0.5 munit/mg protein. The activity was decreased in adrenalectomised rats to a mean of 3.0 ± 0.4 munit/mg protein. The mean activity in rats treated with corticosterone replacement, however, was 4.9 ± 0.6 munit/mg protein, and was not significantly different from controls.

The results, therefore, indicate that adrenalectomy suppressed the maximum catalytic activity of liver lipogenic enzymes (acetyl CoA carboxylase and fatty acid synthetase) with a corresponding reduction in fasting plasma triglyceride concentration. Treatment with corticosterone in replacement doses restored the activity of these enzymes and increased plasma triglycerides to within the normal range.

C. Effects of Adrenalectomy on Liver Gluconeogenic Enzymes and Intermediates

Figure (34) shows that fasting plasma glucose in adrenalectomised rats was significantly lower than sham-operated controls. The mean fasting plasma glucose in

control rats was 4.8 ± 0.3 mmol/L, was reduced to 3.1 ± 0.3 mmol/L in adrenalectomised animals and raised to a mean of 5.1 ± 0.4 following corticosterone replacement therapy.

Table (16) shows that removal of both adrenals also leads to a significant decrease in the maximum activity of phosphoenolpyruvate carboxykinase activity (PEPCK) when assayed in liver homogenates of rats fasted for 36 hours and under *in vitro* optimal conditions. The mean enzyme activity in sham-operated control rats was 62 ± 1.8 munit/mg protein and in the adrenalectomised animals was 38 ± 1.6 munit/mg protein. Treatment with corticosterone 5 mg/kg/day for 8 weeks increased the activity of this enzyme to a mean of 59 ± 2.4 munit/mg protein which was not significantly different from that of control rats. These results suggest that adrenalectomy could inhibit gluconeogenesis at PEPCK step and that the reduction in the activity of this rate limiting enzyme could be an important factor in the decrease in fasting plasma glucose. The decrease in the activity of this enzyme, however, was reversible and was restored to normal following the administration of corticosterone replacement.

To further assess the significance of changes in PEPCK as to the *in vivo* situation, hepatic gluconeogenic intermediates were measured in liver extracts. As shown in Table (16) bilateral adrenalectomy resulted in a significant increase in pyruvate and oxaloacetate

concentrations in liver tissue. The concentration of these metabolites in the adrenalectomy group was 148% and 169% of their control values respectively. The phosphoenol pyruvate concentration, on the other hand, was markedly decreased in livers of animals with adrenalectomy; the mean level was 38% of that observed in control rats. The cross over pattern of gluconeogenic intermediates, depicted in Figure (34), therefore, indicates inhibition of gluconeogenesis at the step which separates oxaloacetate from phosphoenol pyruvate. These results confirm the findings with liver enzyme analysis and suggest that adrenalectomy lead to a decrease in catalytic capacity of PEPCK enzyme. The gluconeogenic pattern observed in rats with corticosterone replacement, on the other hand, was similar to normal, suggesting that the changes induced by adrenalectomy were reversed by corticosterone.

D. Effects of Adrenalectomy on Hepatic c-AMP Content and the Response to Exogenous Glucagon

The mean basal c-AMP concentration in liver extracts obtained from sham-operated control rats was 0.31 $\pm$ 0.04 pmol/mg wet weight. As shown in Figure (35) adrenalectomy resulted in a significant decrease in the mean nucleotide level to 0.19 $\pm$ 0.02 pmol/mg wet weight. On the other hand, treatment of the adrenalectomised rats with corticosterone 5 mg/kg/day raised the level to a mean of 0.2 $\pm$ 0.05 pmol/mg wet weight which was not significantly different from control rats. The results, therefore,

Table (13): Effect of Adrenalectomy and Corticosterone Replacement on Basal Levels of Insulin and Glucagon in Portal Vein Blood

Experimental Conditions	Plasma concentration x 10^{-10} M		Insulin/Glucagon Molar Ratio
	Insulin	Glucagon	
Sham-operated Control	3.6 ± 0.32	1.8 ± 0.20	2.0 ± 0.28
Adrenalectomy	1.8 ± 0.15**	2.1 ± 0.25	0.86 ± 0.2**
Adrenalectomy + Corticosterone, (0.5 mg/kg/day)	3.8 ± 0.36	1.85 ± 0.22	2.05 ± 0.26

Corticosterone was given daily for 8 weeks.

Values are Means ± SEM obtained in 6-9 rats.
The probability value for the significance of difference from the sham-operated controls was determined using the student "t" test.

** = significantly decreased ($P < 0.002$).

EFFECT OF ADRENALECTOMY AND CORTICOSTERONE REPLACEMENT ON BASAL LEVELS OF INSULIN IN PORTAL VEIN BLOOD

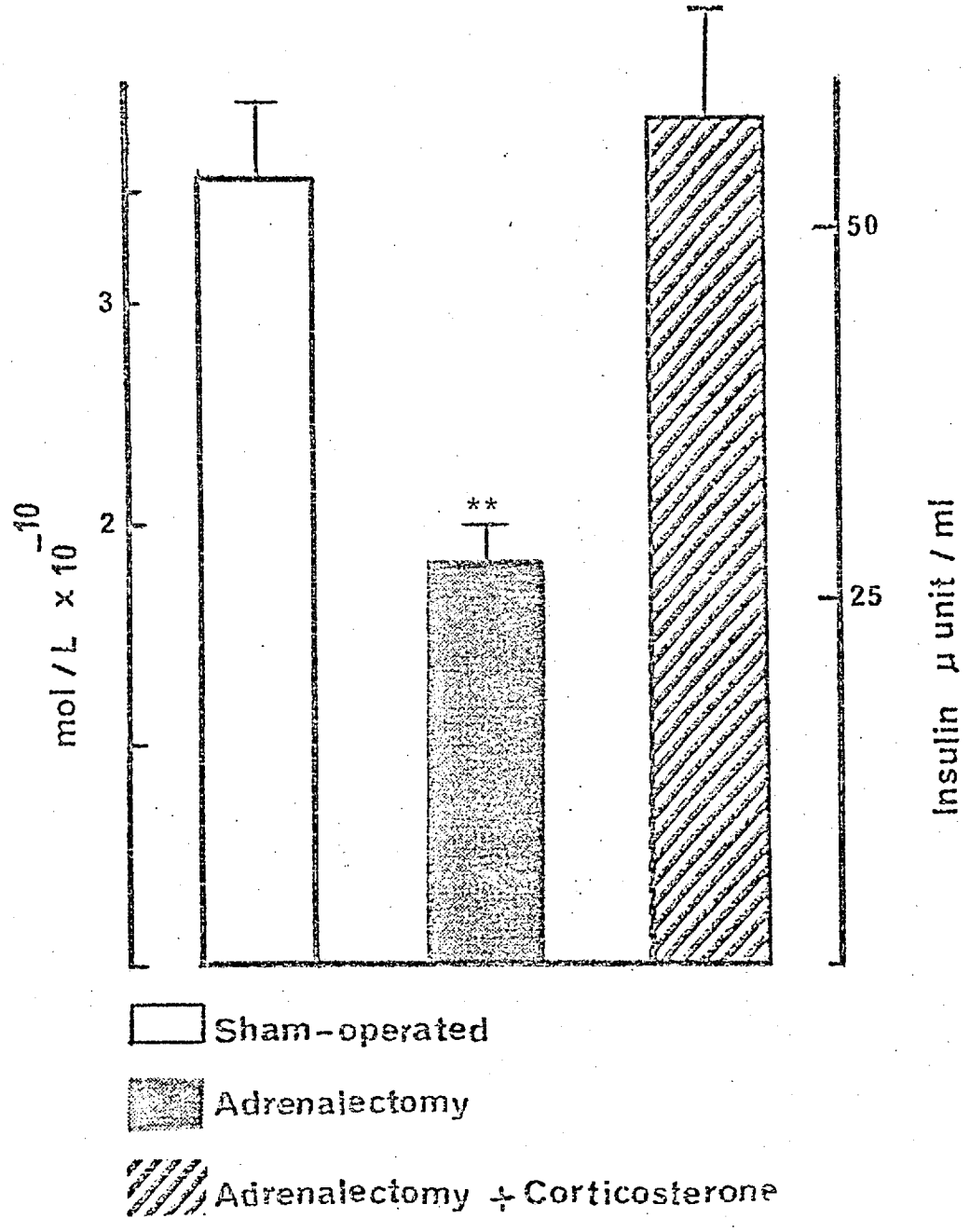


FIGURE (32a)

Corticosterone replacement was given to adrenalectomised rats in a dose of 5 mg/kg/day for 8 weeks. Bars represent the Means and SEM of results obtained in 6-8 rats.

** = Significant decrease as compaired to sham-operated controls ($P < 0.002$).

EFFECT OF ADRENALECTOMY AND CORTICOSTERONE REPLACEMENT ON BASAL LEVELS OF GLUCAGON IN PORTAL VEIN BLOOD

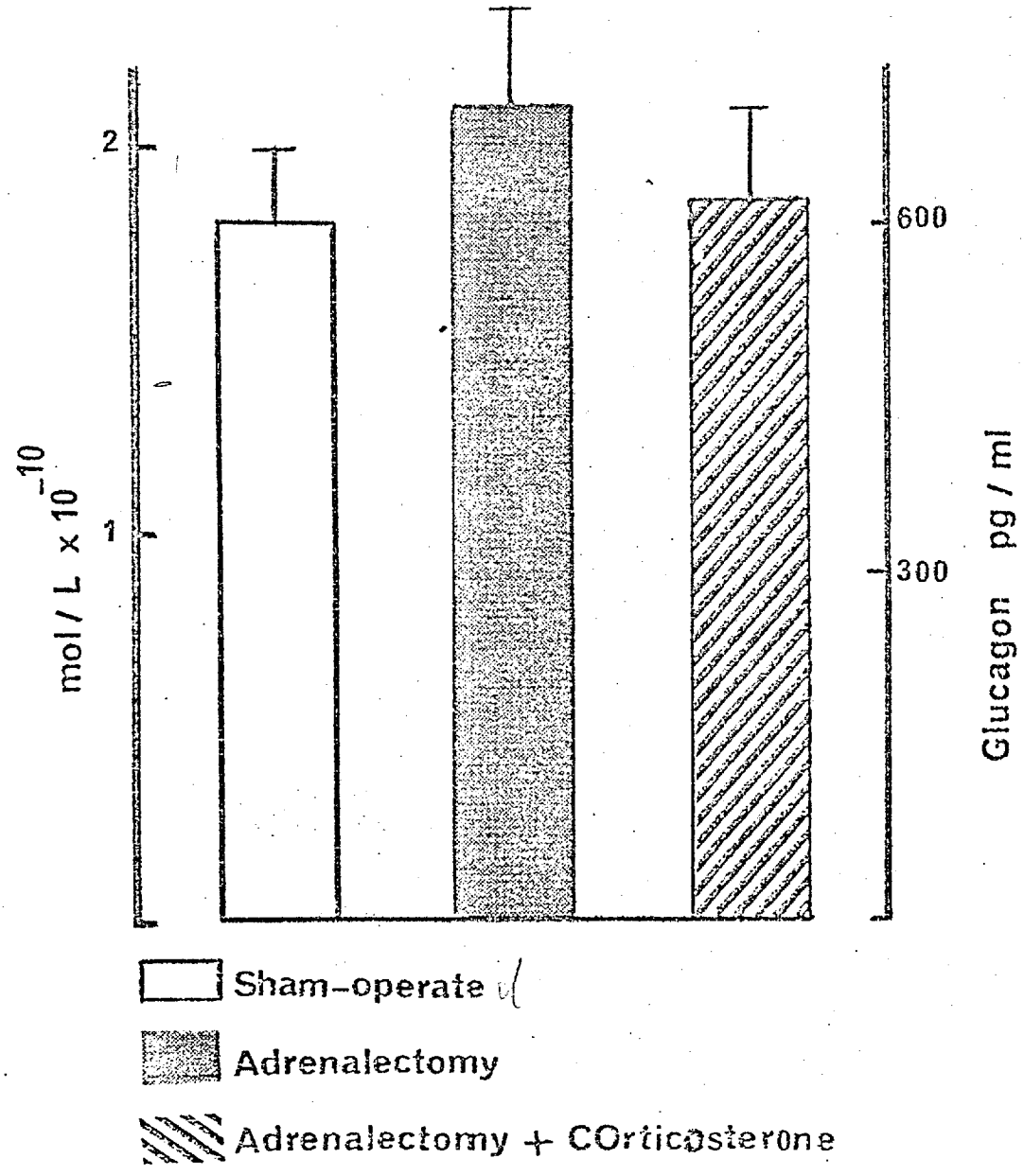


FIGURE (32b)

Corticosterone was given daily in replacement doses of 5 mg/kg/day for 8 weeks. Bars are Means and SEM obtained in 6-8 rats.

