EFFECT OF OVARIAN STEROID HORMONES ON CARBOHYDRATE METABOLISM IN SKELETAL MUSCLE

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A thesis submitted for the Degree of Doctor of Philosophy

The University of Aston in Birmingham

October 1983

For my mother

ACKNOWLEDGEMENTS

I would like to express my sincere thanks to my supervisor Dr. C.J Bailey for his advice, expert guidance and helpful criticisms.

I am also grateful to Dr. T Atkins for advice on Biochemistry, Mrs. Susan Turner for technical assisstance and to John for his help and continous encouragement during the preparation of this thesis.

The financial support of the British Council is gratefully acknowledged.

ABBREVIATIONS

KRB	Krebs Ringer Bicarbonate
TCA	Tricarboxylic acid
BSA	Bovine serum albumin
EDTA	Ethylenediamine-tetracetic acid
LH	Luteinising hormone
FSH	Follicle stimulating hormone
OVX	Ovariectomised
E	Oestradiol
P	Progesterone
P/O	Proestrus/oestrus
M/D	Metoestrus/dioestrus

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Effect of ovarian steroid hormones on carbohydrate metabolism in skeletal muscle

Ovarian steroid hormones play an important role in the regulation of carbohydrate metabolism, particularly at the level of the skeletal muscle. This thesis examines the mechanisms involved, using the female mouse soleus muscle. Groups of bilaterally ovariectomised mice were treated orally for 10 weeks with oestradiol 17β(5 μg/kg/day) and progesterone(1 mg/kg/day) alone and combined. Intact and ovariectomised mice treated with placebo were also studied. Ovariectomy induced insulin resistance and reduced insulin receptor binding. Using ^{14}C-glucose, 3-O-methyl-[U-^{14}C] glucose and 2-deoxy-[1-^{14}C] glucose, it was observed that glucose uptake, phosphorylation, aerobic glycolysis and glycogenesis were impaired by ovariectomy both under basal (in the absence of added insulin) and insulin-stimulated (0.75 mU/ml) conditions. These effects were partially or totally prevented by the oestradiol and progesterone therapies, particularly oestradiol alone. Effects of ovarian steroids at post-receptor sites of insulin action might be attributable in part to actions on gluco-regulatory enzymes. The activities of hexokinase (EC 2.7.1.1), the active I form of glycogen synthase (EC 2.4.1.11) and 2-oxoglutarate dehydrogenase (EC 1.2.4.2) were increased by oestradiol in the presence of insulin, but progesterone did not significantly influence the activities of these enzymes. Progesterone also partly antagonised the effects of oestradiol. To examine the effect of ovarian steroids on the outcome of experimental diabetes, ovariectomised streptozotocin-treated (200 mg/kg) diabetic female mice were treated orally for 10 weeks with oestradiol (5 and 500μg/kg/day) and progesterone (1mg/kg/day). Compared with intact mice, ovariectomy increased the rate of onset and the severity of hyperglycaemia and increased the weight loss induced by streptozotocin. Oestradiol opposed these effects in a dose dependent manner. Progesterone also reduced the extent of hyperglycaemia. The protective effect of ovarian steroids correlated with the extent to which they prevented the fall in plasma insulin and pancreatic insulin content after streptozotocin administration. The effect also correlated with the capacity of the steroids to increase insulin-stimulated glucose uptake into soleus muscle. These studies suggest that ovarian steroid hormones influence glucose metabolism in soleus muscle of female mice by effects at receptor and post-receptor sites of insulin action. Oestradiol exerted prominent effects on several key gluco-regulatory enzymes. Ovarian steroid hormones reduce the severity of streptozotocin diabetes by increasing pancreatic insulin, raising plasma insulin concentrations and enhancing peripheral glucose uptake.

Key words : Ovariectomy oestradiol progesterone
glucose metabolism soleus muscle

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CHAPTER 1

INTRODUCTION

Carbohydrate metabolism forms a vital facet of energy production in the mammal. Glucose is the principal sugar involved, and a complex network of regulatory mechanisms accommodates the fluctuations in nutrient supply and energy demand, to control the availability of glucose for storage and metabolism within the tissues (1). Blood glucose concentrations are normally maintained within close limits. A minimum concentration of 2-3 mmol/l ensures a constant supply of glucose for tissues such as brain and renal medulla that are especially dependent upon this energy source. A maximum concentration of 8-10 mmol/l (in man) prevents the pathological consequences seen in diabetes mellitus (2). Glucoregulation is achieved through the interaction of gluconeogenesis, glycogenesis, glycogenolysis, and aerobic and anaerobic pathways of glucose utilisation (3,4). Both nervous and hormonal events serve to regulate these metabolic processes as described in detail elsewhere (5). In this respect, insulin is recognised as the most important hormone controlling the complex network of metabolic pathways (6,7) Its main function is to maintain plasma glucose at or near normal levels (8-10 mmol/l) by regulating carbohydrate metabolism in insulin-sensitive tissues, particularly skeletal muscle which is a major site of glucose utilisation (8). The action of insulin is known to be modulated by other hormonal secretions, notably glucagon, growth hormone, corticosteroids and ovarian steroid hormones. This thesis will focus on the influence of ovarian steroid hormones on insulin-mediated glucose metabolism in skeletal muscle, looking especially at the cellular mechanisms involved.

Diabetes mellitus describes a heterogenous assembly of disorders in which blood glucose concentrations exceed the normal range. Lesions of both insulin secretion and insulin action

have been observed in the different types of diabetes. Since the effects of ovarian steroid hormones on carbohydrate metabolism have been shown to influence the course of diabetes mellitus, this aspect is also considered herein.

1.1 Literature Review

This chapter presents a review of the pertinent scientific and medical literature concerning female sex steroids and carbohydrate metabolism, with special reference to studies on glucose metabolism in skeletal muscle.

The ovarian sex steroids, oestrogens and progestogens have been shown to influence glucose homeostasis in the female (9-11). The levels of circulating insulin and pancreatic insulin content increase after treatment with these hormones, and during pregnancy when sex steroid titres are raised (12-17). The hyperinsulinaemia is caused by hypertrophy and hyperplasia of the islets of Langerhans, associated with an increase in the number of β cells (18, 19) and an increase in the extent of β cell granulation (20). In recent years, attempts have been made to define the effects of female sex steroids on carbohydrate metabolism with particular regard to oral contraceptive steroids. This was prompted by a large number of reports that some synthetic analogues of natural ovarian steroids used as oral contraceptives caused alterations in glucose tolerance. Exact characterisation of the changes induced by oral contraceptives and interpretation of their significance has proved difficult due to differences associated with the chemical nature of the drugs, effects of different combinations, doses, duration of treatment, mode of administration, physio-pathological condition of the recipients, the methods used to investigate carbohydrate metabolism and the criteria used to assess the results (11). Several excellent reviews on the influence of naturally occurring female sex steroids and their synthetic analogues on carbohydrate metabolism have been published (9, 10,

21,22). This review will attempt to provide an update on these reviews. Special reference will be made to glucose metabolism in muscle, including theories on the mechanism of sex steroid action on glucose metabolism.

Impairment of glucose tolerance and reduction or elevation of plasma insulin levels have been reported during oral administration of oestrogen-progestogen contraceptive preparations (23). There have been conflicting statements concerning the effects caused by these oral contraceptives on carbohydrate metabolism. Beck and Wells (24) reported that there was no effect while several other reports claim that there are abnormal blood glucose patterns (25-27). In general, the recent literature supports earlier indications of a diabetogenic stress among users of oral contraceptives. The extent to which glucose tolerance deteriorates appear to depend on the existing state of the pancreatic β cells :individuals with an adaptive ability to secrete more insulin show minimal changes in glucose tolerance whereas very occasionally, individuals with a compromised adaptive ability, notably potential diabetics, may develop subclinical diabetes (10). This acquired form of subclinical diabetes is in most cases, reversible after the contraceptive agents are discontinued (28,29). In subclinical diabetic women, contraceptive agents commonly precipitate overt diabetes, especially after treatment with combinations which include mestranol (30,31). This is usually accompanied by an endogenous insulin deficiency (24,32). Similar observations have been made in animals treated with mestranol (33). In women with overt diabetes mellitus, there is further deterioration in carbohydrate tolerance after exposure to oral contraceptives (34-36). Thus, it is evident that whereas naturally occurring ovarian sex steroids produce a generally beneficial effect on carbohydrate metabolism at physiological concentrations, the synthetic analogues used in contraceptive preparations have generally detrimental effects, especially in individuals with a predisposition to impaired glucose tolerance. Nevertheless, it should be noted that Gershberg (37) has demonstrated

an improvement in glucose tolerance in many maturity onset diabetics receiving oral contraceptive agents. Hence, under certain circumstances, in particular when low dose contraceptive preparations are used, some beneficial effects on carbohydrate tolerance may occur.

The mechanisms responsible for both the detrimental and beneficial effects of sex steroids on carbohydrate metabolism are still under speculation. In contrast to synthetic steroids which often impair carbohydrate metabolism, many investigators have reported that carbohydrate tolerance improved in a majority of their subjects (both diabetic and non-diabetic) during treatment with oestradiol or oestriol (38, 39). These results corroborate the earlier work of Houssay and his co-workers (22) who showed that oestradiol had an antidiabetogenic effect in diabetic rats. Additional investigations revealed an inhibitory effect of natural oestrogens on hepatic gluconeogenesis while accumulation of liver glycogen was concomitantly increased (40). Others have shown that oestrogens increase the sensitivity of skeletal muscle to insulin action (41, 42). Hence, it seems that natural oestrogens improve carbohydrate metabolism by reducing hepatic glucose production and increasing peripheral glucose utilisation by insulin-sensitive tissues.

Progesterone, in large doses (16 mg/kg body wt) caused glycosuria in partially pancreatectomised rats (43). However, the results of a recent in vivo study (44) confirmed earlier reports that treatment with progesterone at much lower doses led to an increase in the plasma insulin response to glucose in intact rats (14, 17). This stimulatory effect of progesterone on glucose induced insulin secretion was only small. Further work with progesterone is required especially on ovariectomised animals, to investigate the proposal that progesterone may oppose the hypoglycaemic action of insulin (45) possibly by antagonising the facilitation of glucose uptake conferred by oestrogens.

There is evidence to indicate that oestrogens and

progestogens can alter the peripheral action of insulin on glucose uptake by skeletal and diaphragm muscle (41, 42). Skeletal muscle forms an important link between in vitro and in vivo work on sex steroids. Animals can be pretreated with sex steroids and the muscle removed and examined in vitro for glucose uptake and glycogen deposition. The study by Shamoon and Felig (42) showed that short-term administration of oestradiol resulted in a significant increase in insulin-mediated glucose uptake by skeletal muscle. They suggest that oestrogens increase the sensitivity of skeletal muscle to insulin-stimulated glucose uptake rather than increasing endogenous insulin secretion as previously suggested (22, 37). Mckerns et al (41) investigated the effects of in vivo administered oestrogens on glucose metabolism in diaphragm muscle. They found that administration of ethinyl oestradiol for 12 days in rats increased glucose utilisation and glycogen formation in diaphragm muscle, especially in the presence of insulin. This is compatible with earlier reports suggesting that oestrogens have a protective effect on the development of diabetes (22). Furthermore, oestrogen synergism of insulin action has been indicated by a decreased insulin requirement in some diabetics receiving oestrogens.

It is of interest that the action of oestrogens on carbohydrate metabolism by uterine myometrium (smooth muscle) involves an oestrogen activated carrier-mediated system for glucose transport (46). Although there have been studies on progesterone and glycogen content in uterus and muscle (47, 48), there are no studies on progesterone and glucose uptake and metabolism in skeletal muscle.

Although the mechanism by which sex steroids alter carbohydrate metabolism has not been resolved, it is widely believed that metabolic alterations in the liver and muscle are important. Indeed, the metabolism, transformation and degradation of most steroids reside in hepatic tissue (49) and this tissue,

along with muscle play a major role in carbohydrate homeostasis (8).

Several mechanisms for the development of impaired glucose tolerance in oral contraceptive users have been postulated. The glucose intolerance in oral contraceptive users was thought to be due to growth hormone but this is now considered to be an unlikely mediator of the anti-insulin effects observed in many oral contraceptive users (23). There is speculation that the glucose intolerance might be caused by an elevation of cortisol levels(50), a relative deficiency of vitamin B6 (51) or altered tryptophan metabolism (52). There is also a possibility that synthetic sex steroids may promote adipose tissue resistance to insulin (53)., however, Gilmour and Mckerns (54) showed that natural hormones such as oestradiol enhanced glucose uptake by adipose tissue in rats.

Sex steroids may affect glucose metabolism in liver, muscle and other tissues and affect the secretory activity of endocrine glands that influence glucose homeostasis. These hormones are known to act by enzyme induction (55). There is evidence that the steroid hormone enters the cytoplasm of the cell by diffusion across the plasma membrane. It interacts with cytosolic receptors to form a hormone-receptor complex. This leads to an alteration in the structure of the receptor, and the complex enters the nucleus (56). The hormone-receptor complex activates a process of selective transcription resulting in the synthesis of enzyme.

The influence of sex hormones upon the incidence of diabetes has been demonstrated in white rats. Following subtotal pancreatectomy, (removal of about 95% of the gland), the frequency of diabetes is higher in male than in female rats (57). Ovariectomy increased the incidence of pancreatic diabetes and castration of male rats decreased it (58). The sex difference is due to the protective action of the ovary since restitution of the ovaries by means of a graft in ovariectomised animals decreased the frequency of diabetes induced by subtotal pancreatectomy (59). Administration

of diethylstilboestrol, oestrone and ethinyl oestradiol to alloxan diabetic rats resulted in a transient hyperglycaemia followed by a permanent protective action with normal values of glycaemia(60). The protective effect of oestrogens is thought to be due to increased pancreatic insulin concentrations (61) and compensatory hypertrophy of the islets of Langerhans (60).

It would appear, therefore, that natural oestrogenic substances have a beneficial influence on experimental diabetes by inducing hyperinsulinaemia, depressing hepatic glucose release, increasing peripheral glucose utilisation of substrate and generally protecting against diabetogenic stress. The action of progesterone remains uncertain. There may be synergism or antagonism between the oestrogens and progestogens in their various effects on glucose metabolism. The mechanism of action of sex steroids on carbohydrate metabolism remains speculative but hyperplasia of the pancreatic islet cells is involved. Sex steroids might affect glucose metabolism directly via enzyme induction in liver, skeletal muscle, uterus and other tissues. There is still speculation surrounding the effects caused by combinations of oestrogens and progestogens on carbohydrate metabolism. The effects of progesterone in particular, have not been examined in detail. Thus, the present study has been undertaken to examine the effects of ovarian sex steroids, singly and in combination on glucose metabolism in skeletal muscle.

CHAPTER 2

MATERIALS AND METHODS

2.1 Short review of *in vitro* methods for the study of glucose uptake and metabolism by skeletal muscle.

Insulin exerts a hypoglycaemic effect by promoting glucose uptake into certain tissues, primarily skeletal muscle. (62). This hormone also affects the metabolism of glucose within these tissues, partly as a consequence of enhanced glucose uptake and partly through its selective effects on certain metabolic activities within the cell. (61,62). Since skeletal muscle is a major site of insulin action and glucose metabolism (8), various experimental procedures both in vivo and in vitro have been developed to study glucose uptake and metabolism by this tissue. Particular attention has centred on *in vitro* methods since the isolated tissue offers greater potential and versatility for experimental manipulation, and provides selected specificity. Several comprehensive reviews of the early literature on these methods have been published (8, 63, 64). *In vivo* methods for studying glucose metabolism by muscle using the perfused human forearm (65-68) and perfused rat hind limb (69, 70) have been described. These methods offer certain advantages for the study of dynamic aspects of glucose uptake and metabolism under conditions very close to the normal physiological state. However, there are several limitations of in vivo perfusion methods. In particular, these methods lack tissue specificity and there are difficulties in the quantitation and interpretation of the data that they provide (66, 68). In view of these disadvantages, together with the added problem of the size of most laboratory animals, *in vivo* methods were not considered for the present investigation. This review is restricted to recent methodological developments for studying glucose uptake and metabolism in muscle tissues *in vitro*.

The choice of muscle tissue is especially important in in vitro experiments since the validity of these experiments will depend on the selection of a tissue that is representative of the majority of skeletal muscles in the body. In addition, the tissue should ideally show optimum metabolic performance in an artificial (in vitro) environment. The majority of in vitro investigations have used muscles isolated from rodents (71-73). Most of the early in vitro studies on skeletal muscle used the isolated rat diaphragm. After the pioneer studies by Meyerhoff and Himvich (74), the isolated rat diaphragm became the standard preparation for in vitro studies on glucose uptake (71-73). This preparation has the advantage that the muscle is thin and therefore easily oxygenated; it is available in comparatively large amounts; it can be excised very rapidly and divided into two similar segments (left and right hemidiaphragms), one of which can serve as the test preparation and the other as control (75). However, several shortcomings of this preparation have been recognised. Removal of the diaphragm entails cutting the muscle fibres. This disrupts the cell membranes and permits leakage of cell organelles, substrates and metabolites into the incubation medium (76). This may lead to artifactual results. Various modifications have been described to minimise cell leakage such as incubation of the tissue with its osseous attachments intact (77, 78). However, the adhering central tendon, cartilage and cut intercoatal fibres represent a new source of inaccuracy since tissues take up and metabolise glucose. A general criticism of the diaphragm preparation is that the muscle may not be metabolically typical of skeletal muscle because it is rythmically contracting until its isolation, and has a high proportion of collagenous connective tissue (79). Thus, data obtained from diaphragm muscle should be evaluated with caution and only extrapolated to the entire skeletal mass with reservations.