EFFECT OF ADRENALECTOMY AND CORTICOSTERONE REPLACEMENT ON PORTAL VEIN PLASMA INSULIN : GLUCAGON MOLAR CONCENTRATION RATIO (I/G)

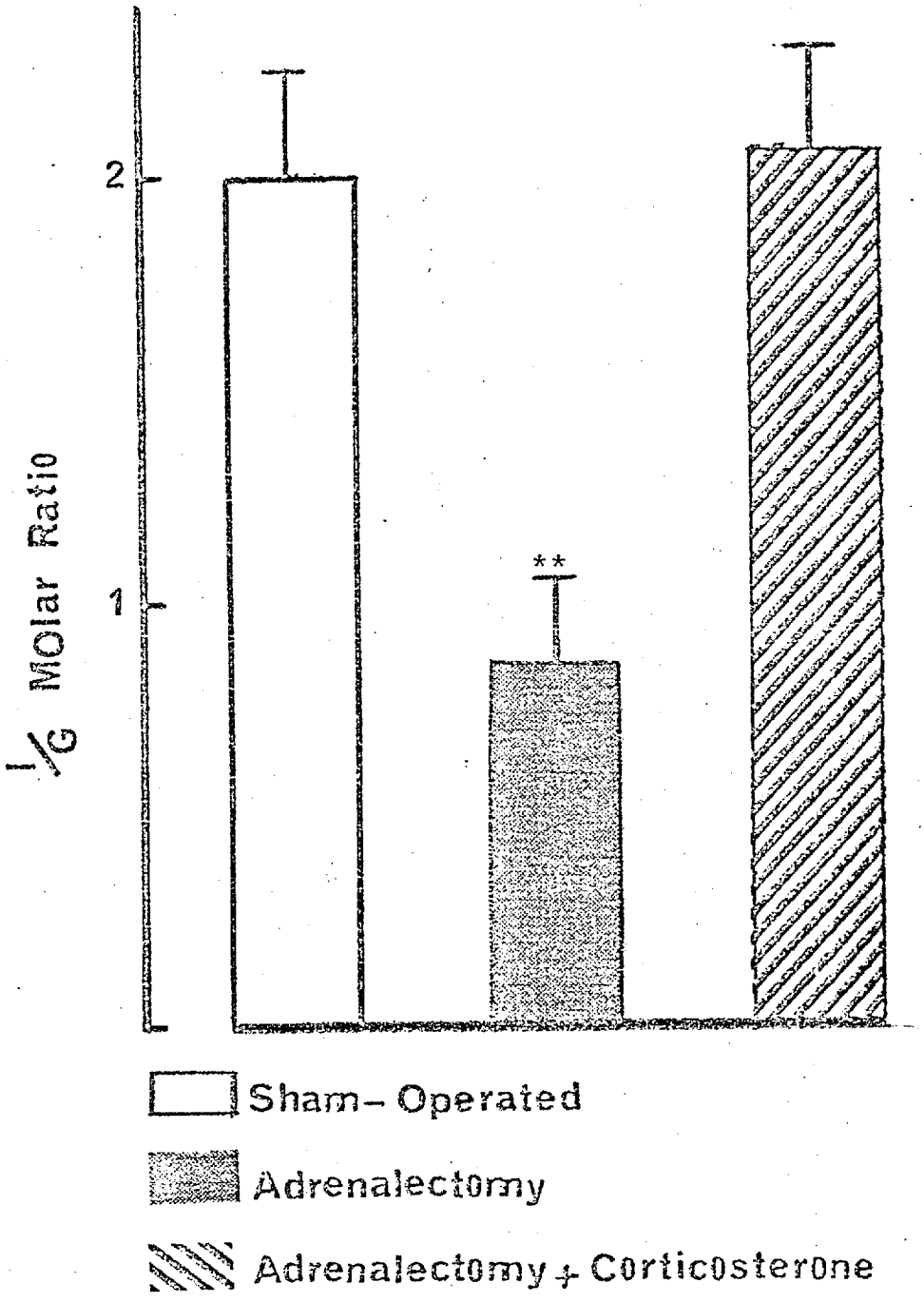


FIGURE (32c)

The I/G ratio was calculated from portal vein insulin and glucagon levels each expressed in its molar concentration (Figures 32 a & b). Bars are Means and SEM obtained in 6-9 rats.

** = Significant decrease as compared to sham-operated controls ($P < 0.002$).

Table (14): Effect of Adrenalectomy and Corticosterone Replacement on the Pancreatic Islets Response to Glucose or to Alanine Infusion

Experimental Conditions	Insulin Concentration x 10^{-10} M		Glucagon Concentration x 10^{-10} M	
	Saline	Glucose	Saline	Alanine
Sham-operated Control	4.2 ± 0.26	14.7 ± 0.80 (250%)	2.2 ± 0.23	9.24 ± 0.60 (320%)
Adrenalectomy	1.2 ± 0.11	3.5 ± 0.10** (130%)	1.9 ± 0.15	8.28 ± 0.46 (336%)
Adrenalectomy + Corticosterone, (0.5 mg/kg/day)	3.6 ± 0.20	13.1 ± 0.62 (264%)	2.0 ± 0.12	8.34 ± 0.38 (317%)

Values are Means ± SEM in 6-8 rats in each group.

Rats were infused with glucose (5.5 mmol/kg) or alanine (1 mmol/kg) via a femoral vein cannula for 15 minutes. Control animals from each group received a saline infusion instead.

Blood for hormonal measurements was obtained from the portal vein at the end of infusion.

Values in parentheses show percentage increment above basal.

** Significantly lower than in sham-operated control rats ($P < 0.005$).

Table (15): Effects of Adrenalectomy and Corticosterone Replacement Therapy on the Activity of Liver Lipogenic Enzymes and Fasting Plasma Triglyceride Level

Experimental Group	Hepatic Lipogenic Enzymes (munit/mg protein)		Fasting Plasma Triglyceride (mmol/L)
	Acetyl CoA Carboxylase	Fatty Acid Synthetase	
Sham-operated Control n = 8	2.6 ± 0.3	5.6 ± 0.5	0.64 ± 0.08
Adrenalectomy n = 6	**1.75 ± 0.2	**3.0 ± 0.4	**0.40 ± 0.06
Corticosterone Replacement (5 mg/kg/day) n = 6	2.8 ± 0.4	4.9 ± 0.6	0.70 ± 0.10

Corticosterone was given daily for 8 weeks.

Values are Means ± SEM of values obtained in 6-8 rats in each group.

The probability for the significance of difference from the sham-operated controls was determined using the student "t" test.

** = significant decrease ($P < 0.02$).

n = number of rats used in each experiment.

EFFECT OF ADRENALECTOMY AND CORTICOSTERONE REPLACEMENT ON PLASMA TRIGLYCERIDE CONCENTRATION, HEPATIC ACETYL CoA CARBOXYLASE AND FATTY ACID SYNTHETASE

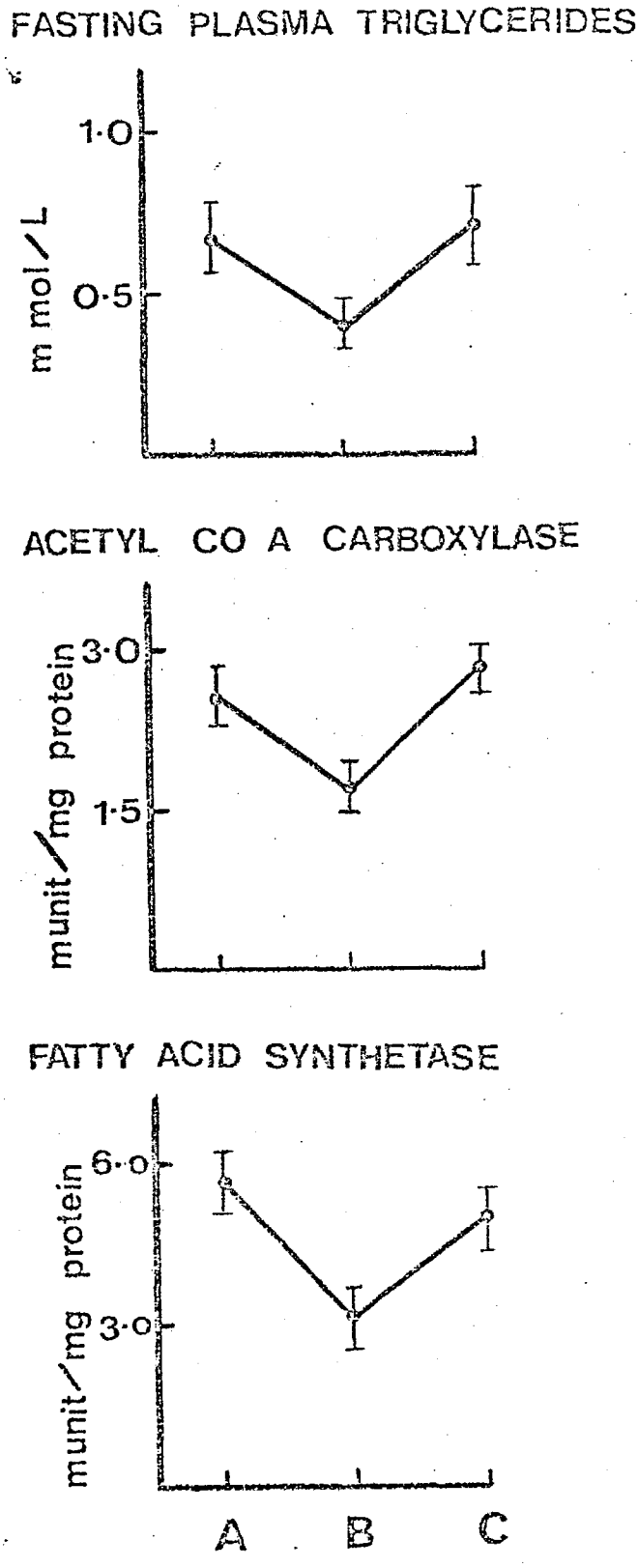


FIGURE (33)

A = sham operated controls, B = adrenalectomy,
C = adrenalectomy & corticosterone replacement (5 mg/kg/day).
Plasma triglyceride was measured in overnight fasted rats.
Hepatic lipogenic enzymes were assayed under optimal conditions to reveal their maximal activities. Results are Means ± SEM on 6-8 rats.

Table (16): Effect of Adrenalectomy and Corticosterone Replacement Therapy on Liver Phosphoenolpyruvate Carboxykinase (PEPCK) and Gluconeogenic Intermediates

Experimental Conditions	PEPCK Activity (mU/mg protein)	Gluconeogenic Intermediates (μmoles/kg wet weight liver)							
		Pyr	Oxal	PEP	2-PG	DAP	FDP	G-6P	Glu
Sham-operated Control (n = 9)	62 ± 1.8	40 ± 2	5.8 ± 0.8	52 ± 2	42 ± 3	28 ± 1.8	24 ± 1.8	280 ± 4	7800 ± 59
Adrenalectomy (n = 11)	** 38 ± 1.6	* 63 ± 1.8	* 10.2 ± 1	** 15 ± 1	43 ± 2	29 ± 1.6	25 ± 1.2	288 ± 6	7176 ± 98
Adrenalectomy + Corticosterone, (0.5 mg/kg/day) (n = 9)	59 ± 2.4	41 ± 1.6	6.5 ± 0.6	58 ± 1.6	42 ± 2	28 ± 1.4	26 ± 1.6	287 ± 8	7960 ± 72

Corticosterone was given intramuscularly, each dose solubilised in 0.2 ml corn oil for 8 weeks. The sham-operated control and the adrenalectomised animals received daily injections of corn oil.

Values are Means ± SEM. * denotes significant increase ($P < 0.05$) when compared to sham-operated control. ** indicates significant decrease than control values ($P < 0.002$).

Abbreviations: pyr, pyruvate; oxal, oxaloacetate; PEP, phosphoenol pyruvate; 2-PG, 2-phosphoglycerate; DAP, dihydroxyacetone phosphate; FDP, fructose 1-6 diphosphate; G-6P, glucose-6-phosphate; glu, glucose.

(n) = number of animals in each group.

EFFECT OF ADRENALECTOMY AND CORTICOSTERONE REPLACEMENT ON LIVER GLUCONEOGENIC INTERMEDIATES AND FASTING PLASMA GLUCOSE

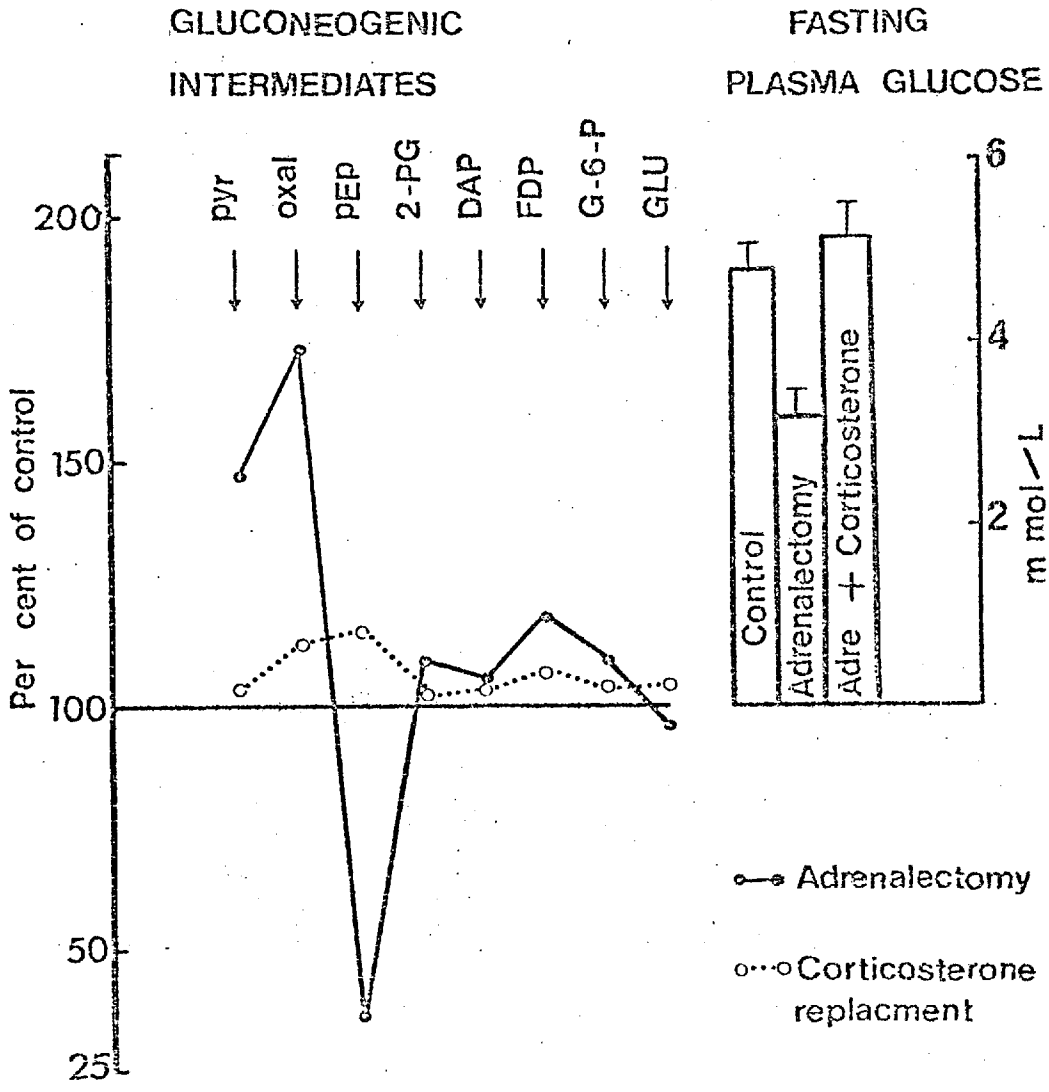


FIGURE (34)

Liver extracts from untreated adrenalectomised rats and from rats receiving corticosterone replacement were assayed for gluconeogenic intermediates. Results are expressed as percentage of values obtained in a similar group of sham-operated control rats. Each point represents the mean of values obtained in 6 rats. (For abbreviations see legend of Table 16).

Table (17): Effect of Adrenalectomy and Corticosterone Replacement Therapy on Basal and Glucagon Stimulated c-AMP Content of Rat Liver

Experimental Conditions	Hepatic c-AMP Content (pmol/mg/wet weight)	
	Basal	Glucagon Stimulated
Sham-operated Control n = 8	0.31 ± 0.04	1.73 ± 0.12
Adrenalectomy n = 6	**0.19 ± 0.02	**0.52 ± 0.07
Corticosterone Replacement (5 mg/kg/day) n = 6	0.32 ± 0.05	1.66 ± 0.20

Basal c-AMP content was determined in liver extracts of overnight fasted rats.

Stimulated levels of c-AMP were estimated in tissue samples obtained 30 sec. following the intraportal injection of 1 µg of glucagon 1 kg body weight.

Values are Means ± SEM.

The probability value for the significance from the control was determined using the student "t" test.

** = significantly decreased from control ($P < 0.002$).

n = number of rats used in each experiment.

EFFECT OF ADRENALECTOMY AND CORTICOSTERONE REPLACEMENT ON BASAL AND GLUCAGON-STIMULATED LIVER c-AMP CONTENT

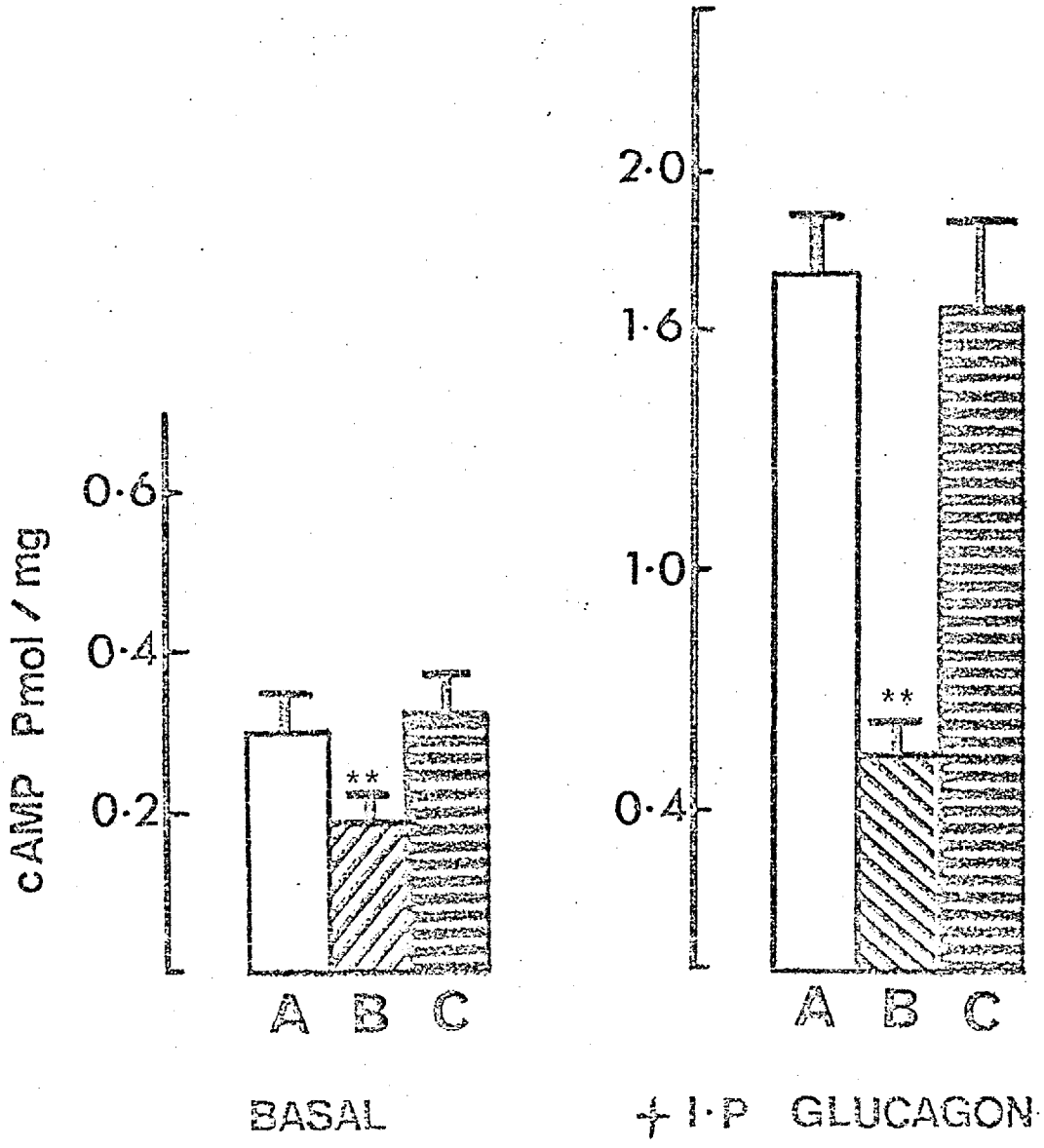


FIGURE (35)

Hepatic c-AMP concentration was measured under basal conditions and following glucagon stimulation as described in Figure (23). Bars represent Means and SEM obtained in 6-8 rats.

A, B and C as in Figure (33).

** = Significant decrease as compared with sham-operated controls ($P < 0.002$).

indicate that adrenalectomy lead to a decrease in basal hepatic c-AMP level, and that treatment of these animals with corticosterone restored the nucleotide level to normal.

To examine the effect of adrenalectomy upon the responsiveness of liver cells to glucagon the increment in hepatic c-AMP content in response to the intraportal injection of glucagon was assessed. As shown in Figure (35) 30 seconds following the intraportal administration of 1 µg/kg of glucagon to sham-operated control rats the level of hepatic c-AMP was 600% above its basal value. In the adrenalectomised animals, on the other hand, exogenous glucagon produced a much smaller increase in hepatic c-AMP level. Thirty seconds after the glucagon injection, the liver c-AMP content of adrenalectomised rats was 260% of its basal value. The increment in hepatic c-AMP following corticosterone replacement, however, was similar to that observed in sham-operated controls (Table 17).

II. Effects of Pharmacological Doses of Corticosterone

A. Effects of Corticosterone on Immuno-reactive Insulin and Glucagon Concentrations in Portal Vein Blood

1. Basal Levels

As shown in Table (18) the mean basal insulin level in portal vein blood of overnight fasted pair-fed control

rats was 3.8 ± 0.41 x 10^{-10}M. The mean glucagon level was 1.7 ± 0.41 x 10^{-10}M and the insulin : glucagon molar concentration ratio avaraged 2.24 ± 0.20.

Increasing doses of corticosterone given for a period of 8 weeks produced progressive increases in basal insulin concentrations in animals receiving 5, 10, 15, 20, 25 and 50 mg of corticosterone/kg/day which were 6.6 ± 60, 16.2 ± 0.92, 18.6 ± 0.80, 19.4 ± 0.62, 22.8 ± 0.68 and 21.6 ± 0.86 x 10^{-10}M respectively (Table 18 and Figure 36a).

The fasting plasma glucagon level in portal vein blood of overnight fasted rats treated with small doses of corticosterone (5 mg/kg/day) was slightly higher but not significantly different from that on control rats. As with insulin, higher doses of corticosterone produced a significant increase in portal vein glucagon. The mean glucagon concentrations in animals treated with 10, 15, 20, 25 and 50 mg corticosterone/kg/day were 3.6 ± 0.18, 3.8 ± 0.12, 4.0 ± 0.15, 4.2 ± 0.20 and 4.0 ± 0.11 respectively (Table 18 and Figure 36b).

As shown in Table (18) the increase in portal vein blood insulin in response to pharmacologic doses of corticosterone was much greater in magnitude than the increase in glucagon. Hence, the insulin : glucagon molar concentration ratio was increased. The molar ratio in animals receiving the highest dose of corticosterone (50 mg/kg/day) was 5.4 ± 0.25 which was approximately double the ratio found in control rats (Figure 36c).

2. Response to Alanine

The capacity of alpha cell to secrete glucagon was assessed by measuring the increment in the portal vein glucagon during an intravenous infusion of alanine (1 mmol/kg) over a period of 15 min. The unstimulated level was measured in animals from each subgroup receiving saline infusion instead. Table (19) shows that in the pair-fed control rats the glucagon level following the alanine infusion was 335% above basal. The administration of corticosterone in a dose of 5 mg/kg/day did not alter the magnitude of glucagon response to alanine. However, treatment with moderate doses of corticosterone (15 mg/kg/day) markedly enhanced the glucagon response to alanine and a further enhancement was observed with larger doses of the steroid (50 mg/kg/day).

The results suggest that moderate and large doses of corticosterone administered to non-adrenalectomised rats modified the pancreatic alpha cells to enhance their capacity to secrete glucagon in response to an alanine stimulus (Figure 37a).

3. Response to Glucose

During glucose infusion to pair-fed control rats the peak portal vein insulin level was $14.9 \pm 0.96 \times 10^{-10}$M which corresponds to an increment of 285% above basal value. Administration of corticosterone in doses of 5, 15 or 50 mg/kg/day increased the secretory capacity of beta cells and enhanced the insulin response to

glucose. The increments in portal vein insulin levels in these rats were 386%, 528% and 496% respectively (Table 19 and Figure 37b).

B. Effects of Corticosterone on Liver Lipogenic Enzymes

As depicted in Figure (38) the administration of corticosterone produced a dose dependent increase in fasting plasma triglyceride concentrations. The mean plasma level in overnight fasted pair-fed control rats was 0.91 ± 0.10 mmol/L. Corticosterone in a dose of 10 mg/kg/day produced a significant increase in plasma triglyceride level to a mean of 1.42 ± 0.19 mmol/L. The plasma concentrations in animals treated with 15, 25 and 50 mg respectively were 1.55 ± 0.12, 1.75 ± 0.17 and 2.3 ± 0.21 mmol/L. The triglyceride level in animals receiving the high doses of corticosterone was 2-3 times that of the controls (Table 20).

The activity of the lipogenic enzymes acetyl CoA carboxylase and fatty acid synthetase were determined in liver homogenates obtained from each animal group. The assays were conducted under optimal conditions meant to express the maximum catalytic activity of these enzymes (see Methodology Section). As shown in Figure (38), corticosterone therapy markedly stimulated the activities of hepatic acetyl CoA carboxylase and fatty acid synthetase. The mean acetyl CoA carboxylase in pair-fed control rats was 2.3 ± 0.32 munit/mg protein. The activity of this enzyme was increased to a mean of 5.4 ± 0.38, and 5.35 ± 0.65, 5.25 ± 0.7 and 5.75 ± 0.47 munit/mg protein respectively

in animals receiving doses of 10, 15, 20 and 25 mg of corticosterone/kg/day. Higher doses of the steroid (50 mg/kg/day) produced a further increase in the activity of acetyl CoA carboxylase to a mean of 6.3 ± 0.38 munit/mg protein (Table 20).

Increasing doses of corticosterone produced a dose dependent rise in fatty acid synthetase activity. The mean fatty acid synthetase activity in control rats was 5.5 ± 0.9 munit/mg protein. Homogenates obtained from the rats receiving 5, 10, 15, 20, 25 and 50 mg of corticosterone/kg/day showed that the enzyme activity was 6.9 ± 1.1, 10.5 ± 1.1, 11.7 ± 1.2, 12.9 ± 0.9, 13.5 ± 1.1 and 13.9 ± 1.2 munit/mg protein respectively (Table).

The results, therefore, suggest that pharmacological doses of corticosterone stimulate the maximum catalytic activity of liver acetyl CoA carboxylase and fatty acid synthetase and the magnitude of stimulation is sufficient to account for the increase in fasting plasma triglyceride concentrations.

C. Effects of Corticosterone on Liver Gluconeogenic Enzymes and Intermediates

Figure (39) shows that the administration of corticosterone to rats produced paradoxical changes in fasting plasma glucose level. With moderate doses of corticosterone (15 mg/kg/day), the mean fasting plasma glucose was significantly decreased while large doses of corticosterone (50 mg/kg/day) increased its level.

Table (21) shows that the administration of increasing doses of corticosterone to rats also produced paradoxical changes in phosphoenolpyruvate carboxykinase (PEPCK) when the activity of this enzyme was assayed in liver homogenates under optimal conditions. In control animals the mean activity was 54 $\pm$ 1.2 munit/mg protein. With moderate doses of corticosterone between 10 and 20 mg/kg/day the activity of PEPCK was reduced. The mean value in animals treated with the 15 mg dose was 42 $\pm$ 1.8 munit/mg protein. On the other hand, with higher doses of the steroid (25 to 50 mg/kg/day) the activity of this enzyme was significantly increased above controls. The mean activity measured in rats treated with the 50 mg dose was 86 $\pm$ 3.8 munit/mg protein.

To determine the significance of the changes in PEPCK activity, the concentration of liver gluconeogenic intermediates was measured in extracts obtained from animals treated either with moderate or high corticosterone (15 or 50 mg/kg/day respectively). As shown in Table (21) the administration of the 15 mg corticosterone dose led to a decrease in the concentration of phosphoenol pyruvate to a level approximatley 55% of the control value. The concentration of pyruvate and oxaloacetate on the other hand were increased to 140% and 125%. These results suggest the inhibition of *in vivo* gluconeogenesis at the step separating pyruvate and phosphoenol pyruvate. The 50 mg dose of corticosterone increased the hepatic concentration of phosphoenol pyruvate to 235%, while causing a fall in pyruvate to 92% and in oxaloacetate to 82% of

control values suggesting that the large dose of steroid lead to the stimulation of gluconeogenesis at the PEPCK step.

The cross over pattern plotted in Figure (39) thus confirms that treatment of rats with 15 mg corticosterone/kg/day inhibits the PEPCK while the 50 mg corticosterone dose stimulates this step. These changes are parallel to the effects of these doses of steroid upon the fasting plasma glucose concentration.

D. Effect of Corticosterone on Hepatic c-AMP Content and Response to Exogenous Glucagon

The mean basal c-AMP concentration in liver extracts obtained from pair-fed control rats was 0.37 ± 0.025 pmol/mg wet weight. As shown in Figure (40) and Table (22), the administration of moderate doses of corticosterone (15 mg/kg/day) to rats with intact adrenals resulted in a significant decrease in the mean nucleotide level to 0.25 ± 0.023 pmol/mg wet weight. On the other hand, treatment of rats with high doses of corticosterone (50 mg/kg/day) produced marked increase in basal c-AMP, the mean level being 0.65 ± 0.05 pmol/mg wet weight. These results indicate a paradoxical effect of corticosterone on hepatic c-AMP content. Moderate doses of corticosterone lead to a decrease, while high doses of the steroid lead to an increase in the nucleotide level.