The limitations of diaphragm muscle necessitated a search for alternative tissue preparations. One preparation

which initially appeared attractive was the isolation of individual muscle cells by mechanical or enzymatic separation of fibres from large mammalian muscles (71). However, it was found that there was an appreciable loss and considerable variability of insulin responsiveness.

In 1965, Arvil and Ahren (80) found that the levator ani muscle from immature male rats could be isolated intact without any cell damage. These investigators initiated a series of experiments with this preparation (80,81). However, the results obtained with this preparation in the study of glucose uptake and metabolism were variable and the response to insulin was poor. Subsequently, the levator ani muscle has received little attention.

In the late nineteen sixties, the isolated rat soleus, a slow twitch red skeletal muscle was examined for in vitro work. There are many commendable points about the soleus muscle. It is cylindrical and terminates at each end in a well defined tendon such that the intact muscle may be removed without severing the muscle fibres (82). As each animal has a pair of these muscles, inherent variation between animals can be minimised by using an experimental design of paired controls (82,83). The biochemical properties of the soleus muscle were examined by Fitts, Brooke and Kaiser (84), and the muscle proved to be very satisfactory for in vitro studies on glucose uptake and metabolism and a large number of researchers have adopted this preparation (82-86). There is considerable evidence that this preparation provides a representative example of skeletal muscles in general (84) but it remains advisable to take into account information gained from other muscles when attempting to devise a complete picture of skeletal muscle glucose metabolism.

The subsequent section of this review will consider techniques available to assess glucose uptake and metabolism by muscle tissues in vitro. The techniques adopted by different research groups vary, but each group has either added radio-

labelled substrates to the incubation medium and assessed the products formed by autoradiography, or measured the dissapearance of non-radioisotopic substrates from the incubation medium and/or the appearance of metabolites in the incubation medium.

The use of radioisotopes is deemed to be more accurate and specific and has been used extensively (85,88-92). The choice of radioisotopes include ^{3}H and ^{14}C. Le Marchand (85,91) and Czech (89) prefer to use tritiated glucose (5-^{3}H-glucose) while Davidson (75,93) has consistently used labelled (^{14}C) glucose. ^{14}C is a more convenient radioisotope to use with glucose and carbohydrates in general. Tritium is labile and is not a good choice to label substrates such as glucose which has numerous hydroxyl groups. Furthermore, it is easier to detect the presence of ^{14}C in liquid scintillation counting because it is radioactively more energetic (158 Kev) than ^{3}H (18 Kev). In experiments using tritiated glucose, the magnitude of glycolysis is assessed from the production of $^{3}H_2O$. The $^{3}H_2O$ has first to be separated from glucose and involves the use of ion-exchange chromatography. ^{3}H-glycogenesis is determined in KOH hydrolysates of muscle to which carrier glycogen has been added (90). The use of ^{14}C provides a much simpler and direct method to assess glucose uptake as the $^{14}CO_2$ and ^{14}C-glycogen are readily separated from ^{14}C-glucose before counting in a liquid scintillation counter.

The radioisotopic methods are preferred to non-radioisotopic methods because they afford a greater degree of accuracy. However, the non-radioisotopic method has been used with apparent success by Chaudry and his co-workers (82,83,86). A major difficulty in evaluating glucose uptake by animal tissues is the retention of this substrate within the extracellular fluid space. Under in vitro incubation conditions, it appears that glucose does not become dispersed throughout the interstitial compartment at the same concentrations as in the incubation medium (73, 82, 94). Furthermore, the interstitial compartment may vary between animal tissues as a result of any treatment to which the isolated

tissue is exposed. These factors may be accounted for by measuring the extracellular fluid volume using a sugar alcohol such as sorbitol or other sugar derivatives which are not metabolised by the cells but which are dispersed in the extracellular compartment in a similar manner to glucose (95).

This review has briefly described the in vitro methods available for the study of glucose uptake and metabolism by skeletal muscle. In vivo methods have received only passing attention because they are not as versatile as in vitro techniques. The advantages and disadvantages of different in vitro muscle preparations are discussed and the soleus muscle is deemed to provide more advantages than any other muscle preparation examines to date. The soleus muscle is representative of skeletal muscles in general, and can be removed intact from the hind limbs of small mammals. The use of radiolabelled ^{14}C-glucose to assess aerobic glucose metabolism and glycogenesis by the formation of $^{14}CO_2$ and ^{14}C-glycogen respectively has produced accurate and reproducible results. Thus the isolated mouse soleus muscle and the use of ^{14}C-glucose have been adopted in the present study.

2.2 Animals

Adult female Theiller Original albino mice (Bantin and Kingman, Hull) from a closed non inbred colony were used in all experiments. The mice were 10-12 weeks old and weighed 25 g at the start of each experiment. They were housed in an air-conditioned room at 22° + 2° C with a lighting schedule of 12 h dark and 12 h light. The mice were fed a standard pellet diet(Mouse breeding diet, Heygate and Sons, Northampton) and allowed free access to tap water.

2.3 Chemicals

The list of chemicals used and their respective sources were as follows : Bovine pancreas insulin (24 IU/mg), oestradiol 17β, progesterone, α-D-glucose, pyruvic acid, 2-deoxy-D-glucose,

3-O-methyl-α-D-glucopyranose, uridinediphosphoglucose, glucose-6-phosphate, coenzyme A, 2-oxoglutarate, NAD^+, $NADP^+$, ATP, creatine phosphate, creatine kinase, glucose-6-phosphate dehydrogenase and streptozotocin from Sigma Chemical Co., Poole ; insulin-free bovine serum albumin (fraction V) from Armour Pharmaceutical Co., Eastbourne; U-^{14}C-glucose (279 mCi/mmol), 2-deoxy-D-[1-^{14}C]-glucose (50 mCi/mmol), 3-O-methyl-[U-^{14}C]-glucose (295 mCi/mmol), UDP-[U-^{14}C]-glucose (240 mCi/mmol) and Na^{125}I (carrier-free, dissolved in sodium hydroxide pH 11, free from reducing agents) from Amersham International, Amersham; porcine monocomponent insulin from Novo Laboratories, Copenhagen; scintillant NE 260 from New England Nuclear, Aberdeen. All other chemicals were of analytical grade and were from BDH (British Drug Houses), Poole.

Experimental Design

2.4 Sex hormone treatment

Mice were bilaterally ovariectomised under sodium pentobarbitone anaesthesia (45 mg/kg body wt, IP) at 12 weeks of age using the surgical procedure described by Armour et al (96). Treatments were begun one week later. Groups of 6 or more mice were treated for 10 weeks with replacement doses of either oestradiol (5 μg/kg/day, progesterone (1 mg/kg/day) or both hormones combined in the same doses as previously. In one study, a dose of 500 μg/kg/day oestradiol was also examined. The hormones were dissolved in 100 μl of 98 % ethanol and made up to the required concentrations in the drinking water. The concentrations were determined on the basis of a preliminary study which showed that an intact female mouse consumes on average, 20 ml of water per day per 100 g body weight. Control ovariectomised mice and intact mice were treated with 100 μl of 98 % ethanol per litre of drinking water. The various treatments are summarised below:

Group A Ovariectomised only

Group B Ovariectomised and treated with oestradiol

Group C Ovariectomised and treated with progesterone

Group D Ovariectomised and treated with oestradiol and progesterone

Group E Intact animals

Since the oestrous cycle influences glucose homeostasis, intact control mice were selected for experiments at either the proestrus/oestrus (P/O) or the metoestrus/dioestrus (M/D) stage of the cycle, as determined by vaginal smear (97). Proestrus/oestrus corresponds with raised circulating titres of gonadal steroids, while late metoestrus/dioestrus corresponds to lower circulating titres of gonadal steroids (98). The doses of sex steroids used herein produce oestradiol and progesterone values within the normal range (G.Willars PhD thesis Univ. of Aston 1984 in preparation)

2.5 Tissue isolation

All experiments were begun at 10.00 h. The mice were fasted for 40 h prior to the experiments. Fasting the mice avoids the influence of short-term changes in metabolic status associated with feeding, and reduces the glycogen content of the muscles(42). Fasted mice were killed by cervical dislocation and the soleus muscles from the right and left hind limbs were removed as described by Maizels, Ruderman and Lau (90). Briefly, the hind limb was excised from the body mass and the overlying skin was removed by a cut around the ankle. The limb was then anchored to a dissecting tray using pins, and the achilles tendon exposed. A ligature was placed around the achilles tendon, the tendon cut distal to the ligature and the gastrocnemius and soleus were pulled away from underlying tissue by traction. A second ligature was placed around the proximal tendon of the soleus and the tendon was severed proximal to the ligature. The soleus muscle was peeled away from the gastrocnemius by blunt dissection after which the gastrocnemius was cut away from the achilles tendon to completely free the soleus. Removing the soleus muscle

in this manner entails severing the tendons at each end of the muscle, leaving the muscle intact. The muscles were weighed and the tendons were tied across the open ends of a polyethylene horseshoe with 4/0 non-capillary braided suture (Armour Pharmaceutical Co., Eastbourne) to maintain the muscle under slight tension.

2.6 Preincubation

Except where indicated, each soleus muscle was preincubated in a 6 cm high flat bottom glass vial containing 2 ml preincubation medium. The preincubation medium consisted of KRB buffer, pH 7.4 supplemented with 2 % bovine serum albumin (insulin-free) and unlabelled glucose at 5 mmol/l. The buffer was gassed with O_2+CO_2, (95 : 5) and maintained at 37°C. Muscles were preincubated for 15 min in a shaking water bath (92 cycles/min). After preincubation, the muscles were rinsed in pre-gassed KRB buffer and transferred to Erlenmeyer flasks for incubation.

2.7 Glucose metabolism

2.7.1 CO_2 production. Muscles were incubated for 120 min in 25 ml Erlenmeyer flasks containing 3 ml of KRB buffer, pH 7.4, 5 mmol/l glucose, 0.5 μCi/ml U-^{14}C-glucose and 2 % bovine serum albumin either in the absence or presence of insulin (0.75 mU/ml). The incubation period of 120 min was selected from the results of a preliminary investigation in which glucose uptake into soleus muscle was determined after incubation for 30, 60, 90, 120 and 150 minutes in the presence of 1 mU/ml insulin. Similarly, the insulin dose was selected following a preliminary investigation in which soleus muscles were incubated in KRB buffer containing a range of insulin concentrations (0.1, 0.5, 1.0, 5.0 and 10.0 mU/ml). An insulin concentration of 0.75 mU/ml was selected for subsequent experiments because it produced a well defined submaximal stimulation of glucose metabolism. All Erlenmeyer

flasks has 1 cm diameter centre wells containing a folded semi-circle of filter paper(2 cm diameter, Whatman no. 30). The two sets of flasks provide an assessment of 'insulin-stimulated' and 'basal' glucose metabolism respectively. Before the start of each incubation period, the flasks were gently gassed with O_2+CO_2, (95 : 5) for 5 min, and then sealed with suba-seal stoppers and incubated for 120 min in a shaking water bath (92 cycles/min) at 37°C. At the end of the test incubation, 0.1 ml of 1 mol/l sodium hydroxide was injected through the stopper onto the filter paper in the centre of the well and the flask was placed on ice for 3 minutes. At the end of the 3 minutes, the muscles were removed from the flasks, frozen in liquid nitrogen and stored at -20°C. The flasks were restoppered and 0.3 ml of 3 mol/l perchloric acid was injected into the reaction chamber. The flasks were left to stand for 1 h at room temperature after which the filter paper was transferred to a scintillation vial containing 10 ml of NE 260 scintillant. All scintillation vials were stored at room temperature for 1 week before they were counted in a liquid scintillation counter, Model Tricarb 2660 (Packard Instrument Co., Illinois, USA) with a counting efficiency of 98 %.

2.7.2 Glycogen formation. Frozen muscle samples were thawed and muscle glycogen was determined after the method of Lo, Siu, Russel and Taylor (99). Briefly, muscle samples were hydrolysed in 1 mol/l sodium hydroxide, the glycogen precipitates extracted with 66 % ethanol and 1 mg carrier glycogen. 0.5 ml of the precipitate in water was transferred to 10 ml of scintillant and counted in a liquid scintillation counter.

2.8 Glucose transport

Soleus muscles were incubated at 37°C in 3 ml of incubation medium containing KRB buffer supplemented with 1 mmol/l 3-O-methyl-α-D-glucopyranose, 0.1 μCi/ml 3-O-methyl-[U-^{14}C]-glucose, 0.1 μCi/ml ^{3}H-inulin, 2 mmol/l pyruvate and 2 % bovine serum

in the absence and presence of insulin (0.75 mU/ml). Pyruvate was added to the incubation medium to provide an energy source for the tissue (100) since 3-O-methyl-α-D-glucopyranose impairs glucose metabolism (101). After 15 minutes, the muscles were removed and hydrolysed in 0.5 ml of 1 mol/l sodium hydroxide and transferred to 10 ml of scintillant (NE 260) for ^{3}H, ^{14}C counting. An incubation period of 15 min was selected because it had previously been shown that within this time period, mouse soleus muscle does not become saturated with 3-O-methyl-α-D-glucopyranose at the concentration used (100). ^{3}H-inulin was used as an extracellular marker as discussed in section 2.1.

2.9 Glucose phosphorylation

Soleus muscles were incubated in 3 ml of KRB buffer containing 1 mmol/l 2-deoxy-D-glucose, 0.1 μCi/ml 2-deoxy-D-[1-^{14}C] glucose, 0.1 μCi/ml ^{3}H-inulin, 2 mmol/l pyruvate, 2 % bovine serum albumin in the absence and presence of insulin (0.75 mU/ml). After an incubation period of 15 min (100) in a shaking water bath (92 cycles/min), the muscles were rinsed in KRB buffer and hydrolysed in 0.5 ml of 1 mol/l sodium hydroxide. The hydrolysates were counted in a liquid scintillation counter as described in section 2.7.1. All results are expressed as nmol of glucose metabolised to product per minute per mg muscle protein.

2.10 Insulin receptor binding

2.10.1 Radioiodination of insulin. Porcine monocomponent insulin was iodinated according to Freychet's modification (102) of the procedure of Hunter and Greenwood (103). LP 3 polyethylene tubes (Luckhams Ltd., Burgess Hill,) were used for the reaction. The reaction mixture comprised : 100 μl of 0.5 mol/l phosphate buffer (pH 7.4), 10 μl of 100mCi/ml Na^{125}I solution, 10 μl of 0.25 mg/ml hydrchloric acid and 10 μl of 0.25 mg/ml Chloramine T in 0.05 mol/l phosphate buffer, pH 7.4. The tube was shaken

after each addition and allowed to stand for 17 seconds before the addition of 20 µl of 0.5 mg/ml sodium metabisulphite in 0.05 mol/l phosphate buffer, and 200 µl of 2.5 % bovine serum albumin solution (insulin-free). A 10 µl aliqout was removed at this stage for estimation of ^{125}I incorporation into insulin and the specific activity of the labelled insulin. The labelled hormone was separated from unreacted iodide and damaged insulin by gel filtration, using a 1 x 30 cm column of Sephadex G 50 Fine. Before applying the reaction mixture to the column, the column was equilibriated with 0.05 mol/l phosphate buffer, pH 7.4 containing 0.5 % bovine serum albumin to reduce adsorption of the labelled insulin onto the sides of the column.

The reaction mixture was applied to the column, eluted at a flow rate of 0.75 ml/min and collected in LP 3 tubes at one minute intervals. The activity in each tube was counted (ICN Gamma Set 500) to assess the specific activity. An elution profile was obtained for each iodination. The peak iodoinsulin fractions were pooled. Four samples (10 µl each) were removed to assess the damage to the hormone. This was carried out by estimating 10 % TCA precipitability and adsorption onto charcoal. The pooled fractions were diluted 1 in 3 with a 0.05 mol/l phosphate buffer, pH 7.4 containing 1 % bovine serum albumin. The pH of each fraction was determined using indicator strips to confirm values in the range 7.0-7.5. The radioactive tracer was stored at -20°C in 250 µl aliqouts.

2.10.2 Insulin binding. Both fed and 40 h fasted mice were used in this experiment. The muscles were individually rinsed in 0.9 % saline before preincubation in glass vials containing 2 ml KRB buffer (pH 7.43) supplemented with 2 % bovine serum albumin (104) and 2 mmol/l pyruvate (100). The muscles were preincubated for 15 minutes at 20°C. A temperature of 20°C was selected because studies in this laboratory and elsewhere (85) have shown that the amount of ^{125}I-insulin specifically bound was more stable at 20°C. After the preincubation period, the muscles were

incubated for 4 h at 20°C in 1.5 ml of KRB buffer with 2 % bovine serum albumin, 2 mmol/l pyruvate and ^{125}I-insulin at 1ng/ml (0.17 nmol/l) in the absence (total binding) or presence (non-specific binding) of unlabelled insulin at 8μmol/l. A 4 h incubation period was selected after a preliminary time course study indicated that a steady state of insulin binding was achieved after four hours. The incubation medium was oxygenated with O_2+CO_2, (95:5) for the first ten minutes of the incubation period. After incubation, each muscle was washed five times with 3 ml of ice-cold saline containing 5 mg/ml bovine serum albumin. A single washing step consisted of a 5 min incubation period. Each muscle was then hydrolysed in 0.3 ml of 1mol/l sodium hydroxide and counted for ^{125}I radioactivity on a Gamma spectrometer (ICN Gammaset 500). A 100 μl aliqout was removed from each sample and frozen for protein assay. The results of the counts from the total and non-specific binding were expressed as a percentage of ^{125}I-insulin bound per mg of muscle protein. Protein was assayed according to the method of Lowry (105) (See section 2.16).