To examine the effect of glucocorticoids upon the responsiveness of liver cells to glucagon the increment

in hepatic c-AMP following the intraportal administration of glucagon was examined. As shown in Figure (40) the intraportal administration of 1 ,ug/kg of glucagon to pair-fed control rats resulted in an increase in hepatic c-AMP to a mean of 1.54 ± 0.1 pmol/mg wet weight. With moderate doses of corticosterone (15 mg/kg/day) the administration of exogenous glucagon produced increase in hepatic c-AMP content to a mean of 1.5 ± 0.17 pmol/mg wet weight which is not significantly different from the level achieved in control rats. High doses of corticosterone (50 mg/kg/day), on the other hand, produced a more marked increase in hepatic c-AMP response, the mean level achieved 30 seconds following glucagon injection was 2.3 ± 0.04 pmol/mg wet weight, which was significantly higher than that observed in control rats. These results suggest that the responsiveness of hepatic cells to exogenous glucagon was not remarkably altered by the administration of moderate doses of corticosterone while large doses of the steroid increased the sensitivity of liver cells and produced an exaggerated response to glucagon.

Table (18): Effect of Corticosterone Therapy on the Basal Levels of Insulin and Glucagon in Portal Vein Blood

Experimental Conditions	Plasma Concentration x 10^{-10} M		Insulin/Glucagon Molar Ratio
	Insulin	Glucagon	
Pair-fed Controls	3.8 ± 0.41	1.7 ± 0.22	2.24 ± 0.20
Corticosterone (mg/kg/day)			
5	6.6 ± 0.60*	2.1 ± 0.24	3.14 ± 0.26*
10	16.2 ± 0.92*	3.6 ± 0.18*	4.50 ± 0.32*
15	18.6 ± 0.80*	3.8 ± 0.12*	4.89 ± 0.29*
20	19.4 ± 0.62*	4.0 ± 0.15*	4.85 ± 0.18*
25	22.8 ± 0.68*	4.2 ± 0.20*	5.43 ± 0.26*
50	21.6 ± 0.86*	4.0 ± 0.11*	5.40 ± 0.25*

Corticosterone was given daily for 8 weeks.

Values are Means ± SEM obtained in 6-8 rats.

* = significantly increased above controls ($P < 0.01$).

EFFECT OF CORTICOSTERONE ADMINISTRATION ON BASAL LEVELS OF INSULIN IN PORTAL VEIN BLOOD

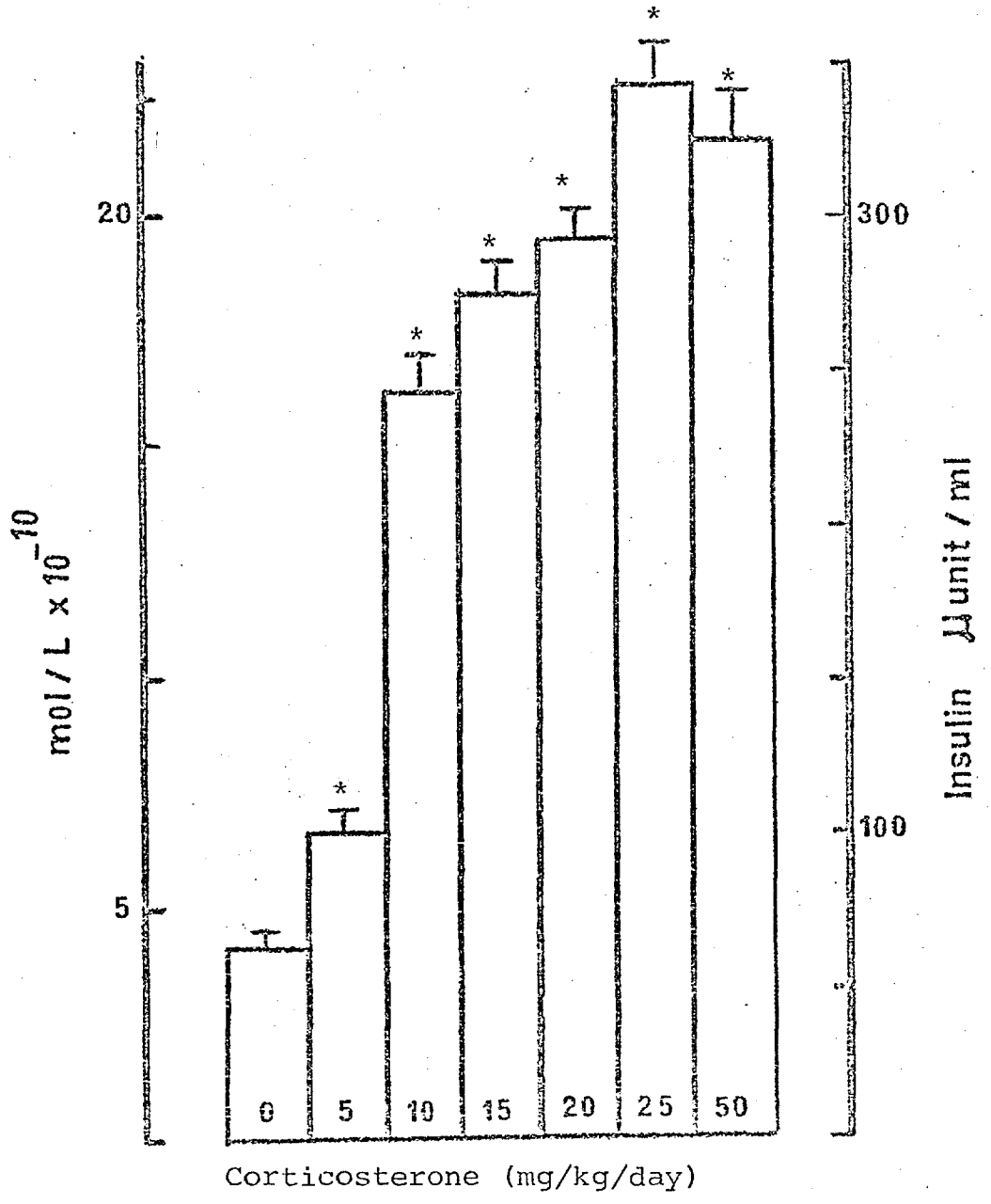


FIGURE (36a)

Corticosterone was given daily to rats with intact adrenals as intramuscular injections for 8 weeks. Bars are Means and SEM of results obtained in 6-8 rats.

* = Significant increase from pair-fed control ($P < 0.01$).

EFFECT OF CORTICOSTERONE ADMINISTRATION ON BASAL LEVEL OF GLUCAGON IN PORTAL VEIN BLOOD

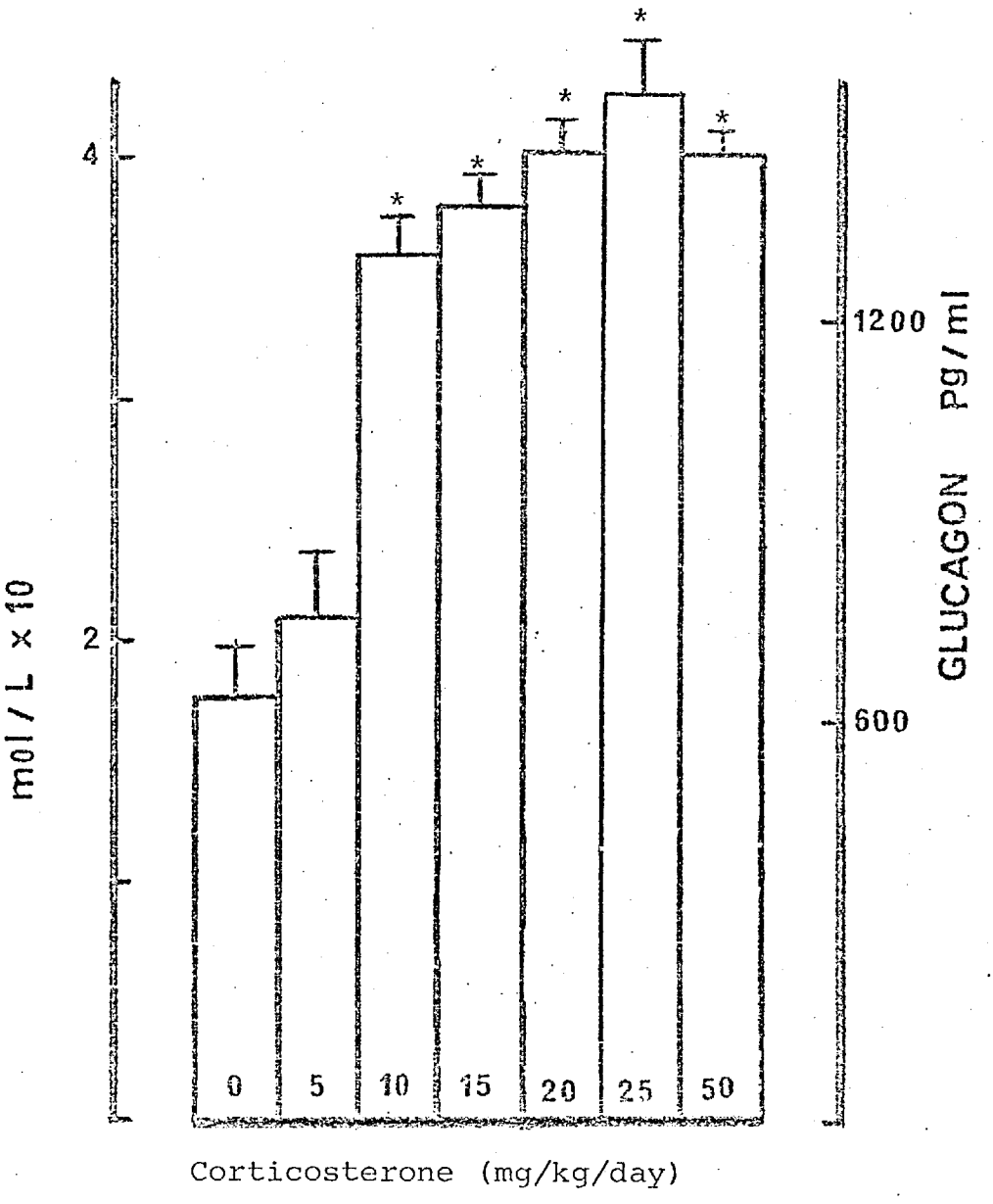


FIGURE (36b)

Corticosterone was given daily to rats with intact adrenals as intramuscular injections for 8 weeks. Bars are Means and SEM obtained in 6-8 rats.

* = Significant increase from pair-fed control ($P < 0.01$).

EFFECT OF CORTICOSTERONE ADMINISTRATION ON PORTAL VEIN PLASMA INSULIN : GLUCAGON MOLAR CONCENTRATION RATIO (I/G)

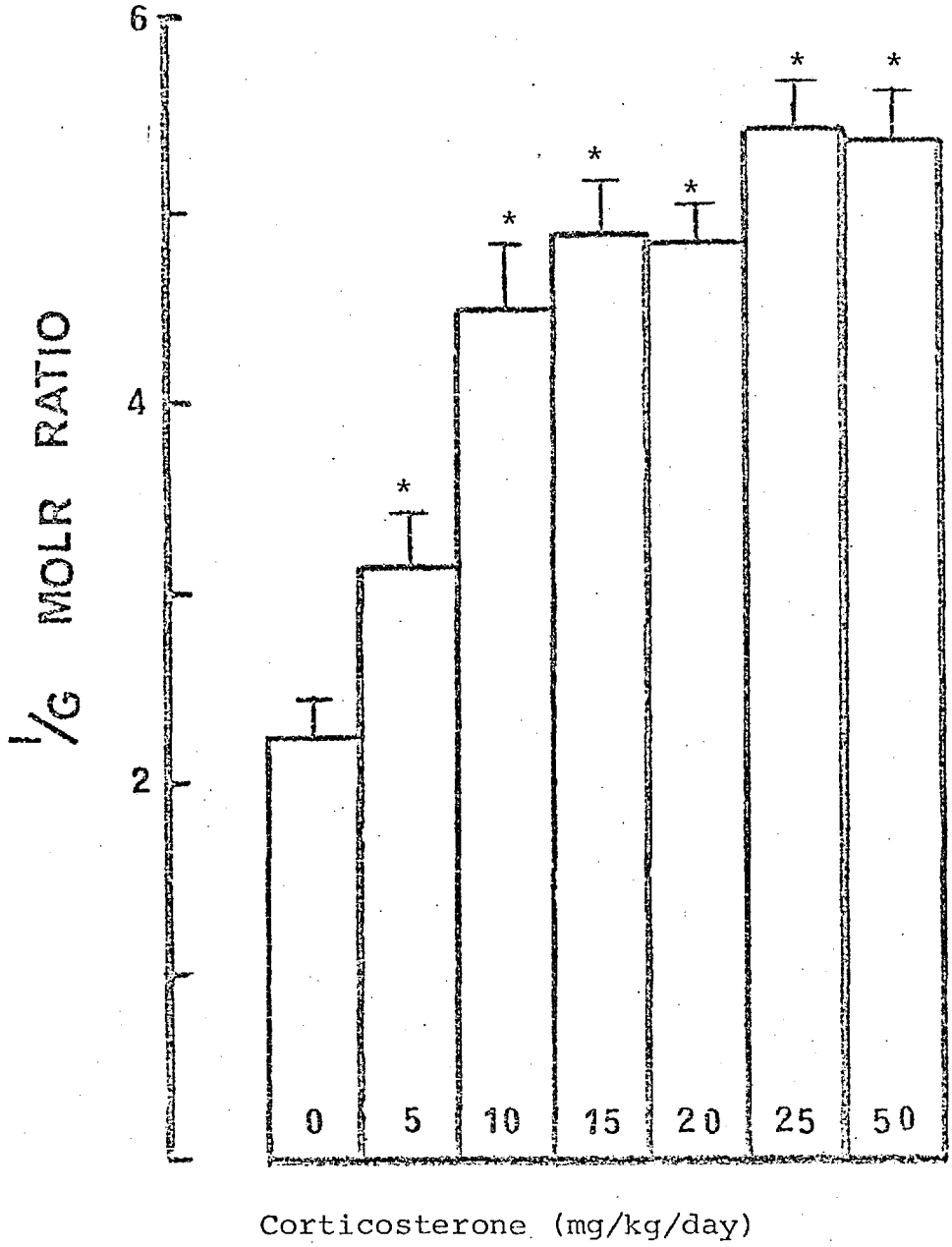


FIGURE (36c)

The I/G ratio was calculated from basal insulin and glucagon levels in portal vein blood each expressed in its molar concentration (Figure 36 a, b).
Bars represent Means and SEM of results obtained in 6-8 rats.

* = Significant increase from pair-fed control ($P < 0.01$).

Table (19): Effects of Corticosterone Therapy on the Pancreatic Islets Response to Glucose or to Alanine Infusion

Experimental Conditions	Insulin Concentration x 10^{-10} M		Glucagon Concentration x 10^{-10} M	
	Saline	Glucose	Saline	Alanine
Pair-fed Controls	3.8 ± 0.24	14.9 ± 0.96 (285%)	2.1 ± 0.22	9.14 ± 0.46 (335%)
Corticosterone (mg/kg/day)				
5	6.4 ± 0.46	31.1 ± 1.2* (386%)	2.0 ± 0.24	8.8 ± 0.38 (340%)
15	16.2 ± 0.94	101.7 ± 4.2* (528%)	4.2 ± 0.36	32.1 ± 1.2 (665%)
50	19.2 ± 0.82	114.4 ± 5.8* (496%)	5.6 ± 0.42	47.7 ± 1.8* (752%)

Rats were infused with glucose (5.5 mmol/kg) or alanine (1 mmol/kg) via a femoral vein cannula for 15 min. Control animals from each group received a saline infusion instead.

Blood for hormonal measurements were obtained from the portal vein at the end of infusion.

For experimental details see text as Table 2.

Values are Means ± SEM obtained in 7-9 rats in each group.

Values in parentheses represent increment above basal value.

* Significantly increased than control rats. ($P < 0.001$).

EFFECT OF CORTICOSTERONE ON PORTAL VEIN GLUCAGON INCREMENT IN RESPONSE TO ALANINE

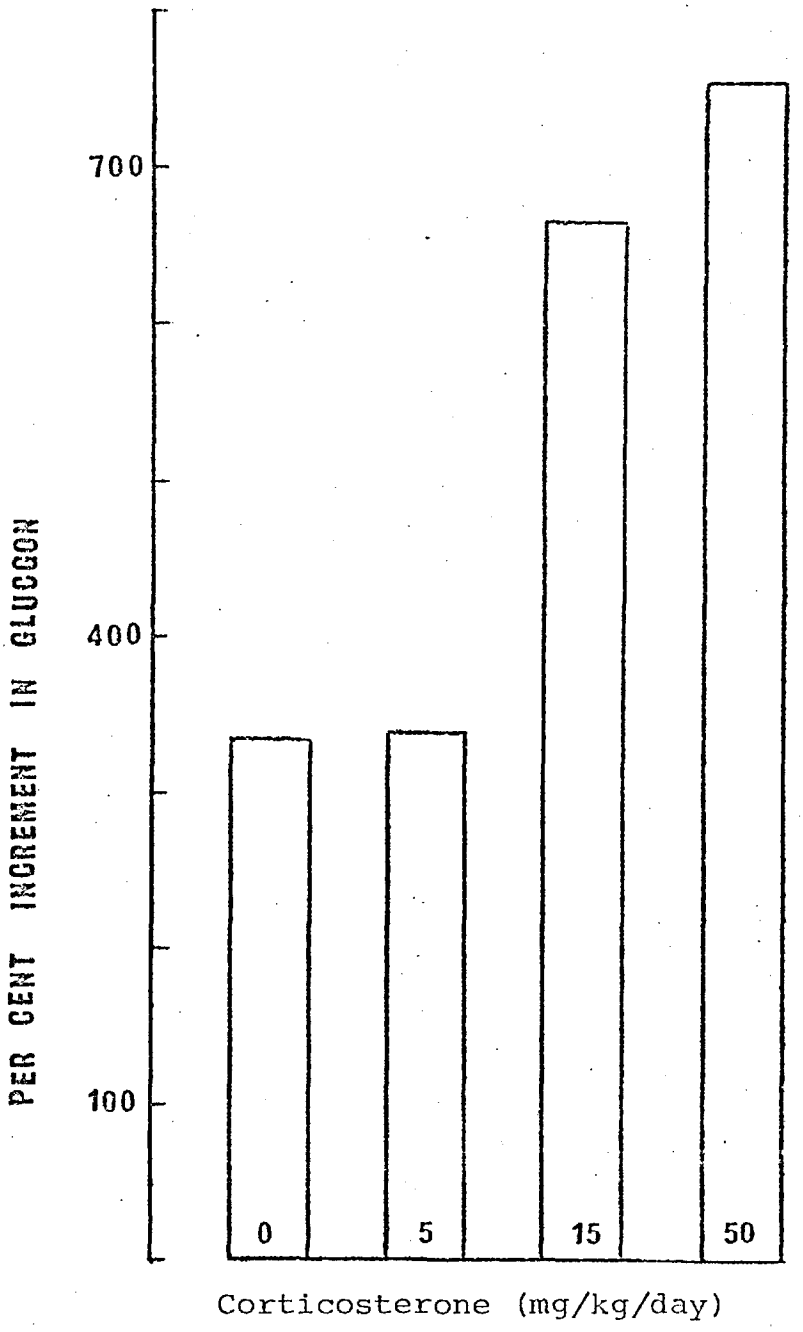


FIGURE (37a)

Animals receiving increasing doses of corticosterone were examined under basal conditions and following stimulation with alanine (see legend to Figure 15). Results were expressed as per cent increment above basal.

EFFECT OF CORTICOSTERONE ON PORTAL VEIN INSULIN INCREMENT IN RESPONSE TO GLUCOSE

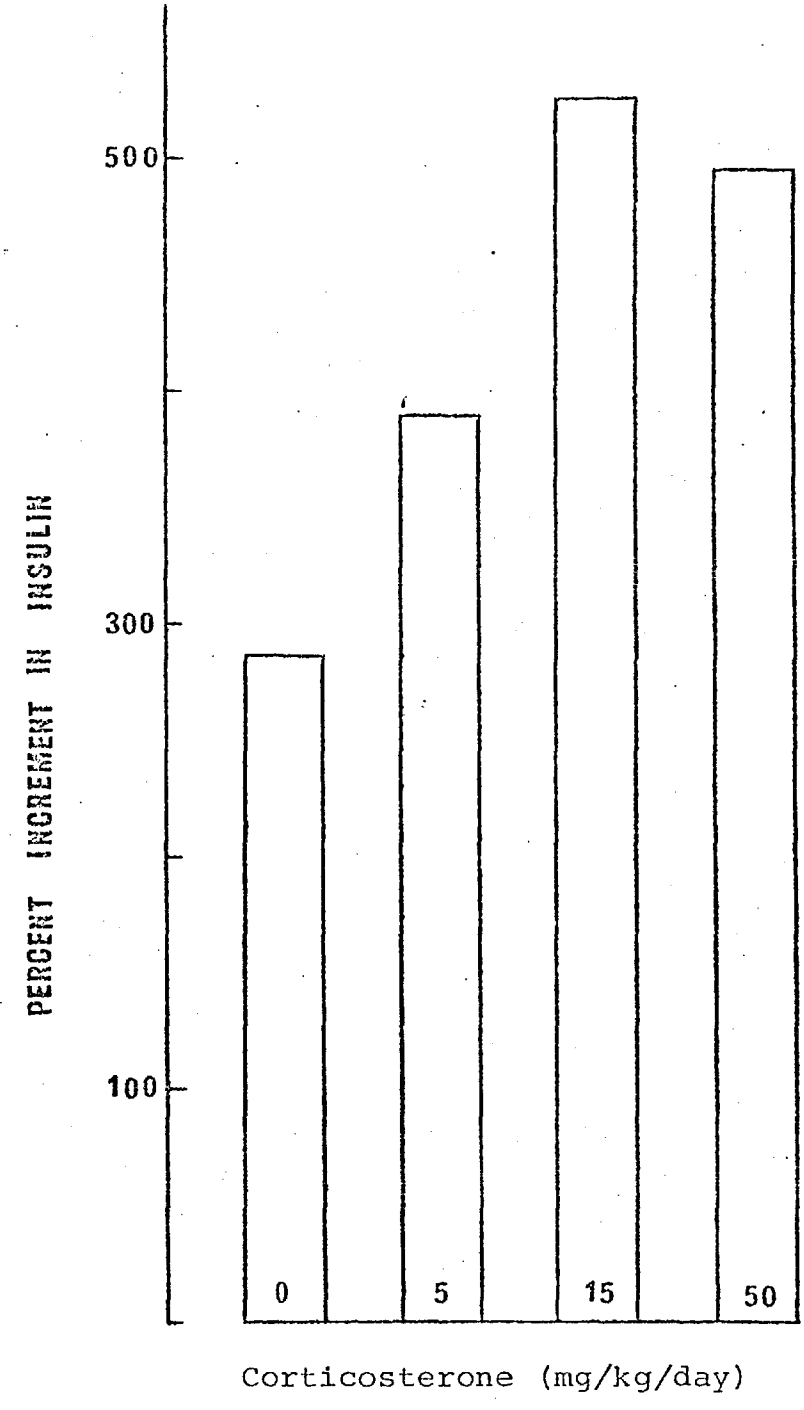


FIGURE (37b)

Animals receiving increasing doses of corticosterone were examined under basal conditions and following stimulation with glucose (see legend to Figure 16).
Results were expressed as per cent increment above basal.

Table (20): Effects of Pharmacological Doses of Corticosterone on Liver Lipogenic Enzymes and Fasting Plasma Triglyceride Level

Treatment Group	Hepatic Lipogenic Enzymes (munit/mg protein)		Fasting Plasma Triglycerides (mmol/L)
	Acetyl CoA Carboxylase	Fatty Acid Synthetase	
Pair-fed Control	2.3 ± 0.32	5.5 ± 0.9	0.91 ± 0.1
Corticosterone 5	2.75 ± 0.4	6.9 ± 1.1	0.91 ± 0.14
10	*5.4 ± 0.38	*10.5 ± 1.1	*1.5 ± 0.16
15	*5.35 ± 0.65	*11.7 ± 1.2	*1.65 ± 0.19
20	*5.25 ± 0.7	*12.9 ± 0.9	*1.7 ± 0.12
25	*5.75 ± 0.47	*13.5 ± 1.1	*1.75 ± 0.17
50	*6.3 ± 0.38	*13.9 ± 1.2	*2.3 ± 0.21

Corticosterone was given as daily intramuscular injection in 0.2 ml corn oil for 8 weeks.

Values are Means ± SEM of values obtained in 6-8 rats. The probability value for the significance of difference from the pair-fed controls was determined using the student "t" test.

* = significantly increased ($P < 0.01$).

EFFECT OF CORTICOSTERONE ADMINISTRATION ON PLASMA TRIGLYCERIDES, LIVER ACETYL CoA CARBOXYLASE AND FATTY ACID SYNTHETASE

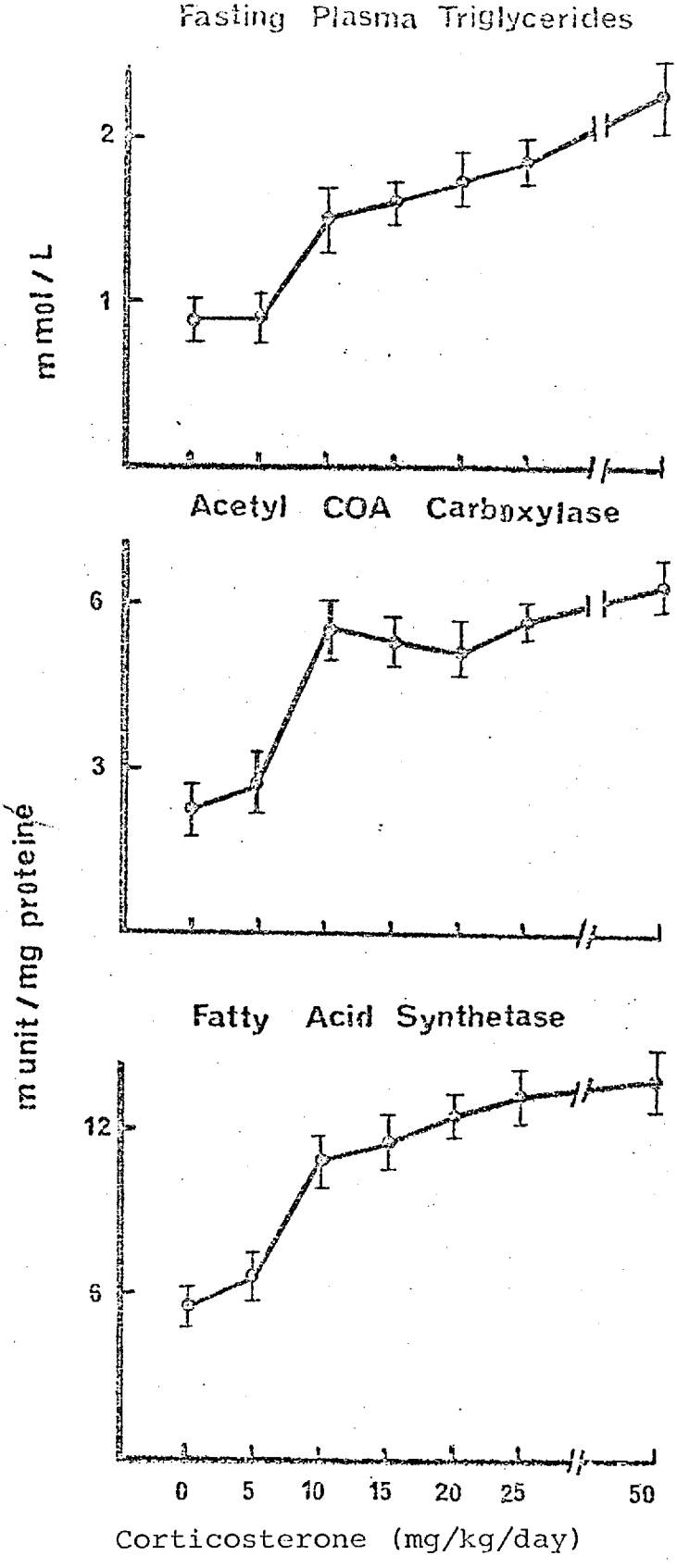


FIGURE (38)

Plasma triglyceride was measured in aortic blood obtained from overnight fasted rats. Liver lipogenic enzymes were assayed under optimal conditions to reveal their maximal activity. Rats were treated with corticosterone for 8 weeks. Values are Means ± SEM of results obtained in 6-8 rats.