2.11 Enzyme assays

2.11.1 Hexokinase (Glucose ATP 6 phospho transferase, EC 2.7.1.1). Muscles were incubated for 120 min at 37°C in 3ml of KRB buffer containing 5 mmol/l glucose and 2 % bovine serum albumin in the absence or presence of insulin (0.75 mU/ml). The incubation medium was oxygenated with O_2+CO_2, (95:5) at 30 min intervals during the incubation period. After incubation, the muscles were prepared for the assay of hexokinase according to the method of Zammitt and Newsholme (106) as described below.

Muscles were removed from their holders and homogenised on ice for 10 min in ground glass homogenisers with 0.2ml of extraction medium. The extraction medium comprised 50 mmol/l triethanolamine, 1 mmol/l EDTA, 2 mmol/l $MgCl_2$ and 30 mmol/l mercaptoethanol adjusted to pH 7.4 with KOH. The homogenates

were centrifuged at 10,000g for 20 minutes in an MSE Superspeed centrifuge using a 10x10ml angle rotor. The assay was carried out in a buffer containing 75 mmol/l Tris/HCl, 7.5 mmol/l $MgCl_2$, 0.8 mmol/ EDTA, 1.5 mmol/l KCl, 4 mmol/l mercaptoethanol, 0.4 mmol/l $NADP^+$, 2.5 mmol/l ATP, 1 mmol/l glucose, 0.1 % bovine serum albumin, 10 mmol/l creatine phosphate, 0.9 U/ml creatine kinase and 0.7 U/ml of glucose-6-phosphate dehydrogenase. $NADP^+$, ATP and the coupling enzymes were added to the assay buffer on the day of the experiment since these reagents are unstable in solution (107). Crystalline glucose-6-phosphate dehydrogenase was reconstituted in 5 mmol/l citrate to obtain maximum stability and total recovery of enzyme activity after reconstitution (Sigma Information bulletin 10-77). 0.1 % bovine serum albumin was included in the assay buffer to avoid inactivation of the enzymes due to dilution (108).

Hexokinase activity was assayed by addition of 25 µl of homogenate to 1.975 ml of the assay buffer described above. The total assay volume was 2 ml and the final pH 7.3. The assay was initiated by the addition of glucose and enzyme activity was quantitated by the spectrophotometric determination of NADPH formed at 340 nm over a 10 min period at 25°C. A preliminary experiment showed that the reaction is linear for at least 12 min.

The data are expressed as units/g wet weight of tissue. One unit of activity is defined as the quantity of enzyme which will generate 1 µmol of NADPH per minute at 25°C. This definition conforms to the recommendations of the Commission on Enzymes of the International Union of Biochemistry (109).

2.11.2 Glycogen synthase (Uridine diphosphoglucose: glycogen α-4-glucosyl transferase, EC 2.4.1.11).

Muscles were preincubated in 2 ml of KRB buffer (pH 7.42) containing 2 mmol/l pyruvate and 2 % bovine serum albumin for 90 minutes at 37°C. The medium was gassed at intervals during the preincubation. At the end of the preincubation period, the

muscles were incubated as described for hexokinase in section 2.11.1. After incubation, the muscles were freeze-clamped in liquid nitrogen and ground with a glass pestle precooled in liquid nitrogen. 0.5 ml of ice-cold buffer (50 mmol/l Tris buffer, pH7.5, 5 mmol/l EDTA and 100 mmol/l potassium fluoride) was added to each muscle homogenate and sonicated on ice for 1 min. (Sonicator A 350G, Ultrasonics Ltd, Leicester). The muscle homogenates were centrifuged at 10,000g for 5 min at 4°C (MSE Superspeed 50, 10 x 10 ml rotor). The supernatants were collected and assayed for glycogen synthase according to the method described by Thomas, Schlender and Larner (110). Samples (30 µl) from the supernatants were added to 60 µl of a solution containing 50 mmol/l Tris buffer pH 7.4, 15 mmol/l sodium sulphate, 20 mmol/l EDTA, 25 mmol/l potassium fluoride, 9 mg/ml glycogen (rabbit liver glycogen, Hopkin and Williams, Cadwell Heath) and 6.7 mmol/l UDP-[U-^{14}C] glucose (20 nCi) in 1 ml polyethylene tubes. The tubes were incubated for 15 min at 30°C. A further 30 µl of the muscle supernatant was added to 60 µl of an identical solution as above, with the addition of 7.2 mmol/l glucose-6-phosphate. This method of assay enables the glycogen synthase active (I) form (incubated without glucose-6-phosphate) to be expressed as a percentage of the total (I+D) synthase activity (incubated in the presence of 7.2 mmol/l glucose-6-phosphate). For each muscle homogenate, an aliquot was removed for protein determination according to the method of Lowry (105) as described in section 2.16. Glycogen was measured as described by Thomas et al (110). Briefly, 60 µl of muscle and enzyme mixture was spotted onto squares (2 x2 cm) of filter paper (Whatman no. 30) and washed immediately in 4 ml of cold 66 % ethanol for 30 min. It has been shown that after 30 min, 96% of the labelled UDP glucose is removed(110). The first wash was followed by two further washes of 10 min with the final wash in acetone. The filter paper was dried under a heat lamp and counted in 10 ml of scintillant.

2.11.3 2-Oxoglutarate dehydrogenase (EC 1.2.4.2)

Muscles were incubated as described for hexokinase in section 2.11.1. At the end of the incubation period, each muscle was homogenised in a ground glass homogeniser with 10 volumes of homogenising medium. The homogenising medium consisted of 50 mmol/l triethanolamine-KOH, 2 mmol/l $MgCl_2$, 1 mmol/l EDTA, 30 mmol/l mercaptoethanol, 2 mol/l glycerol and 1 mmol/l 2-oxoglutarate at pH 7.4. Homogenates were sonicated for 15 seconds and centrifuged for 1 min at 600g in a bench centrifuge. These treatments had no effect on the activity of 2-oxoglutarate dehydrogenase (111). Homogenates were assayed for enzyme activity within 5 min of sonication since 50 % of the enzyme activity is lost 20 min after homogenisation (111).

Oxoglutarate dehydrogenase activity was assayed by measuring the reduction of NAD^+ at 340 nm according to the method of Read, Crabtree and Smith (111). The assay medium consisted of 90 mmol/l Tris-HCl, 12 mmol/l mercaptoethanol, 1 mmol/l potassium cyanide, 0.5 mmol/l coenzyme-A, 2 mmol/l NAD^+ and 0.8 mmol/l 2-oxoglutarate at pH 7.4. 20 µl of homogenate was added to 1 ml of assay medium in a cuvette. The control cuvette contained 1 ml of the same assay medium but in the absence of coenzyme A. Enzyme activity was assayed at 25°C in a spectrophotometer (Beckman SP 30 UV) and values are expressed as µmol of NADH formed per minute per gram wet weight of tissue.

2.12 Streptozotocin-induced diabetes

2.12.1 Comparison of intact and ovariectomised mice.

To determine the influence of endogenous sex hormones on the susceptibility of mice to streptozotocin, a group of age and weight matched female mice were either ovariectomised or sham operated (intact). Both groups of mice were injected (IP) with streptozotocin at a dose of 200 mg/kg body weight, in a volume of 1 ml/kg. The streptozotocin was dissolved in citrate buffer at pH 4.5 immediately prior to use to prevent inactivation of streptozotocin at

neutral pH (112). One week after streptozotocin injection, a small sample of blood was obtained for plasma glucose analysis (see section 2.13) to determine the extent of hyperglycaemia. The mice were selected only if plasma glucose was greater than 16 mmol/l but less than 20 mmol/l. Body weights and plasma glucose were determined weekly for 10 weeks. Plasma insulin was measured periodically during the study and total pancreatic insulin was assessed after the tenth week.

2.12.2 Effect of sex hormones.

Diabetes was induced in ovariectomised mice as described in section 2.12.1. The mice were selected if plasma glucose was approximately 20 mmol/l. Treatment with sex hormones was begun a few days later. Groups of 10 ovariectomised diabetic mice were treated with oestradiol and progesterone as described in section 2.4, with an additional group of mice on a high dose (500 µg/kg body wt) of oestradiol. The mice were treated for ten weeks. Body weights, plasma glucose, plasma insulin and total pancreatic insulin were measured.

2.13 Plasma glucose

Samples of blood were obtained from the cut tip of the tail (113) into heparinised tubes. The blood was centrifuged and plasma was stored at -20°C or used immediately for glucose analysis using a Beckman glucose analyser (Beckman, High Wycombe) (114). Determination of plasma glucose using the glucose analyser is based on the glucose oxidase method. A sample (10 µl) of plasma is added to a solution containing the enzyme glucose oxidase. Dissolved O_2 is consumed from the reaction solution in direct proportion to the concentration of glucose entering the reaction. Measurement of O_2 dissapearance from the solution is achieved with an O_2 sensor electrode and translated to the equivalent glucose concentration. The analyser shows a high degree of accuracy and reproducibility (114) and does not require deproteinated

plasma samples.

2.14 Plasma insulin

Plasma was stored at -20°C and aliquots of 50 µl were used for insulin assay. Plasma insulin was assayed according to the double antibody radioimmunoassay method of Hales and Randle (115), using mouse insulin as standard The insulin standard was dissolved in radioimmunoassay buffer - 0.4 mmol/l phosphate buffer containing 1 % bovine serum albumin, 40 mmol/l $NaH_2PO_4 \cdot 2H_2O$, 0.9% sodium chloride and .025%thiomersalate adjusted to pH 7.4 with aqueous NaOH. The principle of the assay is based on competition between radiolabelled hormone (radioactive antigen or tracer) with unlabelled hormone (antigen) for combination with a specific antibody, to form an antigen-antibody complex. Binding of labelled hormone is progressively diminished by increasing the concentration of the unlabelled hormone, so that the ratio of bound (B) insulin to free (F) insulin decreases as the quantity of unlabelled insulin added to the mixture increases. By comparing the B : F ratio produced by unknown samples containing insulin with that produced by a series of known insulin standards, a quantitative estimation of the amount of insulin in the unknown sample can be derived. The sensitivity of the assay, as defined by Midgley (116) was 0.18 ng/ml and the intra-assay coefficient of variation was 3.58 %.

2.15 Pancreatic insulin

Mice were killed by cervical dislocation, the pancreas was carefully dissected and total pancreatic insulin was extracted by acid-ethanol precipitation (117). Briefly, 5 ml of acidified ethanol (concentrated HCl; 75 % ethanol, 1:7 v/v) was added per gram wet weight of pancreas. The pancreas was minced, homogenised and sonicated on ice for 1 minute. The suspension was allowed to stand overnight at 4°C and centrifuged at 600g for 20 minutes.

The supernatant was stored at -20°C and diluted (1 : 5000 in radioimmunoassay buffer) before insulin assay.

2.16 Protein determination

Muscle protein content was assayed according to the method of Lowry (105). Briefly, 0.5 ml of a solution containing 2 % Na_2CO_3, 1 % $CuSO_4$ and 2 % NaK tartrate was added to 1 ml of diluted samples and allowed to stand for 10 minutes at room temperature after which 0.1 ml of Folin-Ciocalteau reagent (diluted 1 : 1) was added. The tubes were allowed to stand for 0.5 h before the extinction values were read at 750 nm in a spectrophotometer. A standard curve was constructed using bovine serum albumin at 0.5 mg/ml.

2.17 Statistics

Data in this thesis are given $\pm$-SEM (standard error of the estimated mean). The significance of differences between means were analysed by paired and unpaired Student's 't' test as appropriate. p, the probability of difference being due to chance was obtained from tables for 't' (118). Values are significant for $p < 0.05$.

CHAPTER 3

PRELIMINARY STUDIES

3.1 Time course of $^{14}CO_2$ production and ^{14}C-glycogen formation during incubation of mouse soleus muscle with U-^{14}C-glucose.

This section describes a preliminary experiment to examine the time course of $^{14}CO_2$ production and ^{14}C-glycogen formation during incubation of mouse soleus muscle with U-^{14}C-glucose. Since these parameters are known to vary with time (82, 90), the present investigation was undertaken to enable selection of an appropriate incubation time for a detailed study to follow, namely a study of the effect of sex steroids on glucose metabolism in isolated mouse soleus muscles. The parameters of $^{14}CO_2$ production and ^{14}C-glycogenesis after incubation with U-^{14}C-glucose have been selected to provide an indication of glycolysis and glycogen formation from glucose.

3.1.1 $^{14}CO_2$ production

Fig. 3.1 shows $^{14}CO_2$ production with increasing duration of test incubation, both in the absence and presence of insulin. The results show that under basal conditions,(no insulin), $^{14}CO_2$ production increased with increasing periods of incubation. In the presence of 1 mU/ml insulin, $^{14}CO_2$ production also increased with longer incubation periods and consistently exceeded that observed under basal conditions. The increase in $^{14}CO_2$ production in the presence of insulin was relatively greater between 90 and 150 minutes compared with basal, possibly suggesting greater discrimination between basal and insulin-stimulated $^{14}CO_2$ production with longer incubation periods (90 to 150 minutes).

Fig. 3.1 $^{14}CO_2$ production by mouse soleus muscles incubated for increasing duration in the absence (○) and presence (●) of insulin (1.0 mU/ml). Values are expressed as nmoles of glucose converted to CO_2 per mg protein. (n=3)

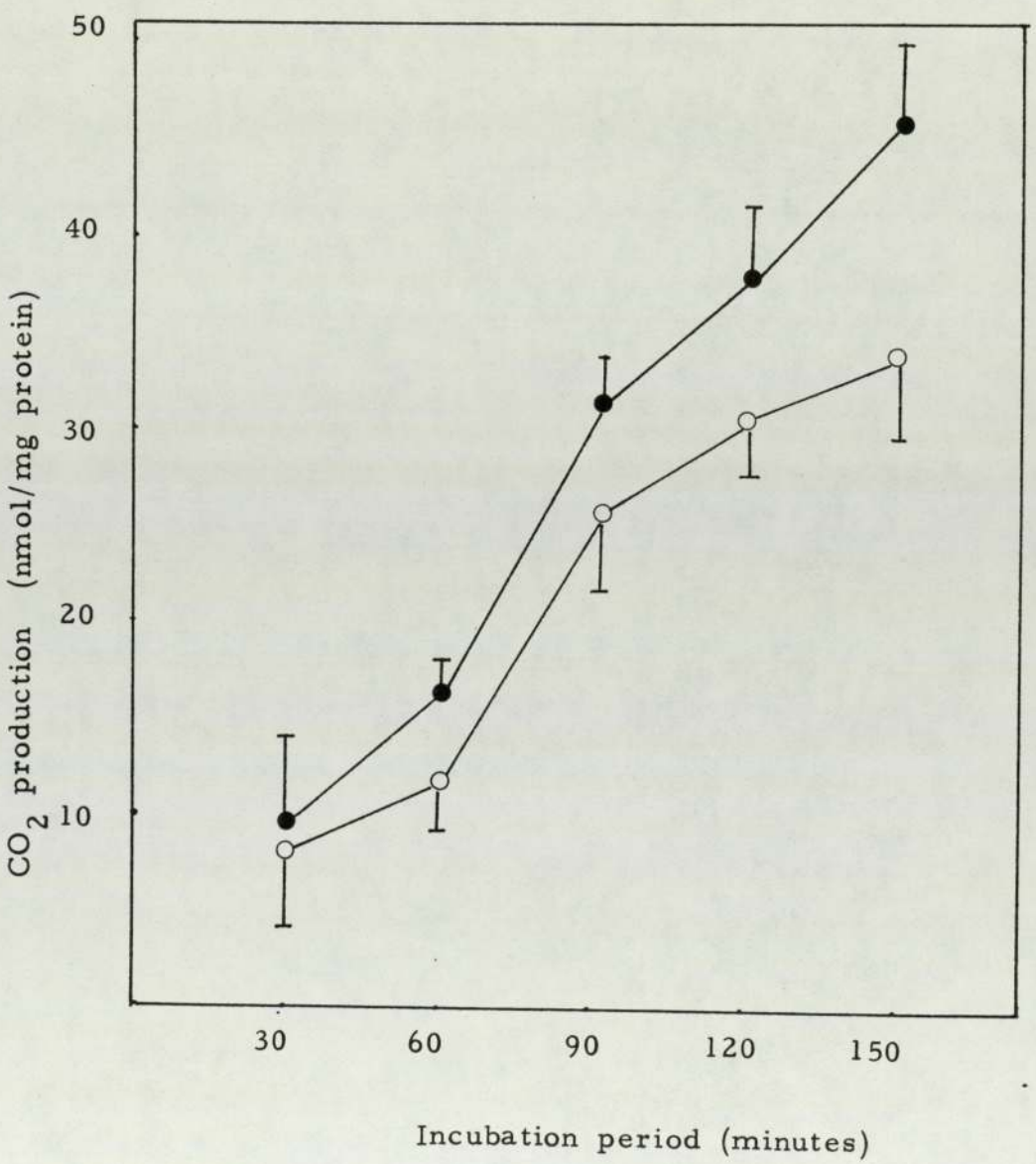
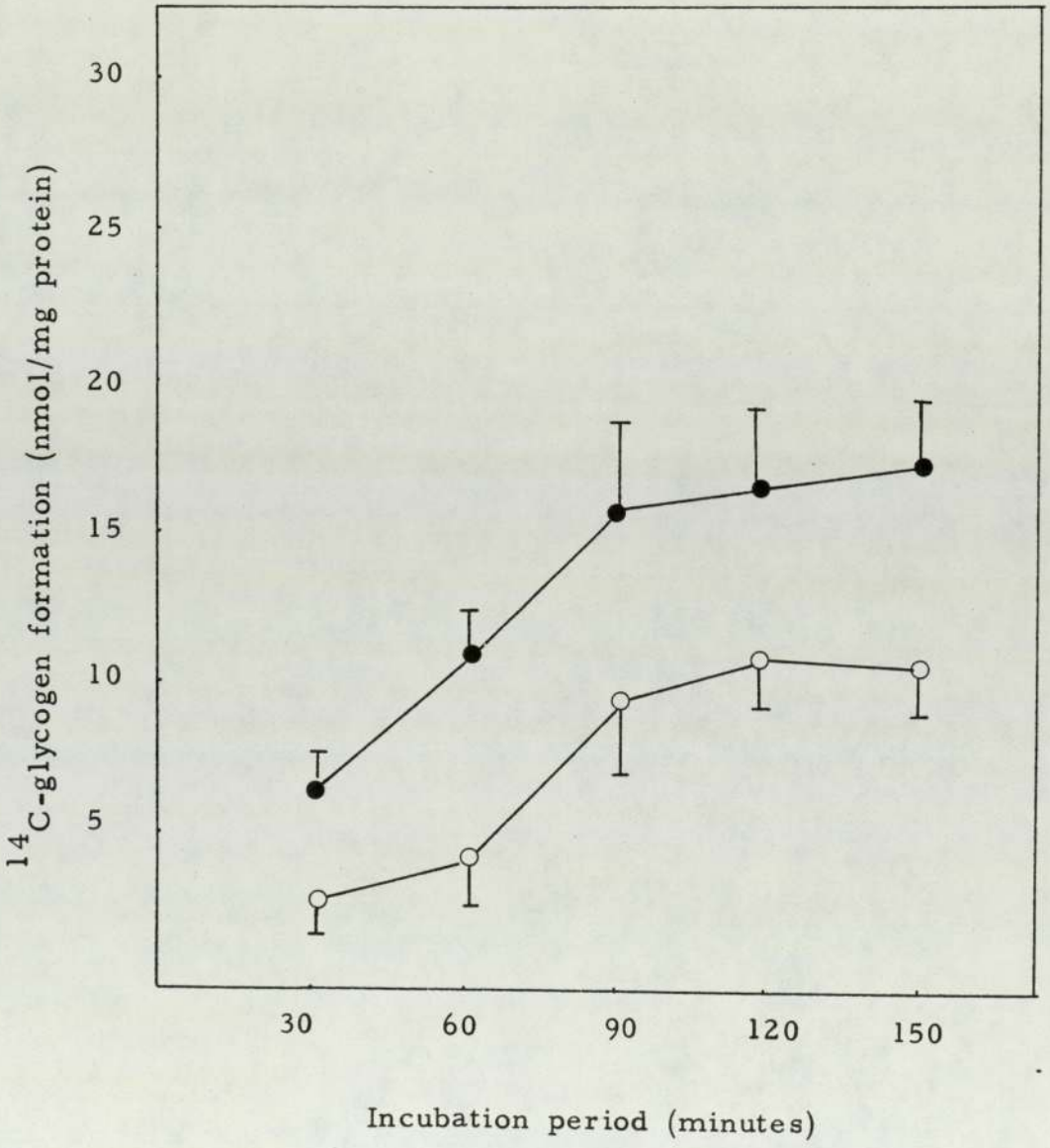