Table (21): Effect of Corticosterone Therapy on Liver Phosphoenolpyruvate Carboxykinase (PEPCK) and Gluconeogenic Intermediates

Experimental Conditions	PEPCK Activity (mU/mg protein)	Gluconeogenic Intermediates (μmole/kg wet weight liver)							
		Pyr	Oxal	PEP	2-PG	DAP	FDP	G-6P	Glu
Pair-fed Control	54 ± 1.2	36 ± 1.2	4.9 ± 0.4	56 ± 3.2	40 ± 2.6	23 ± 2.6	29 ± 2.0	296 ± 4.6	8100 ± 42
Corticosterone (mg/kg/day)									
5	48 ± 2.6	39 ± 0.8	4.6 ± 0.2	52 ± 2.4	36 ± 1.8	20 ± 1.8	32 ± 1.6	285 ± 5.6	8200 ± 46
10	**42 ± 1.8								
15	**38 ± 2.8	*51 ± 1.8	*6.6 ± 0.6	**32 ± 2.4	35 ± 1.6	25 ± 1.9	33 ± 2.3	294 ± 6	8302 ± 28
20	**40 ± 1.4								
25	*69 ± 1.6								
50	*86 ± 3.8	**27 ± 2.1	**3.5 ± 0.4	*128 ± 3.9	*85 ± 2.8	24 ± 1.2	32 ± 2.4	337 ± 8.2	9316 ± 38

Corticosterone was given as daily intramuscular injections in corn oil for 8 weeks.
Values are Means ± SEM obtained in 4-6 rats in each group.
* denotes significant increase ($P < 0.01$). ** indicates significant decrease ($P < 0.02$) as compared to control values.
For abbreviations see Table (16).

EFFECT OF CORTICOSTERONE ADMINISTRATION ON LIVER GLUCONEOGENIC INTERMEDIATES AND FASTING PLASMA GLUCOSE

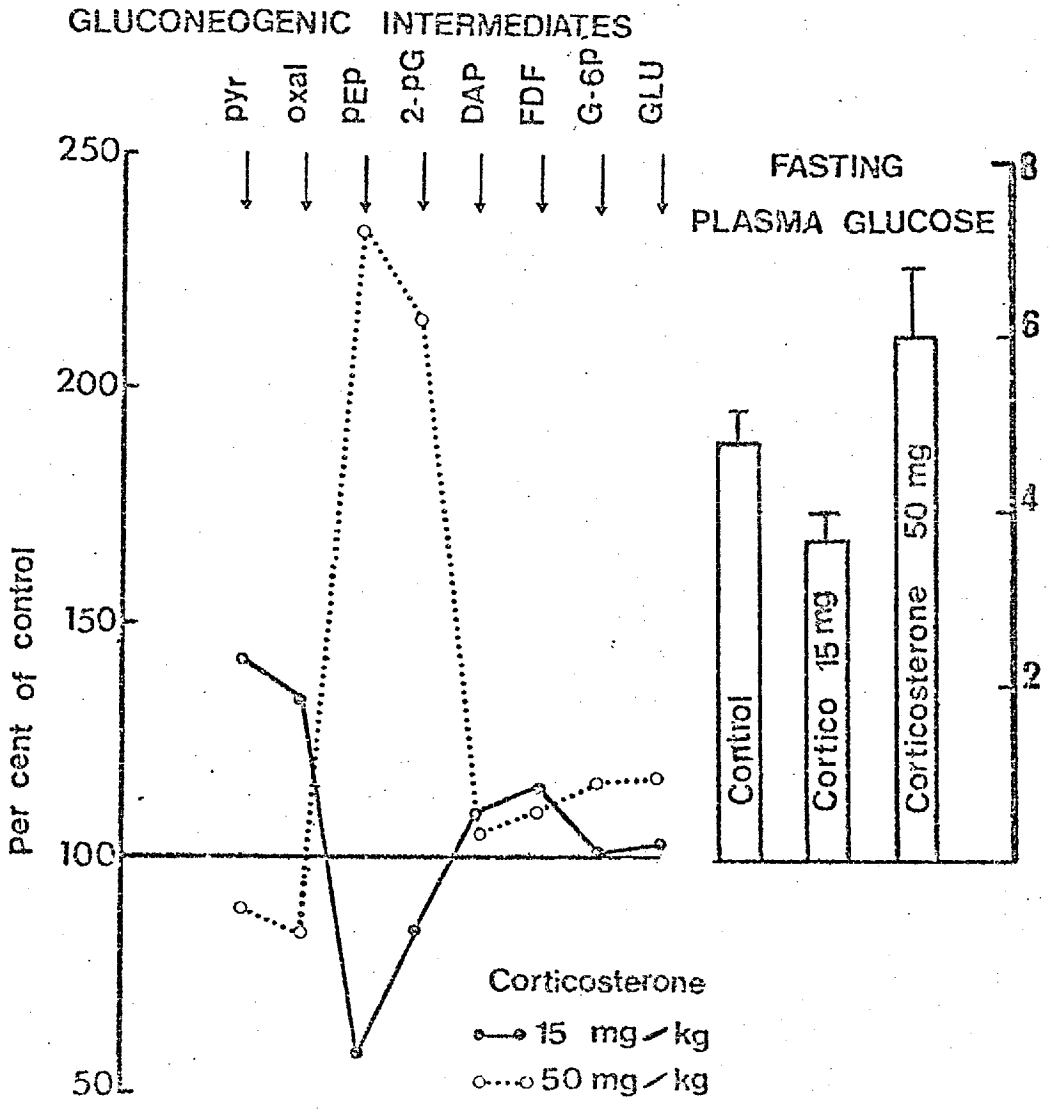


FIGURE (39)

Rats were treated with either 15 or 50 mgs of corticosterone/kg/day for 8 weeks. Livers were then extracted and assayed for gluconeogenic intermediates. Results are expressed as percentage of similar measurements performed in pair-fed control animals.
Each point represents the means of values obtained in 6 rats. (For abbreviations see legend to Table 21).

Table (22): Effect of Pharmacological Doses of Corticosterone on Hepatic Basal and Glucagon Stimulated c-AMP Content

Experimental Conditions	Basal c-AMP pmol/mg wet weight	Stimulated c-AMP pmol/mg wet weight
Pair-fed Control (n = 8)	0.37 ± 0.025	1.54 ± 0.1
Corticosterone 15 mg/kg (n = 6)	**0.25 ± 0.23	1.5 ± 0.17
Corticosterone 50 mg/kg (n = 6)	*0.65 ± 0.05	*2.3 ± 0.04

Corticosterone was solubilised in corn oil and given as daily intramuscular injection for 8 weeks. Basal c-AMP content was determined in liver of overnight fasted rats. Stimulated levels of c-AMP were estimated in liver samples obtained 30 sec. following the intraportal administration of glucagon (1 µg/kg).

Values are Means ± SEM. The probability value for the significance from the control was determined using the student "t" test.

n = number of animals in each experimental group.

** = significantly decreased from control ($P < 0.01$).

* = significantly increased above control ($P < 0.002$).

EFFECT OF CORTICOSTERONE ADMINISTRATION ON BASAL AND GLUCAGON-STIMULATED LIVER c-AMP CONTENT

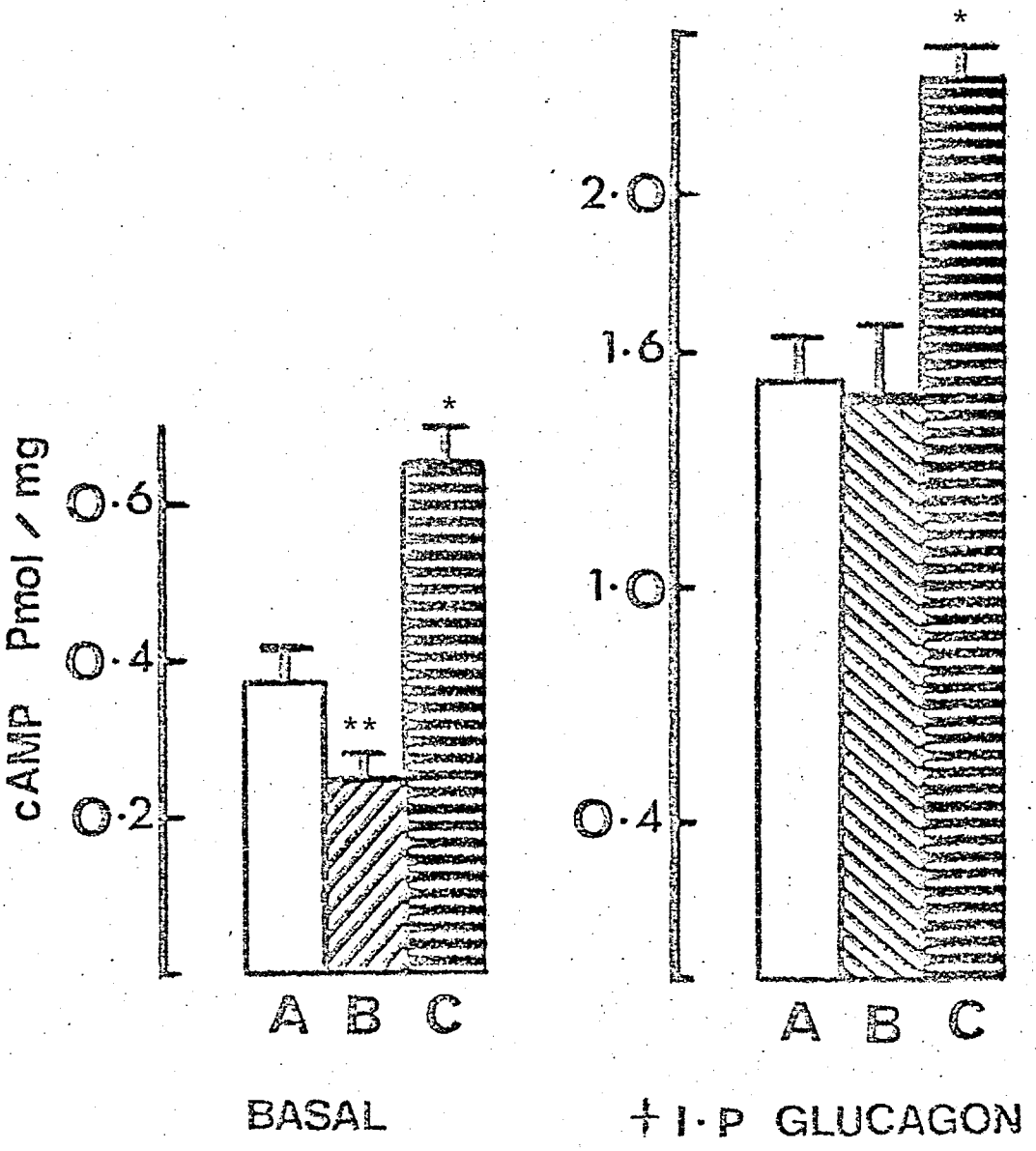


FIGURE (40)

Animals were treated with either 15 or 50 mgs of corticosterone/kg/day for 8 weeks.
A = pair-fed control, B = animals treated with corticosterone 15 mg/kg/day and C = the corticosterone dose administered was 50 mg/kg/day. Hepatic c-AMP was measured under basal conditions and following glucagon stimulation as described in the legend to Figure (22). Bars represent Means and SEM of results obtained in 6 rats.
** = Significant decrease from pair-fed control ($P < 0.01$).
* = Significant increase from pair-fed control ($P < 0.002$).

D I S C U S S I O N

The results of the investigation presented in this section suggest that adrenocortical function has long term effects on hepatic enzymes regulating lipogenesis and consequently participates in the homeostatic control of plasma triglyceride levels. Thus adrenocortical hypofunction, as induced by adrenalectomy, was associated with a reduction in hepatic acetyl CoA carboxylase and fatty acid synthetase. Since these two enzymes play a key regulatory role and are probably rate determinant in mammalian hepatic lipogenesis, (Numa et al, 1961 and Chang et al, 1967), the activity of these two enzymes could be responsible for the reduction in serum triglyceride levels in the adrenalectomised animals. The restoration in enzyme activity and plasma triglyceride levels to within normal limits by low dose corticosterone suggest that normal adrenocortical function is essential for maintenance of rates of normal lipogenesis. In contrast, high dose corticosterone administration increased the activity of these enzymes and induced hypertriglyceridaemia, the latter effect having been observed in humans receiving high doses of corticosteroids (Doar & Wynn, 1970; Bagdade et al, 1970 and Kissebah, 1974).

For many years glucocorticoids have been thought of as important inducers of hepatic enzyme synthesis. Thus adrenocortical steroids were found to stimulate hepatic RNA polymerase activity (Barnabei et al, 1965) as well as synthesis of RNA (Feigelson et al, 1965) and certain

enzyme proteins (Kenney, 1962; Feigelson & Greengard, 1962). Hence, it has been suggested that these hormones act on specific chromasomal loci to stimulate the formation of nuclear (messenger) RNA which serves as a template for the synthesis of these enzymes.

The currently accepted model suggesting that glucocorticoids act by influencing gene expression is a powerful one. Several recent reviews have been published to list many of the glucocorticoid induced enzyme systems in a variety of body tissues (Rosen & Nichol, 1963; Pitot & Yatvin, 1973 and Gelehrter, 1973). A well studied example of such a mechanism is demonstrable in the regulation of hepatic tyrosine aminotransferase. Both in vivo (Kenney, 1962) and in tissue culture (Granner, 1968), it has been shown that glucocorticoids increase the rate of synthesis of this enzyme. Chemicals which block RNA or protein synthesis prevent induction of the transaminase, but agents which only prevent DNA synthesis do not (Peterkafsky & Tomkins, 1967). Moreover, incubation of tissue culture cells with glucocorticoids in the presence of a protein synthesis blocker such as cyclohexamide or puramycin cause an accumulation in RNA, and rapid increase in the enzyme activity on removing the blocker from culture media (Peterkafsky, 1967).

As shown in the present study, a similar mechanism might mediate the effects of glucocorticoids on liver lipogenic enzymes. Evidence from isolated perfused rat liver (Klausner & Haimberg, 1967) seems to support our conclusion. Thus the hepatic release of triglycerides was reduced in livers of adrenalectomised rats and was

increased when the rats were given glucocorticoids prior to their sacrifice. More interestingly, however, is the observation that the exposure of liver to glucocorticoids in vitro also enhanced the hepatic production of triglycerides, suggesting a direct effect of the steroid on liver metabolism.

It is not certain, however, how an increase in hepatic lipogenesis, as seen in our rats, could contribute to the hypertriglyceridaemia seen in patients receiving glucocorticoid therapy since in man plasma triglyceride fatty acids are mainly derived from plasma FFA (Boberg et al, 1972). Glucocorticoids increase the plasma flux of FFA and this provides more readily available source of fatty acids for hepatic triglyceride synthesis. Indeed, studies in human subjects receiving glucocorticoids and on normal dietary intake (Kissebah, 1974) have shown a close correlation between the rate of plasma FFA flux and the magnitude of elevation in plasma triglycerides. On the other hand, recent studies in man (Barter et al, 1972; Barter & Nestel, 1973), have indicated that during the intake of a carbohydrate rich diet hepatic lipogenesis from glucose serves as the major source of triglyceride fatty acids. Under these dietary conditions, an increase in hepatic lipogenic enzymes, as seen during glucocorticoid therapy, could lead to increased production of triglycerides and hypertriglyceridaemia.

The effects of glucocorticoids on phosphoenolpyruvate

carboxykinase (PEPCK) activity and gluconeogenic intermediates on the other hand, suggest that not all glucocorticoid effects can be explained by altered gene expression and that some other mechanisms must be involved. Phosphoenolpyruvate carboxykinase (PEPCK) is an important key regulatory enzyme in the control of gluconeogenesis and hepatic glucose release. The activity of this enzyme is effected via an induction process which is determined by the intracellular cyclic-AMP level (Wicks et al, 1969). The paradoxical changes in hepatic c-AMP and the activity of this enzyme seen with pharmacological doses of corticosterone are not consistent with the view regarding these effects to be direct consequences of glucocorticoid action on the liver. Alternatively, these changes could be in parallel with the alterations in insulin and glucagon secretion or action associated with the intake of glucocorticoids. Insulin and glucagon are known to exert antagonistic effects on hepatic c-AMP concentration and hence the ratio between the two hormones in the portal vein blood regulates the activity of PEPCK and consequently the rate of glucose production (Parrilla et al, 1974).

As shown in the present study glucocorticoids appear to modulate the activity of pancreatic beta and alpha cells to alter their secretion of insulin and glucagon. Adrenalectomy lead to a significant fall in basal and glucose stimulated insulin levels while large doses of corticosterone (2-10 times the replacement dose) enhanced

insulin secretion. These changes are similar to those seen in man, showing an increase in peripheral venous blood levels of insulin following the administration of glucocorticoids (Perley & Kipnis, 1966) and in patients with Cushing's Syndrome (Klink & Estrich, 1964)

The demonstration of increased insulin levels in portal vein blood of glucocorticoid treated rats indicate that the effects of the steroid were due to enhanced insulin release rather than a decrease in the hepatic extraction or degradation of the hormone. Indeed, experiments in guinea pigs, rats and rabbits (Kinash & Haist, 1954; Volk & Lazarus, 1959; Vranic, 1965) have shown that cortisol administration results in hyperplasia of the Islets of Langerhans and enhances the release of insulin from pancreatic tissue incubates. On the other hand, the addition of glucocorticoids to pancreatic tissue in vitro did not influence the rate of insulin secretion suggesting an indirect effect of glucocorticoids on beta cell function.

As in man (Wise et al, 1973; Marco et al, 1973) the administration of glucocorticoids to rats increased both basal and amino acid stimulated glucagon levels. On the other hand, adrenalectomy did not affect glucagon levels suggesting that physiological concentrations of the steroid do not influence the secretory capacity of the alpha cells while large doses enhance glucagon secretion. The insulin to glucagon molar concentration ratio in portal vein blood (I/G) was, therefore, decreased in the

adrenalectomised rats but was markedly increased following glucocorticoid therapy.

Bilateral adrenalectomy leads to a decrease in PEPCK activity with accumulation of pyruvate and oxaloacetate relative to phosphoenol pyruvate indicating inhibition of gluconeogenesis at this step. These results confirm previous findings (Klausner & Heimberg, 1967) showing decreased glucose synthesis in liver of adrenalectomised animals and explain the original observation of Houssay and his colleagues (Houssay et al, 1947) demonstrating a fall in fasting blood sugar in such animals. Adrenalectomy also decreased the basal hepatic c-AMP level consistent with the inhibition of PEPCK. The impairment in the nucleotide response to exogenously administered glucagon suggests that adrenalectomy also decreased the hepatic response to this hormone, and consequently enhanced the biological effectiveness of the small levels of circulating insulin. The changes in hepatic c-AMP and gluconeogenic enzymes were therefore similar to an 'excess insulin' pattern despite a decrease in the I/G ratio in portal vein blood.

Moderate doses of corticosterone (2-3 times replacement dose) decreased hepatic c-AMP, PEPCK activity and produced a fall in fasting plasma glucose. The inhibition of gluconeogenesis in these animals could, therefore, be due to the increased I/G ratio in portal vein blood. This 'insulin excess' pattern is similar to that seen in rats

treated with oestradiol in this study. In contrast to the oestradiol therapy where the increase in I/G ratio was due to the marked suppression of glucagon secretion, in the corticosterone treated rats the increase in this ratio was secondary to enhanced insulin secretion.

Animals given larger doses of the steroid, on the other hand, had increased basal c-AMP levels and an exaggerated response to glucagon despite doubling of the I/G ratio. These results suggest that large doses of the steroid could lead to a decrease in the biological effectiveness of circulating insulin and hence result in the stimulation of PEPCK and gluconeogenesis irrespective of the hyperinsulinaemia. The fasting plasma glucose in these animals was, therefore, higher than normal. The mechanism whereby large doses of glucocorticoids could modulate the hepatic responsiveness to insulin remains uncertain. Olefsky et al (1975) have recently reported that large doses of dexamethasone decreased the binding of insulin to its specific receptors in isolated hepatocytes suggesting that glucocorticoids might decrease the affinity of the insulin receptor towards the hormone. However, an effect of glucocorticoids on the adenyl cyclase system cannot be excluded.

In conclusion, the present investigation demonstrates a complex mechanism mediating the effects of glucocorticoids on liver lipid and carbohydrate metabolism. Glucocorticoids induce the activities of acetyl CoA carboxylase and fatty acid synthetase and hence participate in the control of hepatic

lipogenesis and plasma triglyceride concentation. Glucocorticoids, on the other hand, exert pronounced effects on insulin and glucagon secretion, and at the same time modify the liver cell response to the peptide hormones. The consequent changes in phosphoenolpyruvate carboxykinase activity and gluconeogenesis determines the rate of hepatic glucose release.

SECTION 3

GENERAL DISCUSSION AND CONCLUSIONS

The advance in steroidal hormone therapy is almost certainly part of the unbelievable progress achieved in medical sciences within recent years. Nevertheless, one of the major disappointments in this field is the numerous side effects encountered during long term administration of steroidal agents. Among these, perhaps, the most sinister of all is the increased cardiovascular morbidity in otherwise "low risk" individuals.

Alteration in lipid and carbohydrate metabolism are features well documented in patients receiving steroidal preparations and may play an important role in the pathogenesis of the cardiovascular abnormalities. At present, one of the problems causing tremendous interest is the uncertainty concerning the biochemical events by which steroids could lead to the metabolic aberration. Such information could be of the utmost importance in deciding upon the future use of these agents and in stimulating the development of new preparations with minimal side effects.

In the present thesis, an attempt has been made to reveal some of the mechanisms underlying the metabolic effects of female gonadal steroids and glucocorticoids. These agents and their synthetic analogues are now employed as the main constituents of oral contraceptives and immunosuppressive preparations.

To alleviate any bias introduced by alterations in the composition of the steroid as in some synthetic

analogues, the experiments in this study were performed using the naturally occurring hormones (17-β-oestradiol, progesterone and corticosterone). Furthermore, the doses of each steroid administered per kg body weight was chosen to cover the pharmacological range of equivalent preparations used in human subjects. Also pair-fed controls were used during the whole period of the study to distinguish the effects of the steroid independent from alterations in the feeding habits of the animals occurring during the period of therapy.

The initial objective was to select a representative animal model in which the steroidal metabolic effects commonly observed in humans could be reproduced experimentally. As shown in the present study pharmacological doses of 17-β-oestradiol or corticosterone produced a dose dependent rise in fasting plasma triglyceride concentration. Oestradiol and moderate doses of corticosterone also caused a fall in fasting plasma glucose while large doses of corticosterone lead to fasting hyperglycaemia. Progesterone, on the other hand, had no significant effects. These changes were similar to those observed in human subjects receiving pharmacological doses of equivalent preparations and hence justify the extrapolation of observations made in this animal model to the human situation.

Though steroidal hormones are well known to have many effects upon peripheral tissue metabolism, there is now abundant evidence both in man and experimental animals to suggest that the derangement in plasma triglycerides

and glucose levels originates predominantly from their effects on liver metabolism. From the results in this thesis it is also evident that steroids could modulate the activity of liver lipogenic and gluconeogenic rate limiting enzymes correlating with the changes in fasting plasma triglycerideand glucose concentrations.

The long term treatment of rats with 17-β-oestradiol enhanced the activity of liver acetyl CoA carboxylase and fatty acid synthetase, an effect which was dose dependent and was parallel to the rise in plasma triglyceride level. Bilateral adrenalectomy reduced the activity of liver lipogenic enzymes and led to a fall in plasma triglyceride concentration. Pharmacological doses of corticosterone, on the other hand, stimulated the activity of these enzymes and caused an increase in plasma triglycerides.

The fall in fasting plasma glucose observed during 17-β-oestradiol therapy was associated with the inhibition of phosphoenolpyruvate carboxykinase (PEPCK) enzyme and a decrease in the flow of gluconeogenic intermediates through this enzymatic step. Bilateral adrenalectomy also resulted in a decrease in phosphoenolpyruvate carboxykinase and reduction of fasting plasma glucose. Moderate doses of corticosterone administered to rats inhibited phosphoenolpyruvate carboxykinase activity and caused a decrease in fasting plasma glucose concentration. The fasting hyperglycaemia observed in rats treated with large doses of corticosterone, on the other hand, was associated

with enhanced phosphoenolpyruvate carboxykinase activity and increased flow of gluconeogenic intermediates through this step.

During the administration of progesterone to rats no significant changes were observed in the activity of liver lipogenic or gluconeogenic enzymes. Progesterone, therefore, did not influence the fasting plasma triglyceride or glucose concentrations.

The mechanisms underlying the changes in liver lipogenic and gluconeogenic enzymes occurring during steroidal hormone therapy are not yet certain. At least two main mechanisms have been proposed.

First: Steroids might directly influence the synthesis and/or degradation of liver enzymes and consequently determine their concentration and/or activity in liver cells.

Second: Steroids might affect the secretion and/or actions of some other hormones which in turn could modulate the activity of liver enzymes. Of particular interest in this respect are insulin and glucagon, since these hormones are known to play an important role in the regulation of hepatic lipogenesis and gluconeogenesis and their levels in blood seem to be influenced by the exposure to steroid hormones.

There is now sufficient evidence to indicate that oestrogen and glucocorticoids cause the induction of some enzymes and that this effect might mediate important functions of these hormones. Thus in mammalian liver

cells, oestradiol induces the activity of several enzymes including tryptophan oxygenase, tyrosine aminotransferase and alanine aminotransferase. Glucocorticoids seem also to enhance the activity of these enzymes. Indeed, the studies of Braidman and Rose (1971) have suggested that the effects of oestrogen on these enzymes might be mediated by changes in the adrenocortical function in response to oestrogen therapy. This proposal has been further extended by Wynn et al (1972) and Afolabi (1974) to suggest that the effects of oestrogen on hepatic lipogenesis and plasma triglyceride concentration might occur via a similar mechanism involving the adrenal cortex. Their view has been based upon the following findings.

First: The administration of oestrogens increases the adrenal gland weight in rats (Afolabi, 1974) and enhances the secretion rate of cortisol in humans (Burke, 1969).

Second: The long term administration of oestrogenic preparations or glucocorticoids to humans or animals produces similar changes in plasma triglycerides and other metabolites (for references see Introduction).

Third: In adrenalectomised rats the effects of oestrogen on plasma triglyceride concentration were no longer discernible (Afolabi 1974).

The results in the present study showed a reduction in maximal activity of liver lipogenic enzymes in adrenalectomised rats, which was restored to within

normal range by the administration of corticosterone in replacement doses. These findings suggest that the adrenal cortex might exert a permissive function in maintaining the normal activity of these enzymes. Furthermore, the similarities in the responses of plasma triglycerides and liver lipogenic enzymes induced by either 17-β-oestradiol or corticosterone (Figure 41) do support, but do not establish, the view regarding some of the effects of oestradiol to operate via the adrenal cortex.