Fig. 3.2 ^{14}C-glycogen formation by mouse soleus muscles incubated for increasing duration in the absence (○) and presence (●) of insulin (1.0 mU/ml). Values are expressed as nmoles of glucose converted to glycogen per mg protein. ($n=3$)



3.1.2 ^{14}C-glycogen formation

Fig. 3.2 shows ^{14}C-glycogen formation from U-^{14}C-glucose after incubation of mouse soleus muscles for increasing duration (30 to 150 minutes). Glycogen formation increased with increasing periods of incubation and was apparently maximal after 120 minutes. This was observed under both basal and insulin-stimulated conditions. Insulin at 1 mU/ml consistently increased glycogen formation but greater discrimination between basal and insulin-stimulated glycogenesis was observed with longer incubation periods.

3.2 $^{14}CO_2$ production and ^{14}C-glycogen formation during incubation of mouse soleus muscle with increasing insulin concentrations (0.1, 0.5, 1.0, 5.0 and 10.0 mU/ml).

This experiment examines the insulin dose response for selected parameters of glucose metabolism in the isolated mouse soleus muscle. The experiment enabled a suitable insulin dose to be selected for use in subsequent experiments, namely studies on the effects of sex steroids on glucose metabolism in the mouse soleus muscle. Since insulin affects glucose uptake by soleus muscle, the parameters $^{14}CO_2$ production and ^{14}C-glycogen formation were measured after incubation of soleus muscles with insulin concentrations of 0.1, 0.5, 1.0, 5.0 and 10.0 mU/ml in the presence of 5 mmol/l U-^{14}C-glucose. ^{14}C-glycogenesis and $^{14}CO_2$ production provide indices of glycogen formation and oxidative phosphorylation respectively, from glucose.

3.2.1 $^{14}CO_2$ production

Fig. 3.3 shows that $^{14}CO_2$ production by mouse soleus muscles increased linearly between an insulin dose of 0.1 and 1.0 mU/ml, after which $^{14}CO_2$ production became almost constant. In the absence of insulin, $^{14}CO_2$ production by mouse soleus muscles was lower than that observed in the presence of insulin. The p values (from paired 't' tests) showed that there was no significant difference between basal and insulin-stimulated $^{14}CO_2$ production

at insulin concentrations 0.1 and 0.5 mU/ml. This may be accounted for by the wide variation in response between individual muscles and the small number of muscles used (n=3). However, Fig. 3.3 does show that increasing concentrations of insulin stimulate $^{14}CO_2$ production up to an insulin concentration of 1.0 mU/ml when $^{14}CO_2$ production is apparently maximal. A significant difference was observed between basal and insulin-stimulated $^{14}CO_2$ production at insulin concentrations of 1.0, 5.0 and 10.0 mU/ml ($p < 0.05$). The Eadie-Hofstee plot was constructed to enable selection of the half-maximal insulin concentration for use in subsequent experiments. Fig. 3.4 shows that the Km value was 0.774 mU/ml. Hence, under the conditions used in this experiment, $^{14}CO_2$ production is half maximal at an insulin concentration of 0.774 mU/ml.

3.2.2 ^{14}C-glycogen formation

Fig. 3.5 shows ^{14}C-glycogen formation by mouse soleus muscles in the absence and presence of insulin (0.1, 0.5, 1.0, 5.0 and 10.0 mU/ml). ^{14}C-glycogen formation increased with increasing concentrations of insulin, particularly between 0.1 and 1.0 mU/ml, and was apparently maximal at 1.0 mU/ml. In the absence of insulin, glycogen formation was lower than in the presence of insulin. However, there was no significant difference between basal and insulin-stimulated ^{14}C-glycogen formation at insulin concentrations of 0.1 and 0.5 mU/ml. A significant difference was observed at insulin concentrations of 1.0 and 5.0 mU/ml. As noted with CO_2 production, this reflects the wide variation between individual muscles and the sample size (n=3). The Eadie-Hofstee plot (Fig. 3.6) shows that ^{14}C-glycogen formation is half-maximal at an insulin concentration of 0.631 mU/ml.

Fig. 3.3 $^{14}CO_2$ production by mouse soleus muscles incubated in the absence (○) and presence (●) of increasing insulin concentrations (0.1, 0.5, 1.0, 5.0 and 10.0 mU/ml). Values are expressed as nmoles of glucose converted to CO_2 per minute per mg protein. (n = 5)

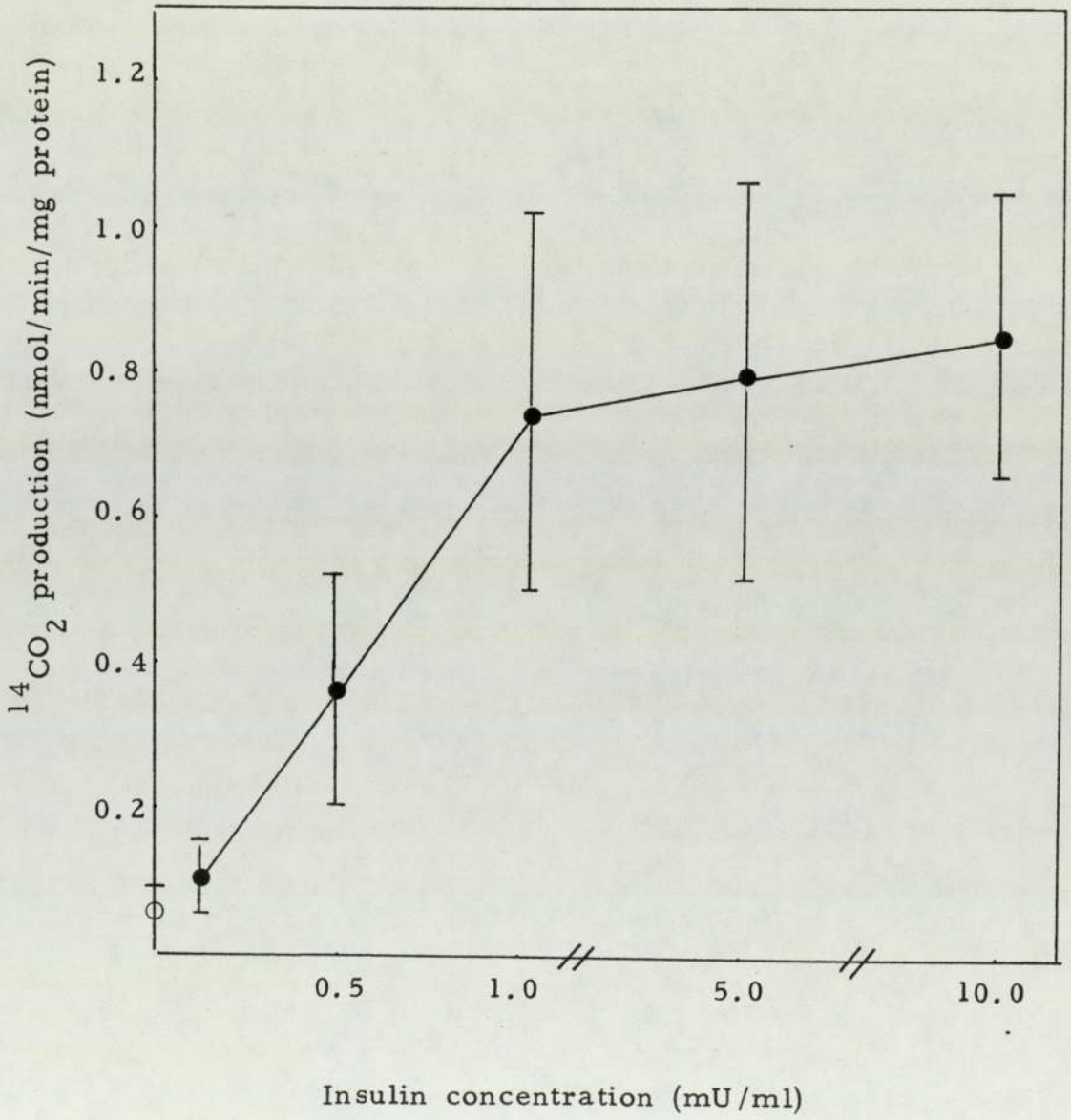


Fig. 3.4 Eadie-Hofstee plot of V_o versus $V_o / [S_o]$ for $^{14}CO_2$ production, where V_o is the rate of $^{14}CO_2$ production (nmol/min/mg protein) and $V_o / [S_o]$ is the product of reaction rate and the reciprocal of the insulin concentration used (nmol/min/mg. ml/mU^{-1}). The intercept on the V_o axis represents V_{max} and the gradient of the line is -1/Km.
(Straight line drawn using analysis of linear regression)

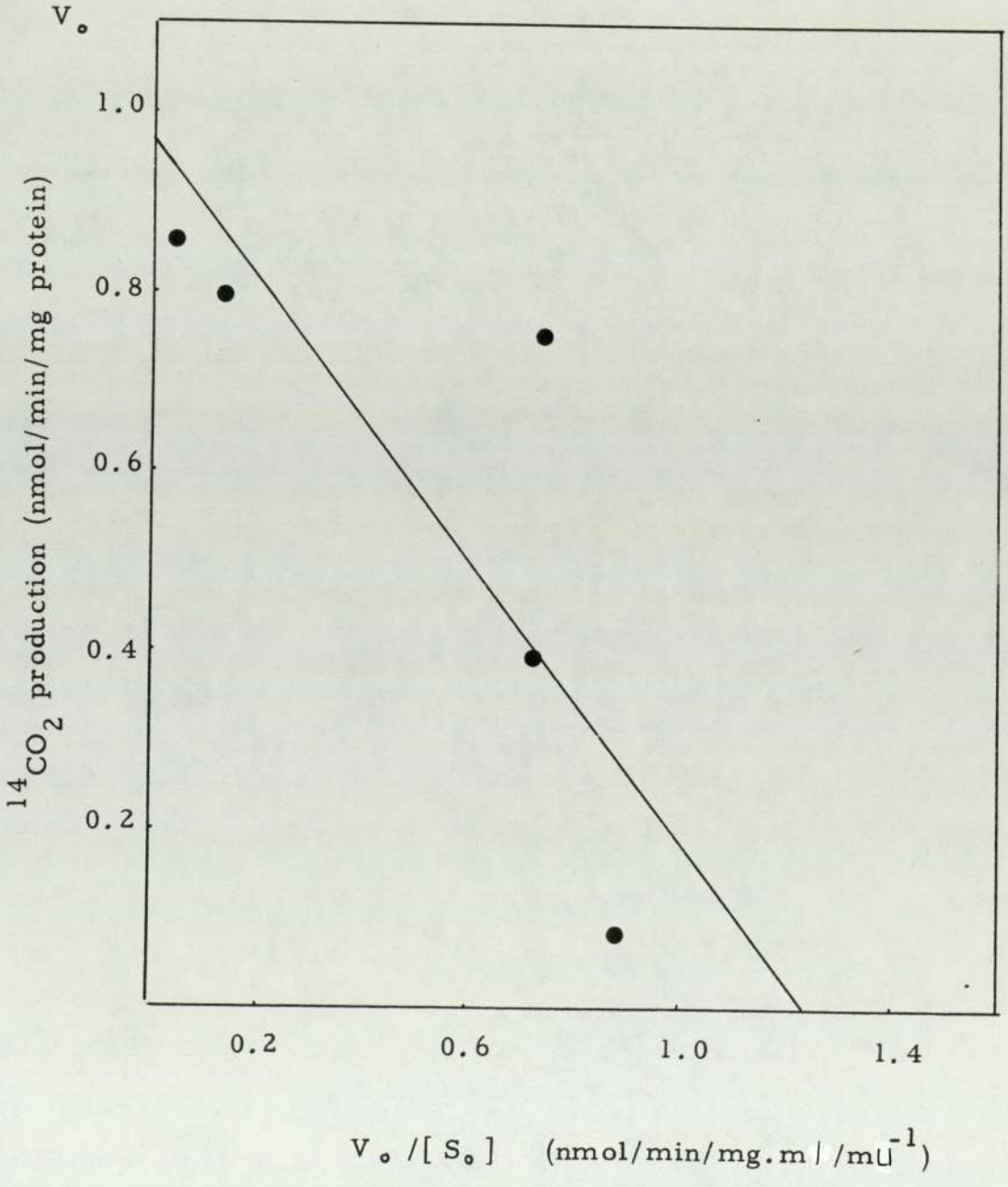


Fig. 3.5 ^{14}C-glycogen formation by mouse soleus muscles incubated in the absence (○) and presence (●) of increasing insulin concentrations (0.1, 0.5, 1.0, 5.0 and 10.0 mU/ml). Values are expressed as nmoles of glucose converted to glycogen per minute per mg protein. (n=5)