In order to reveal the role of altered pancreatic function beta and alpha cell in the metabolic changes associated with steroid therapy we have examined the effects of 17-β-oestradiol, progesterone and corticosterone on insulin and glucagon levels in portal vein blood. Though there has been a tremendous amount of literature concerning the effects of these steroids on insulin and glucagon secretion, none of these studies have been conclusive since hormonal measurements were performed in samples obtained from peripheral venous blood. The entire pancreatic production of hormones has to traverse the liver before reaching the peripheral circulation. Therefore, alteration in the rate of hepatic uptake or degradation as induced by some steroids can produce marked changes in their peripheral level independent of changes in their secretion. To obtain more precise information concerning the basal secretion of these hormones, the studies in this thesis were performed on samples of blood

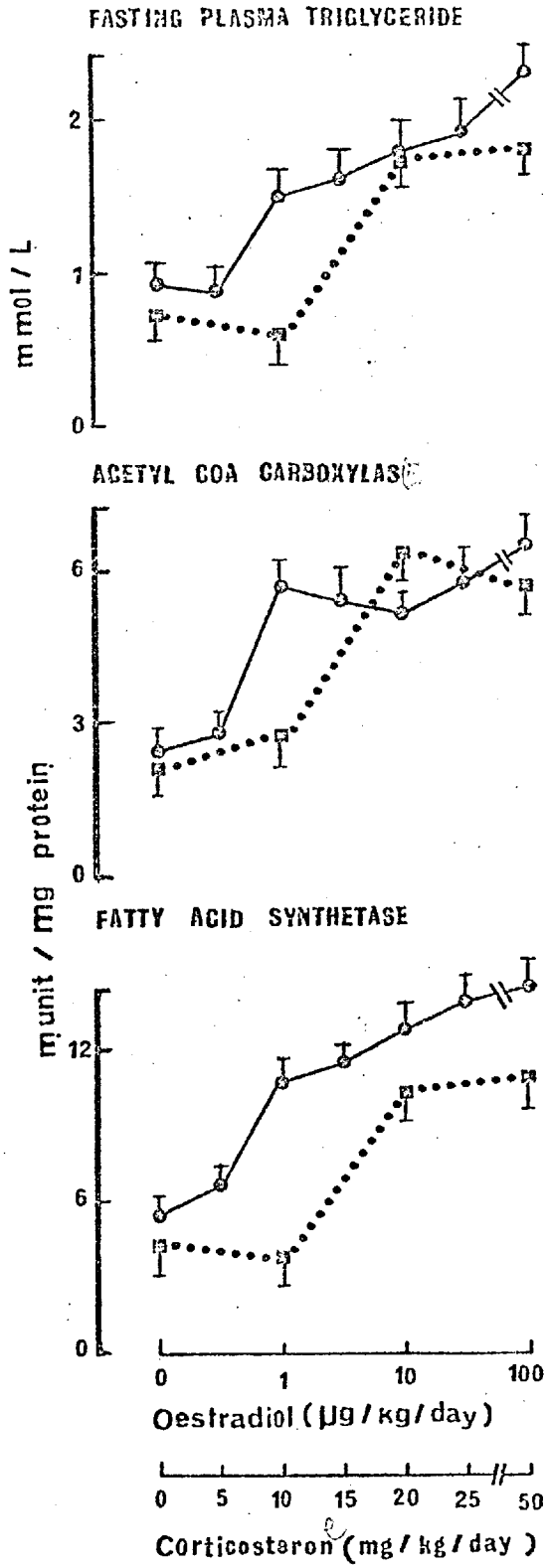

FASTING PLASMA TRIGLYCERIDE
m mol / L
2
1
0
ACETYL COA CARBOXYLASE
m unit / mg protein
6
3
0
FATTY ACID SYNTHETASE
12
6
0
0
1
10
100
Oestradiol (µg / kg / day)
0
5
10
15
20
25
50
Corticosteron (mg / kg / day)

FIGURE (41)

obtained from the main portal vein. Furthermore, the secretory capacity of alpha and beta cells were investigated following maximal stimulation by determining the hormonal levels following infusion of either alanine or glucose respectively.

The data in the present investigation suggest that oestrogen and progesterone modulate the activity of alpha and beta cells of the pancreas to alter their release of insulin and glucagon and that the effects of the two steroids are antagonistic. Oestrogen inhibited the activity of alpha and beta cells while progesterone stimulated both cells. Oestrogen had a more suppressive effect on the alpha cell than the beta cell resulting in a greater reduction in glucagon than insulin levels. In oestradiol treated rats, therefore, the insulin : glucagon molar concentration ratio (I/G) in portal vein blood was increased 2-3 times higher than that of control rats. Progesterone, on the other hand, proportionately increased the activity of both alpha and beta cells stimulating the secretion of insulin and glucagon to the same magnitude and hence the I/G ratio remained unaltered.

Adrenocortical hormones also seem to influence the function of both beta and alpha cells, though the former is more sensitive to the actions of this steroid. Thus bilateral adrenalectomy caused a decrease in basal and glucose stimulated insulin level, while glucagon concentrations were unchanged resulting in a decrease in the I/G ratio. Pharmacological doses of corticosterone,

It is unlikely that the changes in pancreatic hormone levels were secondary to alterations in splanchenic blood flow since the changes in insulin and glucagon induced by steroids were not of equal magnitude.

on the other hand, enhanced the secretion of both insulin and glucagon. Insulin levels were disproportionately higher than glucagon and hence I/G ratio was increased by glucocorticoid therapy.

Though both oestradiol and corticosterone increased the portal vein I/G ratio the changes in the absolute concentration of pancreatic hormones induced by each steroid were contradictory (Figure 42). These findings suggest that it is unlikely for the effects of oestradiol on pancreatic function to be mediated via the adrenal cortex. Indeed, there is now evidence to indicate the presence of a specific receptor protein in mammalian pancreas which could bind with high affinity to oestrogens suggesting that this steroid might have direct actions on islet tissue (Avery et al 1973).

To assess the effects of steroidal hormones on the hepatic responsiveness to pancreatic hormones, the <u>in vivo</u> increment in hepatic c-AMP to the intraportal administration of glucagon was assessed. The nucleotide level in this tissue is normally regulated by the balance between the rates of synthesis by the adenyl cyclase and its degradation via the activity of phosphodiestrase. These enzymes are activated and/or inactivated in response to the binding of pancreatic hormones to liver cells. The level at any particular moment would, therefore, be an adequate representative of the balance in the biological effectiveness of insulin and glucagon (see Introduction).

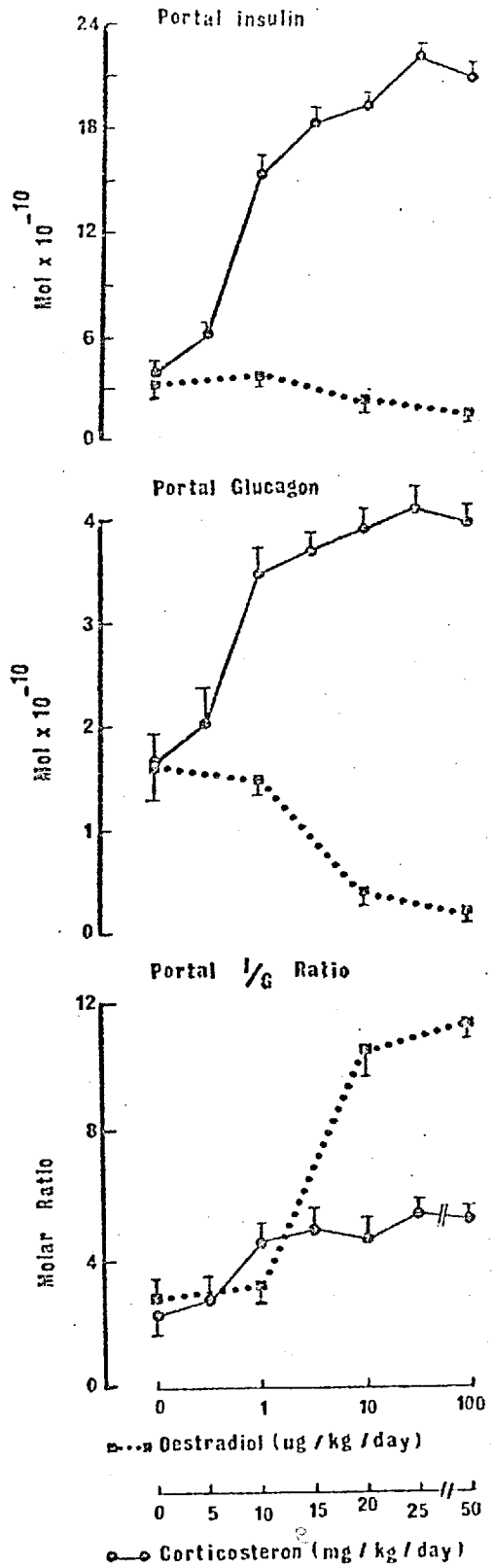

Portal insulin
24
18
12
6
0
Mol x 10^-10
Portal Glucagon
4
3
2
1
0
Mol x 10^-10
Portal I/G Ratio
12
8
4
0
Molar Ratio
0
1
10
100
Oestradiol (ug / kg / day)
0
5
10
15
20
25
50
Corticosteron (mg / kg / day)

FIGURE (42)

It is evident from the results in this study that treatment of rats with 17-β-oestradiol decreases the biological effectiveness of glucagon. The glucagon stimulated c-AMP levels were lower in livers obtained from rats receiving oestradiol. Similarly, corticosterone deficiency as induced by adrenalectomy led to a reduction in glucagon stimulated c-AMP content. On the other hand, large doses of corticosterone (10 times the replacement dose) lead to an exaggerated c-AMP response to glucagon suggesting an increased resistance to the antagonistic hormone (insulin). Progesterone and moderate doses of corticosterone (5 times the replacement dose) did not affect the sensitivity of liver cells to pancreatic hormones.

But how could changes in the secretion and/or actions of pancreatic hormones be held responsible for the altered hepatic metabolism during steroid therapy?

Treatment of rats with oestradiol increased I/G molar concentration ratio in blood perfusing the liver. Oestradiol also impaired the sensitivity of liver cells to glucagon and augmented the biological effectiveness of insulin. Hence the basal c-AMP content in livers of oestradiol treated rats was lower than in pair-fed controls. These alterations can provide an additive and/or an alternative mechanism to explain the enhancement in the activity of lipogenic enzymes in rats receiving oestradiol. However, the altered secretion and action of pancreatic hormones and the consequent reduction in c-AMP level might also account for the inhibition of phosphoenolpyruvate

carboxykinase and gluconeogenesis since the nucleotide operates as an important regulator of this enzyme.

Progesterone increased the secretion of insulin and glucagon equally and hence the I/G ratio remained unaltered. No significant changes were observed in the sensitivity of liver cells to this hormone. The c-AMP content of livers obtained from progesterone treated rats was similar to that of controls. Progesterone therapy, therefore, did not affect the activity of liver enzymes.

Though bilateral adrenalectomy lowered the I/G molar concentration ratio in portal vein blood, liver tissue from these rats was resistant to the action of glucagon. Consequently the biological effectiveness of circulating insulin was increased resulting in a decrease in basal hepatic c-AMP and inhibition of phosphoenolpyruvate carboxykinase activity and gluconeogenesis.

Moderate doses of corticosterone (2-5 times the replacement dose) increased the I/G ratio without alteration in the hepatic response to glucagon. The excess insulin level could then be responsible for reduced basal c-AMP content and the inhibited phosphoenolpyruvate carboxykinase. Large doses of corticosterone (5-10 times the replacement dose), on the other hand, appear to impair the hepatic effectiveness of insulin to antagonise glucagon on hepatic function, consequently the hepatic basal c-AMP content was higher than in pair-fed control rats. Hepatic phosphoenolpyruvate carboxykinase activity and gluconeogenesis were, therefore,

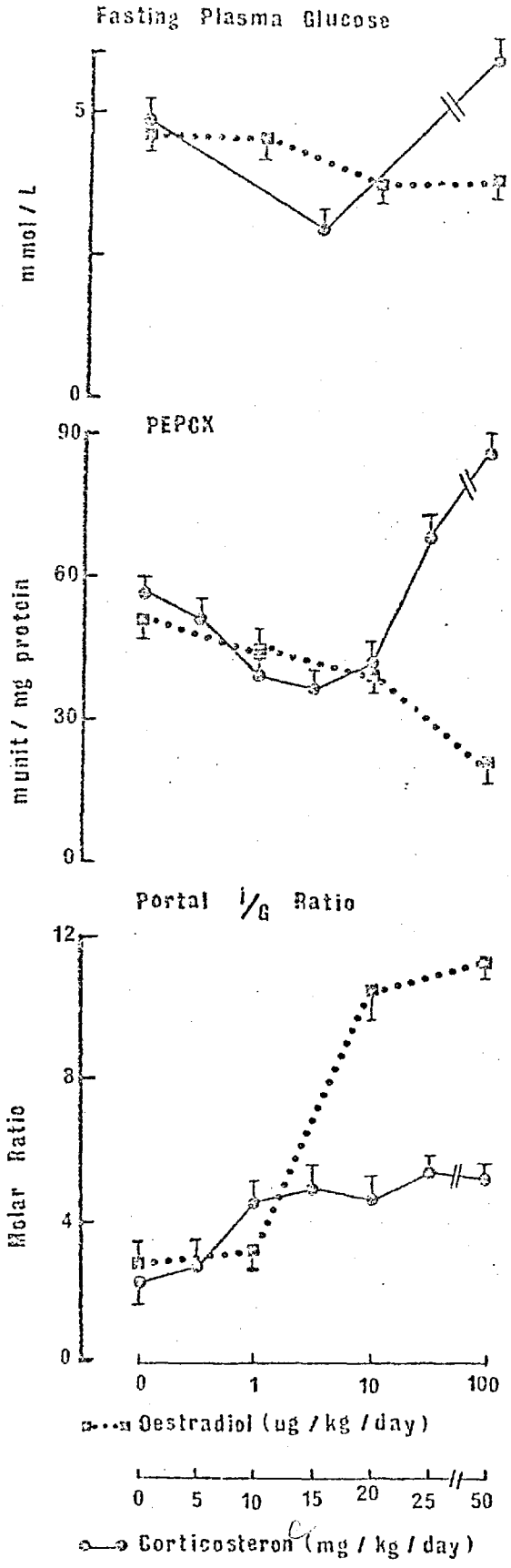

Fasting Plasma Glucose
mmol / L
5
0
PEPCK
munit / mg protein
90
60
30
0
Portal I/G Ratio
Molar Ratio
12
8
4
0
0
1
10
100
Oestradiol (ug / kg / day)
0
5
10
15
20
25
50
Corticosteron (mg / kg / day)

FIGURE (43)

enhanced despite the marked hyperinsulinaemia. These results could satisfactorily account for the paradox between the changes in I/G ratio on the one hand, and the alterations in phosphoenolpyruvate carboxykinase on the other hand (Figure 43).

Finally, the conclusions from the present study suggest that part of the effects of steroidal hormones on lipid metabolism might be due to their direct actions stimulating the activity of rate limiting lipogenic enzymes in the liver. Steroids also produce changes in pancreatic alpha and beta cell functions resulting in alterations in insulin and glucagon levels perfusing the liver. Further, some of the steroids appear to modulate the hepatic response and modifying the biological effectiveness of insulin and glucagon. The changes in the secretion and/or actions of the pancreatic hormones might provide a mechanism to explain some of the alterations in gluconeogenic enzymes and hepatic glucose production. Other mechanisms have also to be considered. The process by which steroids could influence the secretion and/or actions of pancreatic hormones remains obscure.

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PUBLICATIONS FROM THIS THESIS

1. Gonadal Steroid Effects on Plasma Lipoproteins: Alteration in Insulin to Glucagon Molar Ratio.
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2. Mechanism of Oestrogen and Progesterone Effects on Carbohydrate and Lipid Metabolism.
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3. Mechanism of Oestrogen and Progesterone Effects on Lipid and Carbohydrate Metabolism: Alteration in Insulin to Glucagon Ratio and Liver Enzyme Activity.
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4. Mechanism of Glucocorticoid Effects on Lipid and Carbohydrate Metabolism in the Rat: Role of Insulin and Glucagon.
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