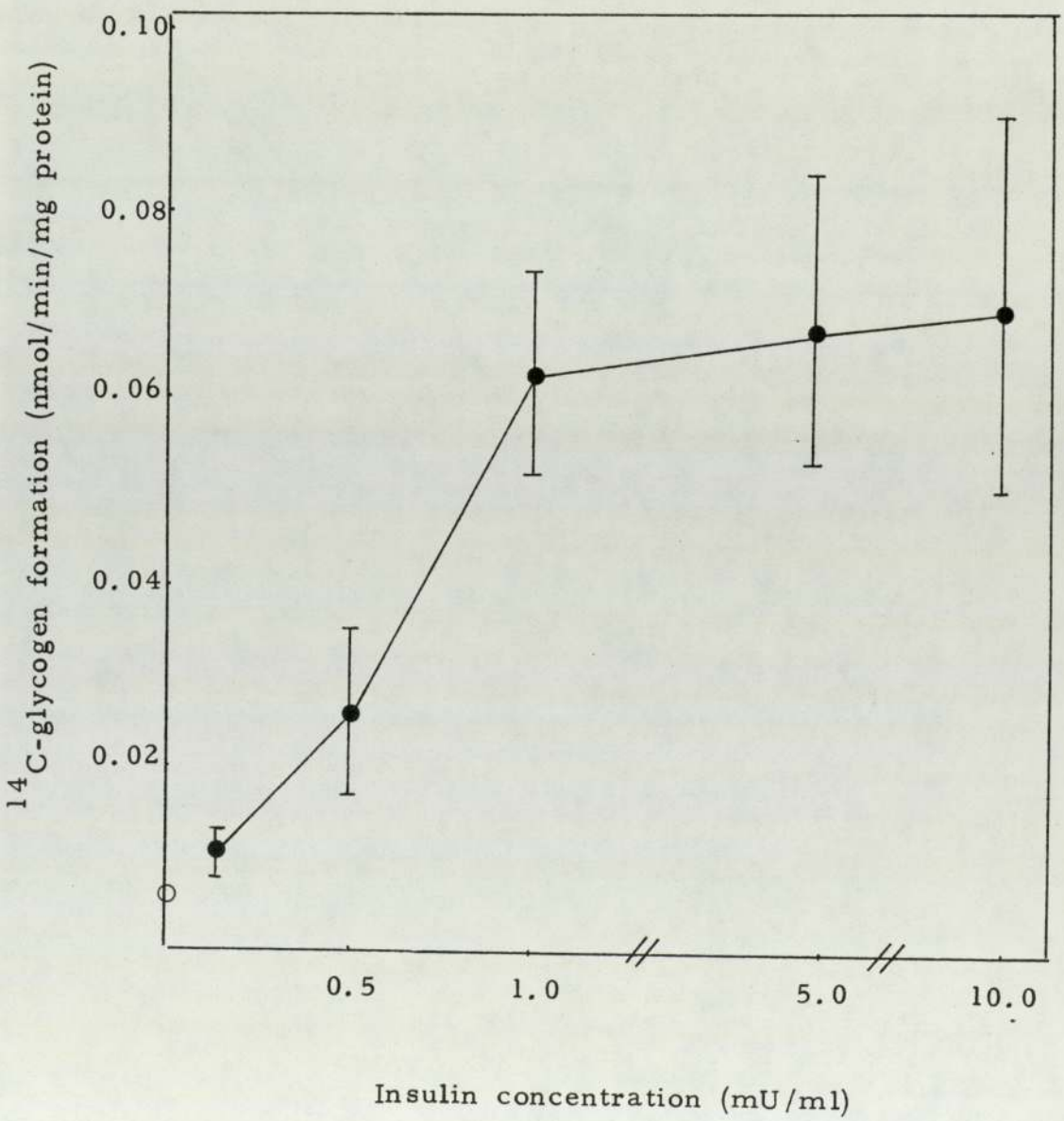
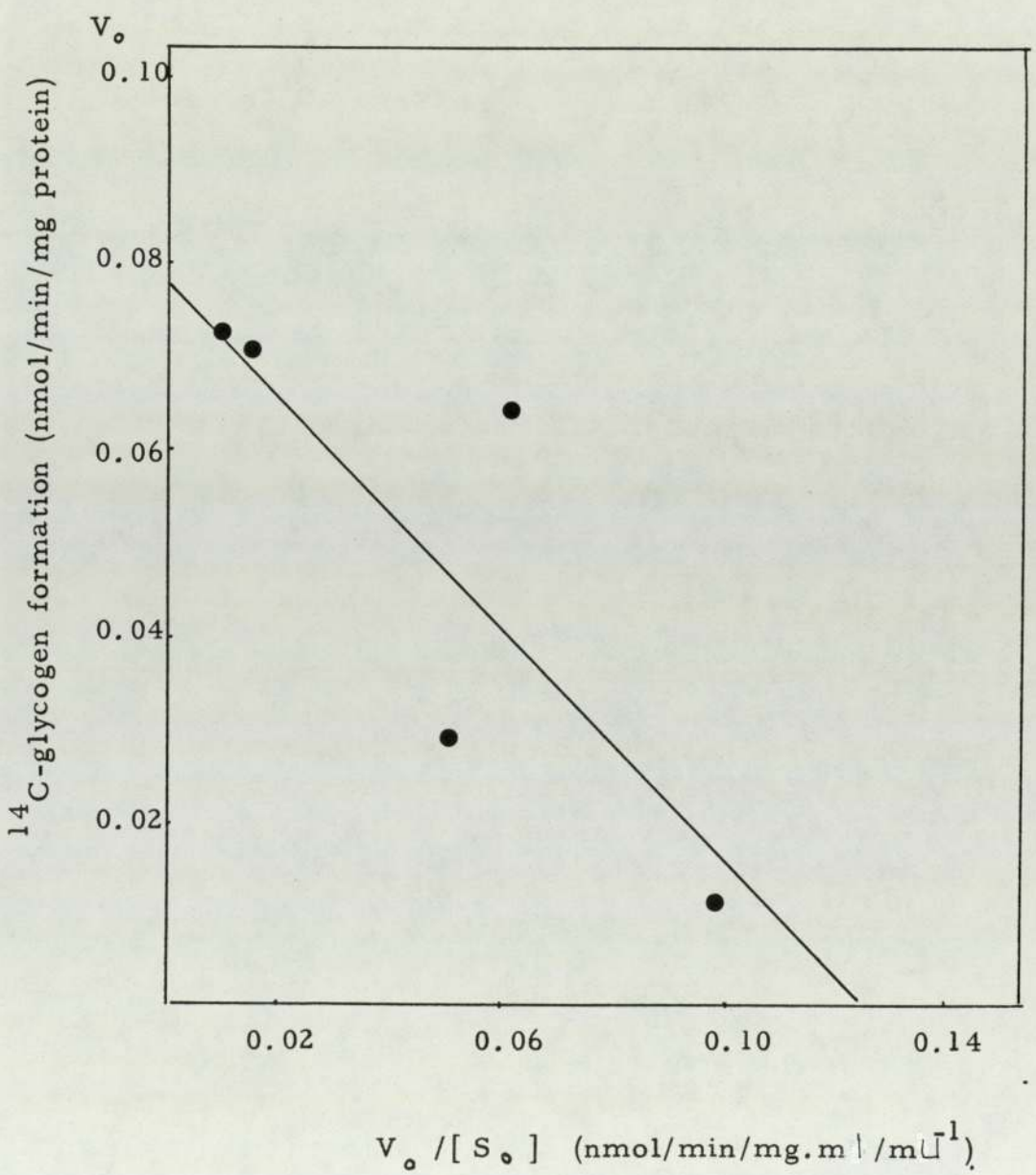


Fig. 3.6 Eadie-Hofstee plot of V_o versus $V_o / [S_o]$ for ^{14}C-glycogen formation, where V_o is the rate of glycogen formation (nmol/min/mg protein) and $V_o / [S_o]$ is the product of reaction rate and the reciprocal of the insulin concentration used (nmol/min/mg.mℓ/mU^{-1}). The intercept on the V_o axis represents V_{max} and the gradient of the line is -1/Km.
(Straight line drawn using analysis of linear regression)



3.2 Discussion

The results obtained in this study indicate that the isolated mouse soleus muscle is a viable preparation under the conditions used. The production of CO_2 and glycogen indicate that glucose was taken up and metabolised by the tissue. This was maintained during incubation periods ranging from 30 minutes to 150 minutes. The known stimulatory effect of insulin on glucose uptake was apparently confirmed by the increase in CO_2 production and glycogenesis resulting from the addition of this hormone to the incubation medium. At a concentration of 1 mU/ml, insulin has been reported to increase glucose uptake by 66 % (117). In the present study, CO_2 production was increased by 30 % in the presence of insulin.

Glucose taken up by the muscle may be anaerobically metabolised to lactate via pyruvate, aerobically metabolised via pyruvate and the tricarboxylic acid cycle or converted to glycogen (95). In this study, glycogen formation was assessed directly and the production of CO_2 was used as an index of oxidative phosphorylation.

CO_2 production increased with longer periods of incubation, particularly between 90 and 150 minutes indicating that aerobic glucose metabolism was maintained throughout the 150 minutes of incubation in vitro. Glycogen formation was also increased with increasing duration of incubation and was apparently maximal after 120 minutes. Since greater discrimination between basal and insulin-stimulated glucose metabolism was observed with longer periods of incubation, it was decided to adopt an incubation period of 120 minutes for subsequent studies.

CO_2 production increased linearly between an insulin concentration of 0.1 and 1.0 mU/ml. Using an Eadie-Hofstee plot, a half-maximal response was produced at an insulin concentration of 0.774 mU/ml. Glycogen formation from glucose also increased with increasing insulin concentrations in the incubation medium and reached constancy at 1.0 mU/ml. The Eadie-Hofstee

plot indicated that an insulin concentration of 0.631 mU/ml produced a half-maximal increase in glycogen formation. For future experiments, an insulin concentration is required which will produce approximately a half-maximal response. This will allow for any synergism of insulin action to be detected in subsequent experiments. Thus, an insulin concentration of 0.75 mU/ml has been selected for subsequent studies.

CHAPTER 4

EFFECT OF SEX HORMONES ON GLUCOSE METABOLISM

4.1 $^{14}CO_2$ production and ^{14}C-glycogen formation from U-^{14}C-glucose in mouse soleus muscle.

As discussed in Chapter 1, previous reports on the effects of sex steroids on glucose metabolism indicate that natural oestrogens tend to improve glucose tolerance (38,59,118), but reports concerning the effects of progesterone, and oestradiol and progesterone in combination have been conflicting (45,119,120). There is evidence that female sex steroids can modify the hypoglycaemic action of insulin (121,122). In particular, physiological concentrations of natural oestrogens appear to improve the hypoglycaemic action of insulin, whereas progestogens may antagonise this effect of oestrogens (40,123). However, this evidence is derived from circumstantial observations in vivo : for example, glucose tolerance tests and insulin hyperglycaemia tests conducted at different stages of the reproductive cycle, during pregnancy or after the menopause, which are associated with physiological alterations of circulating sex hormones, or during sex steroid administration for contraceptive or therapeutic purposes. Evidence derived in this manner is difficult to interpret because it does not distinguish the effects of the individual sex steroids on specific tissues : that is, the in vivo studies indicate only the net effect of the entire sex steroid milieu on glucose homeostasis by the whole organism.

To appreciate the action of individual sex steroids, and defined combinations of sex steroids on glucose metabolism by specific tissues requires an in vitro approach. Since the actions of sex steroids may take days, weeks or even months to become manifest fully (124,125) and since these actions may not be developed in isolation from the in vivo situation (125), it is both desirable and expedient to administer sex steroids to the whole organism. One of the most accurate indication of the effects of

individual female sex steroids and combinations of these steroids can be obtained using ovariectomised animals in which interference from endogenous circulating sex steroids is minimal. Indeed, ovariectomised rodents produce only very limited quantities of oestradiol and oestrone from the adrenal cortex (126, 127). However, the interconversion of androgens within the plasma may occur but this only results in very low circulating levels of oestrogens (128). These levels may be contrasted with the high levels of oestradiol, oestrone and progesterone in intact animals (129, 130). To determine the action of the steroid treatments on glucose metabolism in skeletal muscle, which is quantitatively a major site of glucose metabolism, in vitro studies may be conducted using isolated soleus muscles as described in previous chapters. Thus, the present study was undertaken to assess the effects of ovariectomy and oestradiol-progesterone replacement regimens on CO_2 production and glycogen formation in the isolated mouse soleus muscle

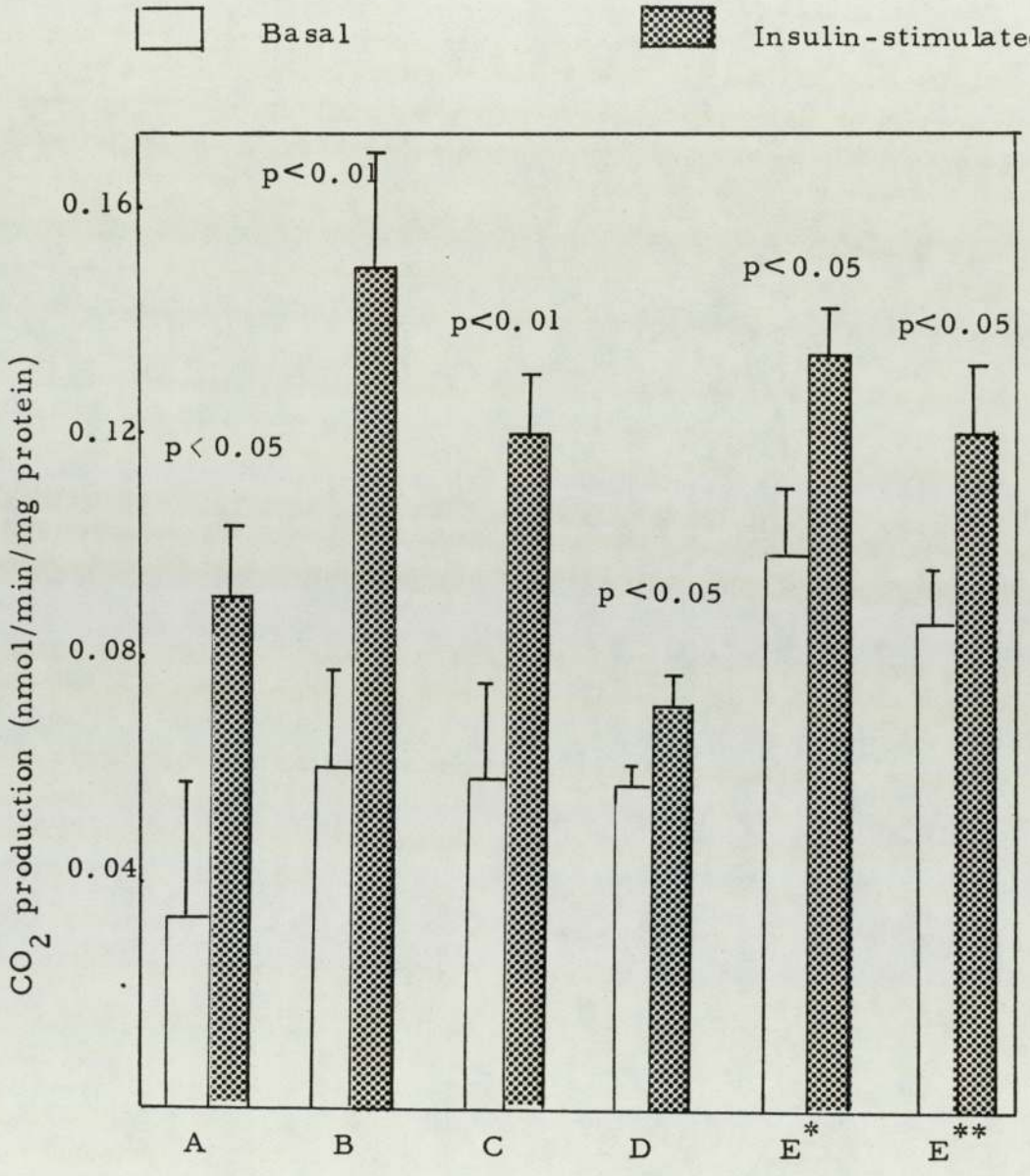
4.1.1 $^{14}CO_2$ production

Fig. 4.1 shows $^{14}CO_2$ production from U-^{14}C-glucose by mouse soleus muscles of intact proestrus/oestrus (P/O), metoestrus/dioestrus (M/D), ovariectomised and ovariectomised steroid treated mice. In all cases, insulin at a concentration of 0.75 mU/ml significantly increased $^{14}CO_2$ production. The individual oestradiol and progesterone treatments resulted in a highly significant increase (29 % and 19 % respectively) in insulin-stimulated $^{14}CO_2$ production compared with ovariectomised mice, while oestradiol and progesterone in combination did not significantly alter insulin-stimulated $^{14}CO_2$ production compared with control mice. The data also show that insulin-stimulated $^{14}CO_2$ production by muscles from intact mice was greater than that from ovariectomised controls. Indeed, $^{14}CO_2$ production from muscles of intact P/O mice was greater than that from M/D mice, which was greater than that from ovariectomised mice.

These data may be associated with the circulating sex steroid levels in these groups (P/O > M/D > ovariectomised mice) (98). Fig. 4.1 also shows that basal $^{14}CO_2$ production is significantly higher in intact M/D and P/O mice than in ovariectomised and ovariectomised hormone treated mice. This indicates that normal physiological concentrations of sex steroids can promote non-insulin mediated CO_2 production. Since replacement regimens of physiological concentrations of oestradiol and progesterone (individually and in combination) in ovariectomised mice did not result in the same effect, it is tempting to suggest that some other ovarian factor, present in intact animals is involved in promoting basal glucose uptake. The ovarian factor could be another hormone which may be acting synergistically or in concert with oestradiol and progesterone. However, this hypothesis remains purely speculative since it is extremely unlikely that replacement regimens of sex steroids, even at physiological concentrations are sufficient to mimic the effects of the entire sex steroid milieu found in intact mice.

The effects of oestradiol and progesterone treatment on insulin-stimulated $^{14}CO_2$ production may be compared directly using the increment in $^{14}CO_2$ production over basal (Fig. 4.2). Insulin at 0.75 mU/ml produced a 12 % increment in $^{14}CO_2$ production in ovariectomised mice and an increase of 36 % and 27 % respectively in oestradiol treated and progesterone treated mice. In contrast to the individual treatments, the increment in insulin-stimulated $^{14}CO_2$ production over basal in the combined oestradiol and progesterone treated group was much lower than in both the individual treatments and in the intact P/O mice. Measurements made using muscles from intact P/O and M/D mice enable a comparison of the effect of the oestrous cycle on $^{14}CO_2$ production in soleus muscles. In P/O mice, the increment in $^{14}CO_2$ production was not significantly greater than M/D mice and both these groups were not significantly different from ovariectomised mice. This supports evidence from the combined oestradiol-progesterone treatment in ovariectomised mice which indicates that whereas

Fig. 4.1 CO_2 production by soleus muscles from control and hormone treated mice, after incubation of muscles in the absence and presence of insulin (0.75 mU/ml). p denotes significance of difference between basal and insulin-stimulated CO_2 production for each group, determined by Student's 't' test for paired data. Values are expressed as nmoles of glucose converted to CO_2 per minute per mg muscle protein and are means ± SEM of 5 or 6 determinations.



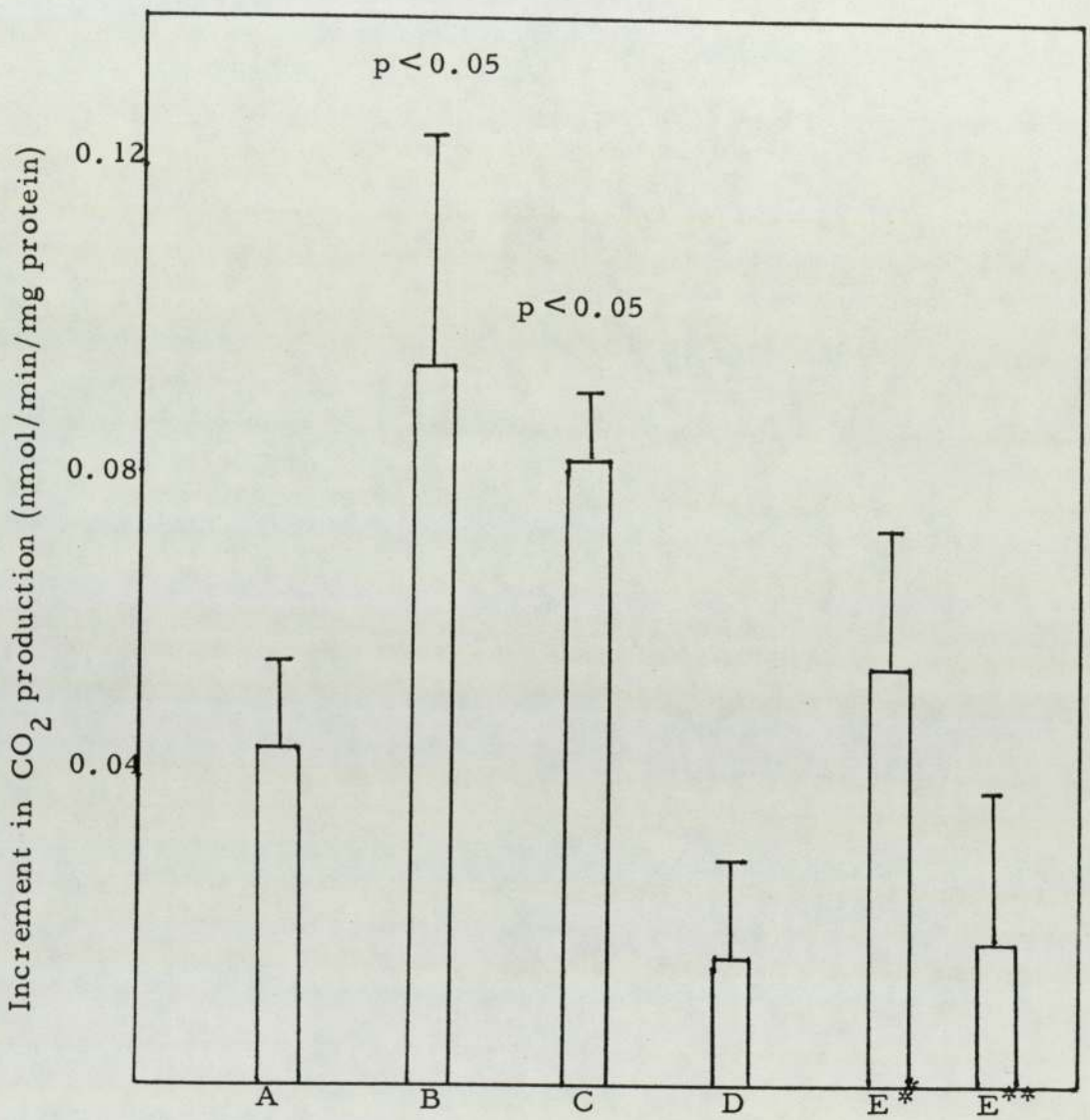
A = Ovx only B = Ovx + Oestradiol

C = Ovx + Progesterone D = Ovx + Oestradiol+Progesterone

E^* = Intact proestrus/oestrus

E^{**} = Intact metoestrus/dioestrus

Fig. 4.2 Increment in insulin-stimulated CO_2 production above basal levels in mouse soleus muscle. p denotes significance of difference compared with ovariectomised mice determined by Student's ' t ' test for unpaired data. (n = 5 or 6)



A = Ovx only

B = Ovx + Oestradiol

C = Ovx + Progesterone

D = Ovx +Oestradiol+progesterone

E* = Intact proestrus/oestrus

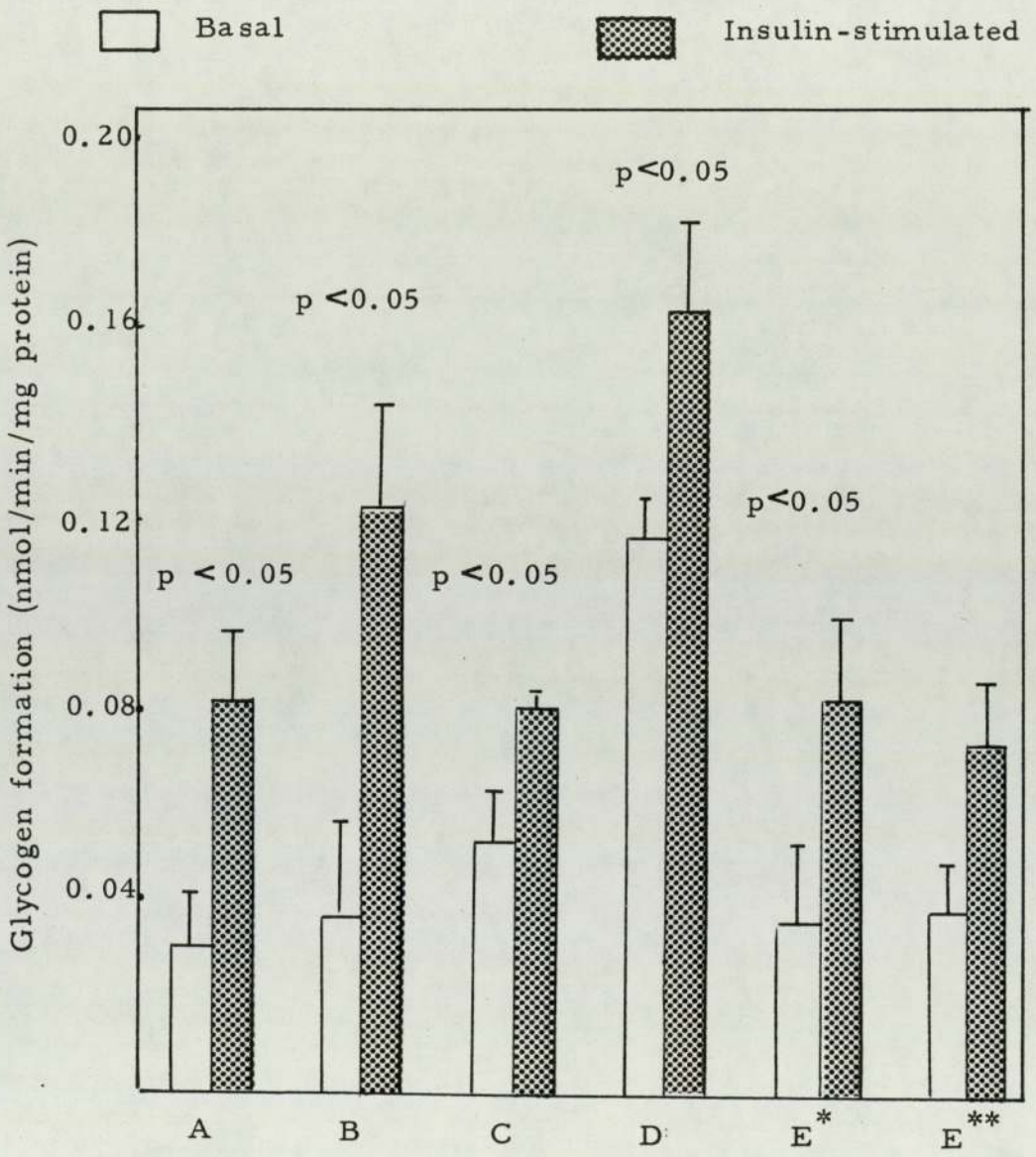
E** = Intact metoestrus/dioestrus

the individual steroids increase insulin-stimulated $^{14}CO_2$ production, the two hormones together antagonise each other. Thus, physiological concentrations of these hormones at either higher (P/O) or lower (M/D) levels do not significantly alter increments in insulin-stimulated $^{14}CO_2$ production by mouse soleus muscles. However, the absolute values for insulin-stimulated $^{14}CO_2$ production were greater in intact P/O mice and progressively less in intact M/D and ovariectomised mice. This is consistent with the progressively lower sex steroid titres in these groups.

4.1.2 ^{14}C-glycogen formation

Fig.4.3 shows ^{14}C-glycogen formation by mouse soleus muscles from intact P/O, M/D, ovariectomised and ovariectomised steroid treated mice. Insulin at a concentration of 0.75 mU/ml significantly increased ^{14}C-glycogen formation over basal in ovariectomised control, oestradiol treated and intact P/O mice over the 120 minute incubation period studied. Insulin-stimulated ^{14}C-glycogen formation was highest in the combined oestradiol and progesterone treated mice. This was twice the value for insulin-stimulated ^{14}C-glycogen formation in ovariectomised mice. In oestradiol treated mice, insulin-stimulated ^{14}C-glycogen formation was twice the value from ovariectomised mice. In contrast to the basal values observed with $^{14}CO_2$ production, physiological concentrations of sex steroids appear not to promote basal ^{14}C-glycogen formation. The increments in insulin-stimulated ^{14}C-glycogen formation over basal values are shown in Fig.4.3. The increment in ^{14}C-glycogen formation was highest in oestradiol treated mice and was significantly greater than the value for ovariectomised mice. The values for basal and insulin-stimulated ^{14}C-glycogen formation were highest in mice receiving the combined oestradiol and progesterone treatment. The increase in ^{14}C-glycogen formation produced by insulin was smaller in the progesterone treated group than in ovariectomised controls. Intact mice showed similar changes to those observed with CO_2 production. The insulin-stimulated

Fig. 4.3 Glycogen formation by soleus muscles from control and hormone treated mice, after incubation of muscles in the absence and presence of insulin (0.75 mU/ml). p denotes significance of difference between basal and insulin-stimulated glycogen formation for each group, determined by Student's 't' test for paired data. Values are expressed as nmoles of glucose converted to glycogen per minute per mg muscle protein and are means ± SEM of 5 or 6 determinations.



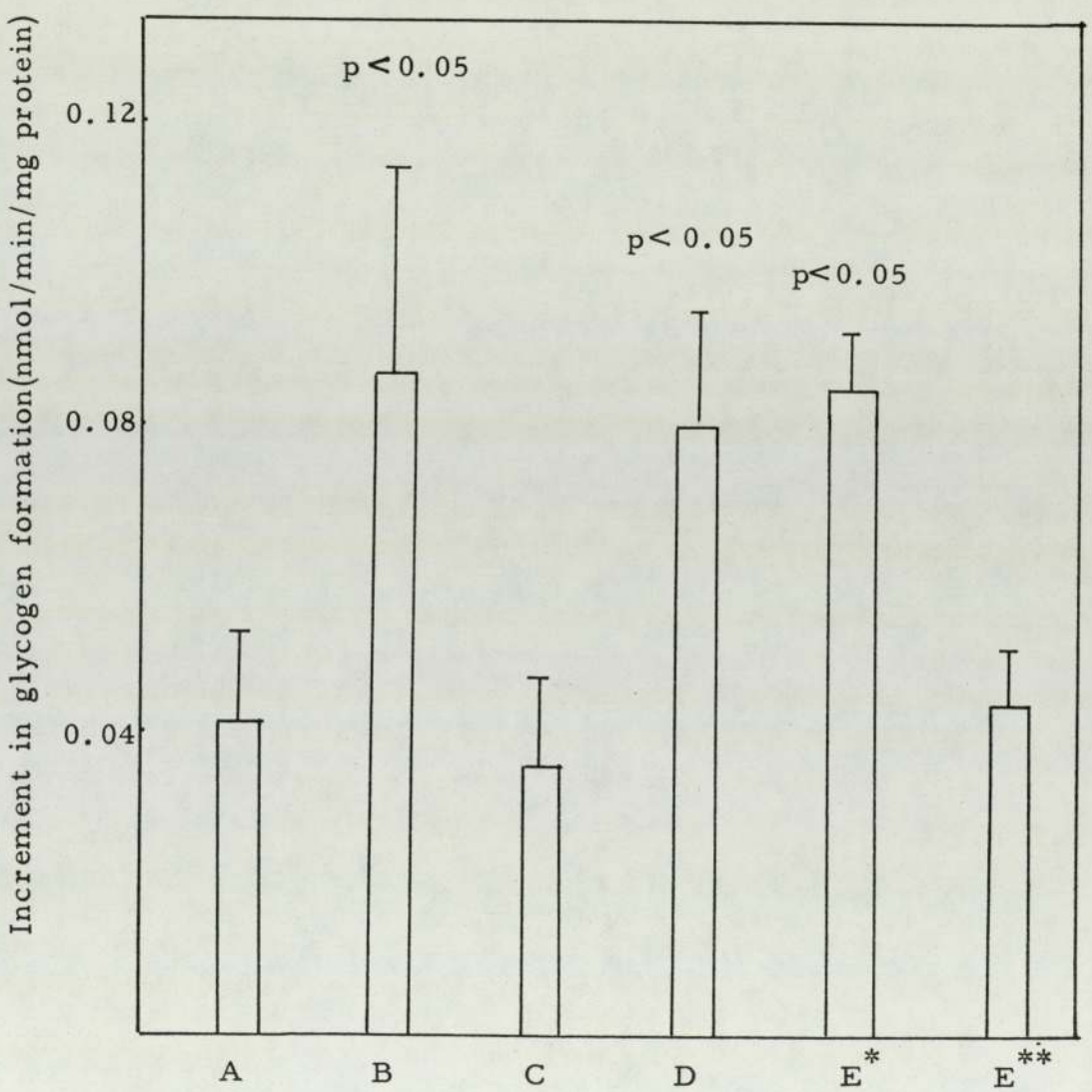
A = Ovx only B = Ovx + Oestradiol

C = Ovx + Progesterone D = Ovx + Oestradiol+Progesterone

E^{*} = Intact proestrus/oestrus

E^{**} = Intact metoestrus/dioestrus

Fig. 4.4 Increment in insulin-stimulated glycogen formation above basal levels in mouse soleus muscles.
p denotes significance of difference compared with ovariectomised mice, determined by Student's ' t ' test for unpaired data.
(n = 5 or 6)



A = Ovx only B = Ovx + Oestradiol
C = Ovx + Progesterone D = Ovx + Oestradiol+Progesterone
E* = Intact proestrus/oestrus
E** = Intact metoestrus/dioestrus

increment in glycogen formation in intact M/D mice was not significantly different from that of ovariectomised mice, but the increment induced by insulin in the intact P/O mice was twice that of ovariectomised mice.

4.2 Discussion

The advent of contraceptive steroids and their reported effects on glucose tolerance has prompted investigations into the effects of natural sex hormones on carbohydrate metabolism (40, 41, 131). While synthetic oestrogens have often been reported to cause a deterioration in glucose tolerance (9, 26), natural oestrogens fail to produce such an effect (122, 132). Indeed, as early as 1940, it was observed that natural oestrogenic preparations decreased the insulin requirement of insulin-dependent diabetic women (38) and more recent evidence from experimental diabetes in rats by Costrini and Kalkhoff (17) and Shamoon and Felig (42) has given support to these observations.

Available evidence on the effects of progesterone on carbohydrate metabolism is not conclusive (133). Matute and Kalkhoff (40) found that progesterone administered in vivo increased plasma insulin levels in 24 h fasted rats and inhibited hepatic gluconeogenesis. The rate of insulin release from rat islets was enhanced after progesterone administration (134). However, there are alternative reports that progesterone does not significantly affect glucose tolerance in the rat, monkey or human despite the induction of hyperinsulinaemia (17, 45, 135). Furthermore, progesterone has been reported to have little influence on the outcome of experimental diabetes (60, 135).

The results obtained in this study indicate that daily replacement in vivo of oestradiol (5 μg/kg) and progesterone (1 mg/kg) in ovariectomised mice increased in vitro glucose uptake and metabolism by soleus muscles in the presence of insulin (0.75 mU/ml). The basal values obtained with CO_2 production indicate that physiological concentrations of sex steroids in intact

mice can promote non-insulin mediated glucose uptake. This was not observed in ovariectomised mice treated with replacement doses of sex hormones and seems to indicate that some other factor, other than oestradiol and progesterone may be responsible for promoting basal (non-insulin mediated) CO_2 production.

In vivo administration of oestradiol and progesterone individually increased the sensitivity of the muscle to insulin. This finding is compatible with the results obtained by Shamoon and Felig (42) who demonstrated that short-term administration of the naturally occurring oestradiol-17β stimulated insulin-mediated uptake of glucose by rat diaphragm muscle. It is of interest that Mckerns and Coulomb (41) found an increase in glucose utilisation and glycogen formation in rat diaphragm muscle in vitro after ethinyl oestradiol was administered in vivo. Combined oestradiol and progesterone (doses as above) suppressed CO_2 production compared with each hormone individually, but significantly stimulated glycogen formation compared with all other groups.

^{14}C-glucose that is taken up by the muscle undergoes the major metabolic fates as shown in Fig. 4.5.

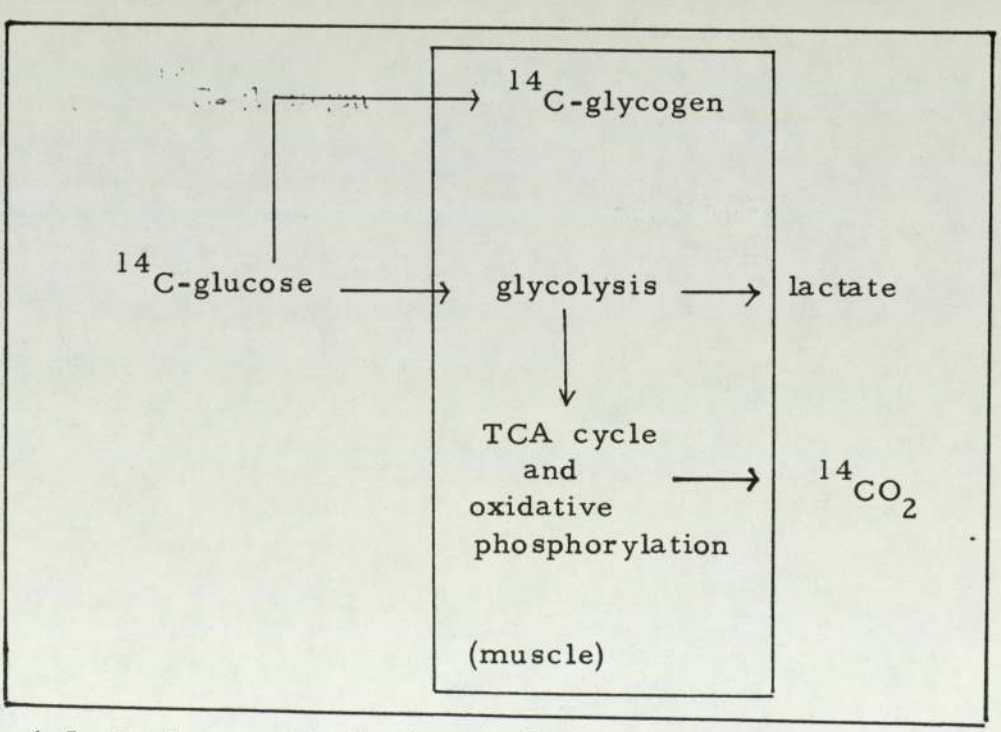


Fig. 4.5 Major metabolic fates of glucose

Thus, it appears that in vivo administration of the combined oestradiol and progesterone regimen directs a greater proportion of the glucose into glycogen, resulting in a relative decrease in CO_2 production. The difference between ovariectomised mice and the intact P/O and M/D mice indicates that a physiological oestradiol-progesterone combination produces the same effect.

The results of this study indicate that sex steroids, in particular oestradiol and progesterone individually synergise the action of insulin on glucose metabolism. In the absence of insulin, physiological concentrations of sex hormones conferred by intact ovarian endocrine function may promote glucose uptake in intact animals, manifested as an increase in CO_2 production. Since ovariectomy and physiological replacement doses of sex hormones do not result in the same effect, it is likely that another ovarian hormone may be acting synergistically with oestradiol and progesterone to promote basal glucose uptake in intact mice. However, it must be borne in mind that it is almost impossible to mimic the delicate hormonal balance found in an intact mouse, despite the use of concentrations of sex steroids very close to the physiological range. Oestradiol and progesterone alone, appear to increase the sensitivity of the muscle to insulin. This would account for reports in the literature that when sex steroids are administered at physiological concentrations to ovariectomised animals, oophrectomised women and post-menopausal women, the hypoglycaemic action of insulin is accentuated (38, 121, 122). This would also account for reports that administration of oestrogen-progestogen combinations or progesterone alone to animals or women with endogenous sex steroids may antagonise the hypoglycaemic action of insulin (23, 31, 136, 137). The increased hypoglycaemic action of insulin produced by physiological oestradiol and progesterone replacement may be attributed in part to stimulation of glycogenesis and increased catabolism of glucose via glycolysis and oxidative phosphorylation.

CHAPTER 5

EFFECT OF SEX HORMONES ON GLUCOSE UPTAKE AND PHOSPHORYLATION

5.1 Extracellular fluid (ECF) space and 3-O-methyl-α-D-glucopyranose (3-O-MG) uptake in mouse soleus muscle.

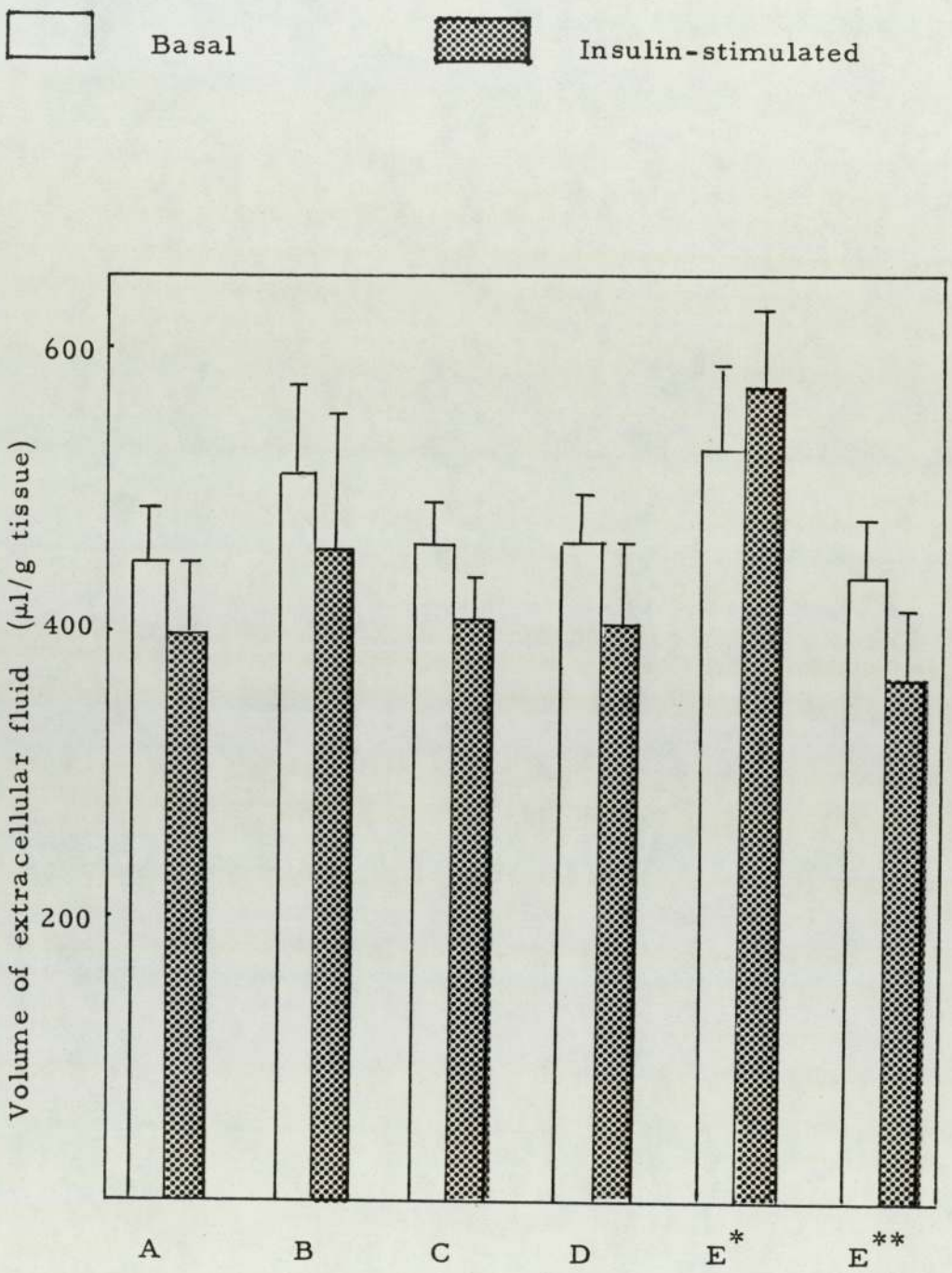
Ovarian steroid hormones, in particular oestradiol have been shown to promote glucose metabolism in the isolated mouse soleus muscle (Chapter 4). The mechanism by which sex steroids exert their influence on glucose metabolism may involve the uptake and phosphorylation of glucose. To investigate the effect of sex steroids on glucose transport per se, the glucose analogue 3-O-methyl-α-D-glucopyranose is frequently used. The accumulation of this analogue within the cells can be used as a direct assessment of glucose uptake. There is evidence that 3-O-methyl-α-D-glucopyranose uses the same membranal carrier mechanism as D-glucose (138) but the analogue is not metabolised further once inside the tissue.

A small proportion of the glucose that is taken up by the muscle accumulates in the extracellular fluid space and is not metabolised. Hence, the volume of the extracellular fluid space must be quantified to give an accurate indication of the proportion of glucose that is taken up by the cells. This study was conducted to investigate the effects of replacement doses of sex steroids in ovariectomised mice on 3-O-methyl-α-D-glucopyranose uptake in the isolated mouse soleus muscle, using ^{3}H-inulin to quantify the extracellular fluid space.

5.1.1 Extracellular fluid space

Fig. 5.1 shows the volume of extracellular fluid in soleus muscles from control and hormone treated mice. Tissue extracellular fluid volume was calculated as described in Appendix II. Long term (10 weeks) ovariectomy and treatment with sex steroids did not significantly change the volume of extracellular fluid, particularly

Fig. 5.1 Volume of extracellular fluid in soleus muscles from control and hormone treated mice. (n = 5)



A = Ovx only

B = Ovx + Oestradiol

C = Ovx + Progesterone

D = Ovx + Oestradiol+Progesterone

E^{*} = Intact proestrus/oestrus

E^{**} = Intact metoestrus/dioestrus

in the combined oestradiol and progesterone treated group (Fig5.1). However, these differences were taken into account in the calculation of 3-O-MG uptake into the cellular compartment. There were no significant variations between the right and left soleus muscles of mice in the same group, indicating that the presence of insulin which was added to the incubation medium containing the right soleus muscle did not significantly affect the extracellular fluid volume.

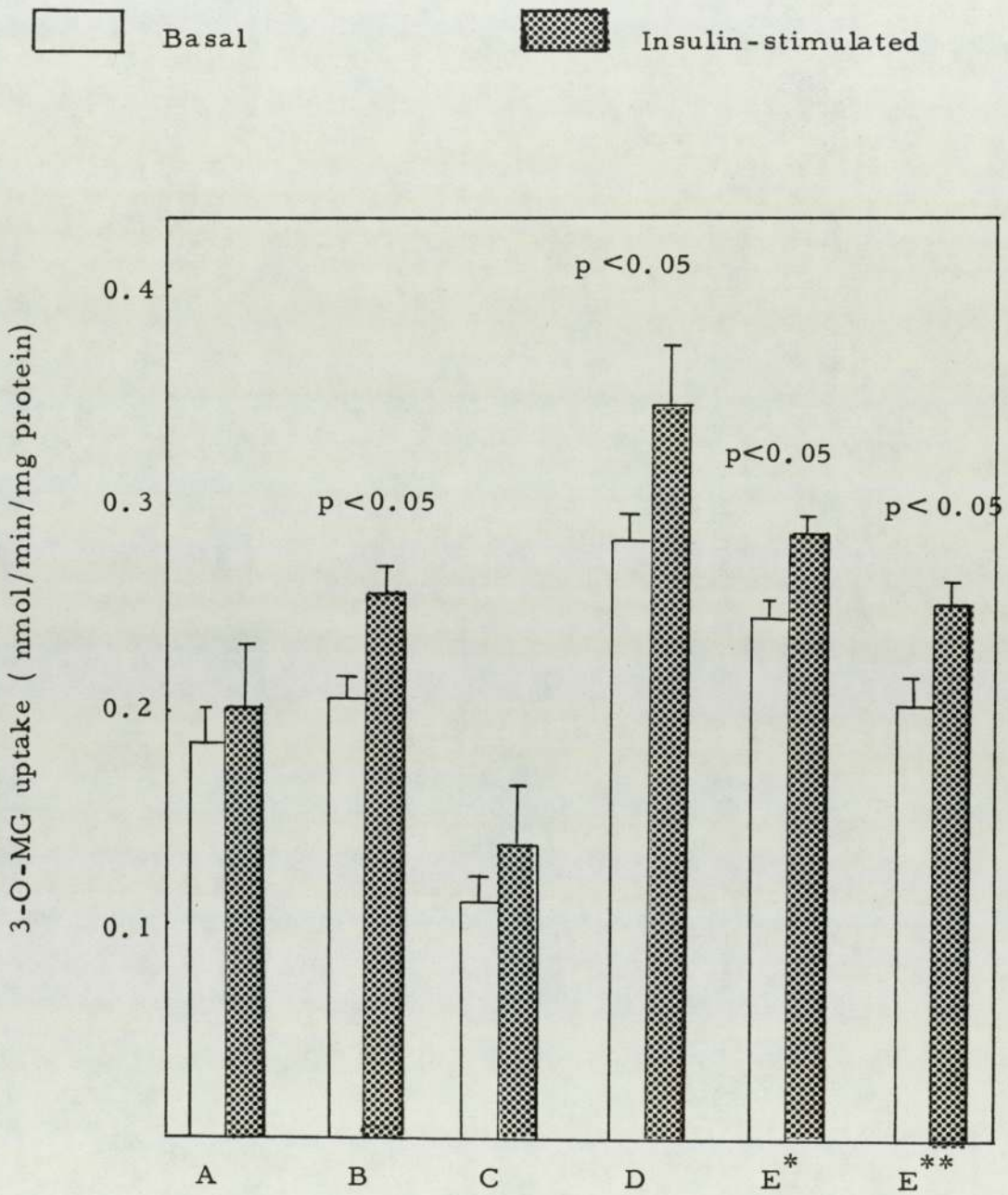
5.1.2 Uptake of 3-O-methyl-α-D-glucopyranose.

Fig. 5.2 shows 3-O-MG uptake by mouse soleus muscles in the absence and presence of insulin (0.75 mU/ml). Insulin at this concentration stimulated 3-O-MG uptake compared to basal in the intact mice and in the mice treated with oestradiol and oestradiol and progesterone combined, but not in the ovariectomised mice and in the progesterone treated mice. Progesterone alone, decreased 3-O-MG uptake under basal conditions, indicating a decrease in glucose transport. In contrast, oestradiol and progesterone in combination resulted in a larger increase in insulin-stimulated and basal 3-O-MG uptake than that observed after treatment with oestradiol alone. The results obtained with intact P/O and M/D mice when compared with ovariectomised mice indicate that the presence of endogenous sex steroids in intact mice does influence glucose transport. The absolute values of insulin-stimulated and basal 3-O-MG uptake in intact P/O mice were greater than from intact M/D mice. This is consistent with the higher titres of sex steroids in intact P/O mice (98). Thus, the results with intact mice at different stages of the oestrous cycle indicate that endogenous sex steroids in intact mice promote basal glucose transport.

The effects of sex steroid treatments on insulin-stimulated 3-O-MG uptake are readily compared in Fig. 5.3 which shows the increment in 3-O-MG uptake over basal for each treatment. While the individual oestradiol and progesterone treat-

-ments did not significantly alter the effect of insulin on 3-O-MG uptake, the combined oestradiol and progesterone regimen produced a large increase. Intact M/D mice showed a significant increase in insulin-stimulated 3-O-MG uptake compared with ovariectomised mice. This is not readily explained in the context of higher titres of endogenous sex steroids in intact P/O mice. However, as shown in Fig. 5.2, the absolute value for basal 3-O-MG uptake in intact P/O mice was much higher than in intact M/D mice. Furthermore, the insulin-stimulated increment in 3-O-MG uptake in intact P/O mice was not significantly different from that shown by intact M/D mice.

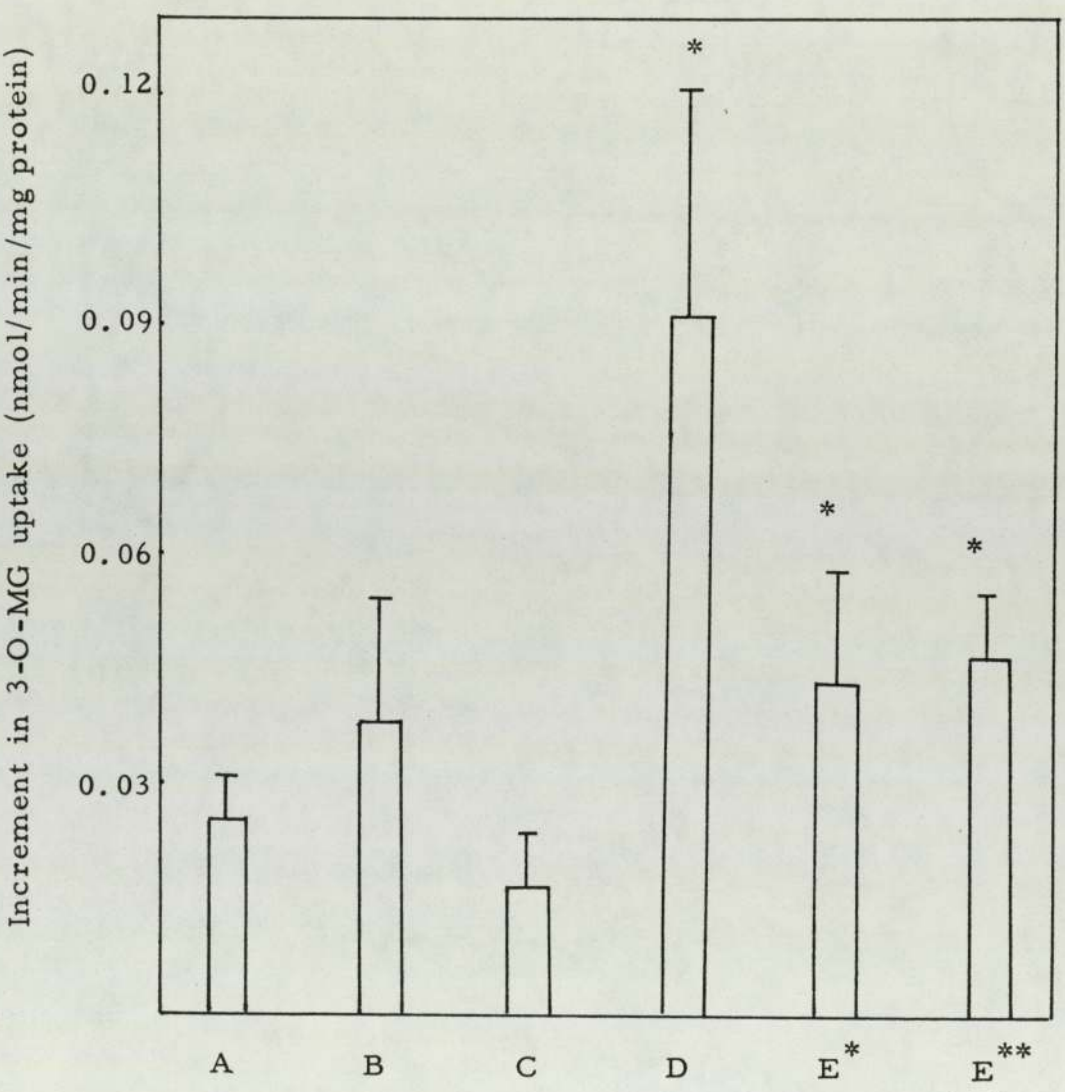
Fig. 5.2 3-O-methyl-α-D-glucopyranose (3-O-MG) uptake by mouse soleus muscles from control and hormone treated mice. p denotes significance of difference between basal and insulin-stimulated 3-O-MG uptake for each group, determined by Student's paired 't' test. Values are means ± SEM of 5 or 6 determinations.



A = Ovx only
B = Ovx + Oestradiol
C = Ovx + Progesterone
D = Ovx +Oestradiol+Progesterone
E^{*} = Intact proestrus/oestrus
E^{**} = Intact metoestrus/dioestrus

Fig. 5.3 Increment in insulin-stimulated 3-O-MG uptake above basal levels in mouse soleus muscle.

* = $p < 0.05$ compared with ovariectomised mice



A = Ovx only

B = Ovx + Oestradiol

C = Ovx + Progesterone

D = Ovx +Oestradiol+Progesterone

E^{*} = Intact proestrus/oestrus

E^{**} = Intact metoestrus/dioestrus

5.2 2-deoxy-D-glucose phosphorylation in mouse soleus muscle.

2-deoxy-D-glucose has been shown to enter the cell using the same membranal carrier as D-glucose (101). Unlike 3-O-methyl-α-D-glucopyranose, 2-deoxy-D-glucose is phosphorylated in the same manner as D-glucose but it is not metabolised thereafter (101). Hence, the accumulation of phosphorylated 2-deoxy-D-glucose within the cellular compartment provides an indication of glucose phosphorylation.

5.2.1 Extracellulr fluid space.

The values for extracellular fluid volume were similar to those obtained previously. These values have been taken into account in the calculations of 2-deoxy-D-glucose uptake . The values of extracellular fluid volume are documented in Table 5.5.

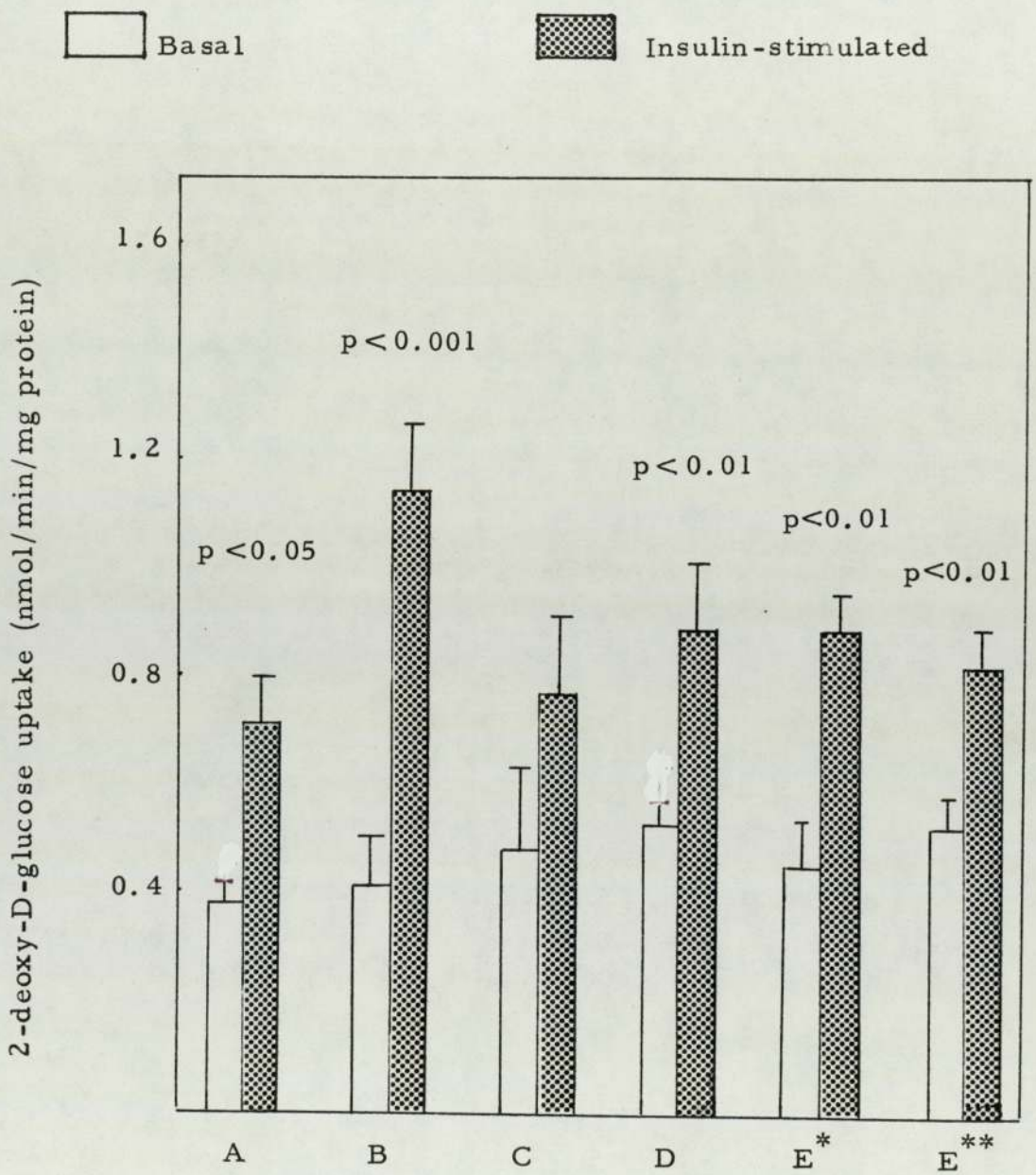
5.2.2 Uptake of 2-deoxy-D-glucose

Fig. 5.4 shows 2-deoxy-D-glucose uptake by mouse soleus muscles from intact P/O, M/D, ovariectomised control and hormone treated mice. In all cases, except in the progesterone treated mice, insulin (0.75 mU/ml) significantly increased 2-deoxy-D-glucose uptake. Oestradiol treatment resulted in a 46 % increase in insulin-stimulated 2-deoxy-D-glucose uptake which was twice the increase observed in ovariectomised mice. Insulin also significantly stimulated 2-deoxy-D-glucose uptake in intact mice and the increment in insulin-stimulated 2-deoxy-D-glucose was greater in intact P/O mice (25 %) than in intact M/D mice (15 %). Fig. 5.4 also shows that physiological concentrations of sex steroids promoted non-insulin mediated (basal) 2-deoxy-D-glucose uptake by soleus muscles. In particular, a combined oestradiol and progesterone replacement regimen in ovariectomised mice resulted a significant increase in basal 2-deoxy-D-glucose uptake compared with ovariectomised mice.

The effect of sex steroid treatments on insulin-stimulated 2-deoxy-D-glucose may be compared directly using the increment

values above basal (Fig. 5.5). The increments in oestradiol treated and intact P/O mice were respectively, approximately three times and two times greater than the value from ovariectomised mice. While oestradiol alone increased 2-deoxy-D-glucose uptake, oestradiol and progesterone in combination appeared to inhibit this effect, suggesting that progesterone antagonises the effect of oestradiol. Progesterone alone did not affect insulin-stimulated 2-deoxy-D-glucose uptake.

Fig. 5.4 2-deoxy-D-glucose uptake by soleus muscles from control and hormone treated mice, incubated in the absence and presence of insulin (0.75 mU/ml). p denotes significance of difference between basal and insulin-stimulated 2-deoxy-D-glucose uptake for each group, determined by Student's paired 't' test. Values are means ± SEM of 5,6 or 7 determinations.



A = Ovx only B = Ovx + Oestradiol

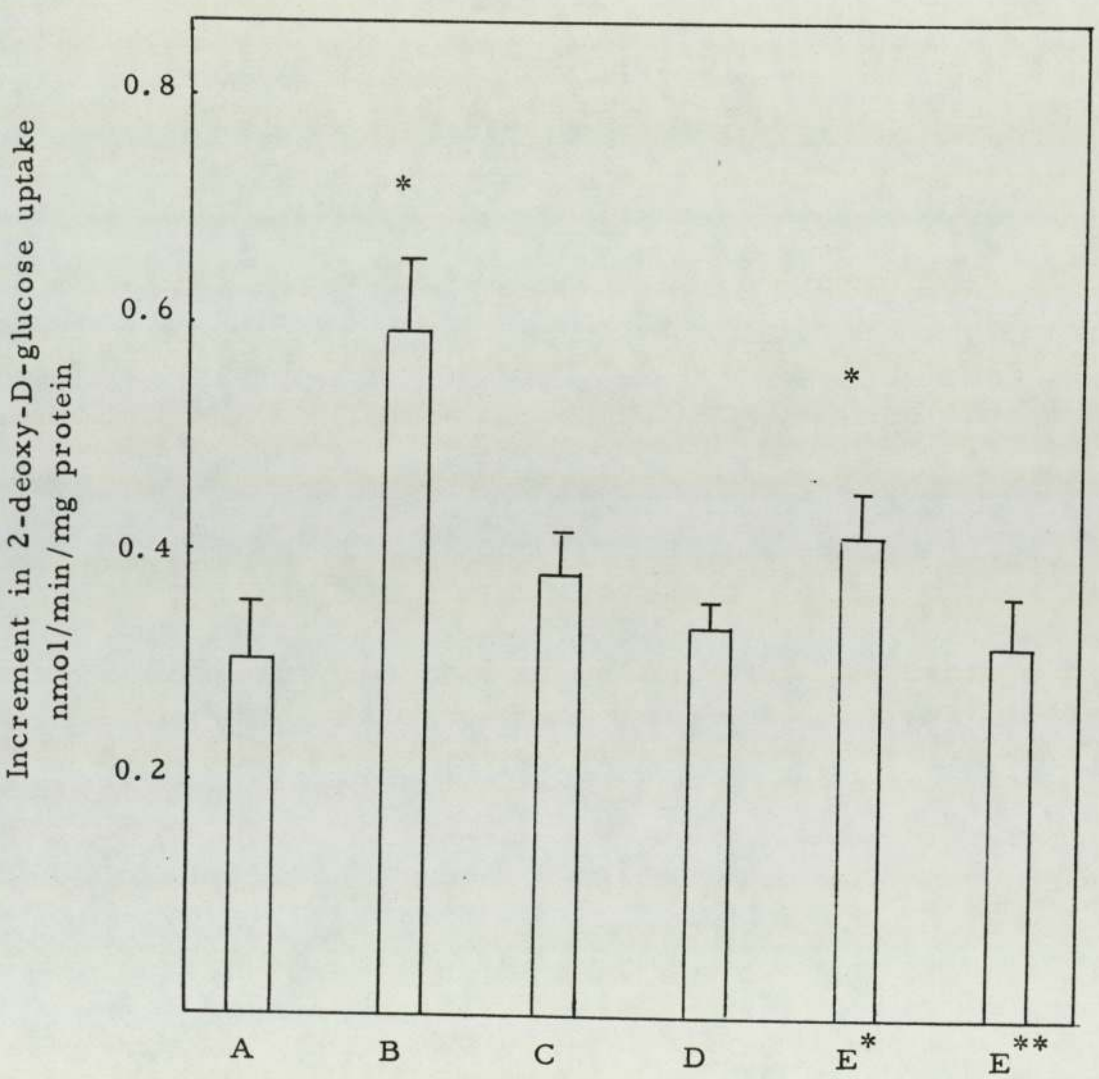
C = Ovx + Progesterone D = Ovx + Oestradiol+Progesterone

E^{*} = Intact proestrus/oestrus

E^{**} = Intact metoestrus/dioestrus

Fig. 5.5 Increment in insulin-stimulated 2-deoxy-D-glucose uptake above basal levels in mouse soleus muscle.
(n = 5, 6 or 7)

* = $p < 0.05$ compared with ovariectomised mice



A = Ovx only

B = Ovx + Oestradiol

C = Ovx + Progesterone

C = Ovx +Oestradiol+Progesterone

E^{*} = Intact proestrus/oestrus

E^{**} = Intact metoestrus/dioestrus

5.3 Discussion

The results from the study described in Chapter 4 indicated that long term ovariectomy and sex steroid replacement can affect glucose metabolism in the mouse soleus muscle. It is also clear that sex steroids modulate the influential role of insulin in the control of glucose metabolism in muscle. However, there was no evidence to indicate whether the alterations in glucose metabolism in the presence of sex steroids were derived from a specific alteration of glucose transport and/or glucose phosphorylation. This study was conducted with two aims in mind : to determine the effect of sex hormone replacement therapy on both glucose transport and glucose phosphorylation using the glucose analogues 3-O-methy-α-D-glucopyranose and 2-deoxy-D-glucose respectively. At the same time, it was deemed desirable to quantify the extracellular fluid volume in the muscle using inulinas a marker.

In the presence of insulin, oestradiol has been shown to stimulate glucose metabolism, manifest by an increase in CO_2 production and glycogen formation. There was no indication however, as to whether the increase in glucose metabolism was a result of a specific stimulation of glucose transport or glucose phosphorylation. The present study shows that 3-O-MG uptake in soleus muscles from oestradiol treated mice was significantly increased in the presence of insulin (0.75 mU/ml). Phosphorylation of 2-deoxy-D-glucose was also increased, both under basal conditions and in the presence of insulin. The results indicate that oestradiol synergises the action of insulin in promoting glucose metabolism partly by enhancing glucose transport and phosphorylation in mouse soleus muscle.

Physiological replacement doses of progesterone to ovariectomised mice increased glucose metabolism via a stimulatory effect on CO_2 production (Chapter 4). Glycogen formation was not increased, indicating that under the influence of progesterone, a greater proportion of the glucose taken up by the

muscle is directed towards glycolysis and oxidative phosphorylation rather than glycogen storage. Progesterone did not significantly alter 3-O-MG uptake and 2-deoxy-D-glucose phosphorylation in the presence of insulin. Taken together, these data suggest that the fate of glucose within the cell is adjusted by progesterone at a site beyond the level of glucose phosphorylation.

Previous reports concerning the effect of combined oestradiol and progesterone treatment on glucose metabolism have been controversial. In general, in vivo observations of changes in plasma glucose homeostasis (119, 120) suggest that the two hormones together tend to antagonise each other in this respect. The results of experiments reported in this thesis (Chapter 4) showed that oestradiol and progesterone in combination appeared to direct a greater proportion of cellular glucose towards glycogenesis with a relative decrease in CO_2 production. The increase in glycogen formation may be partly explained by the increase in insulin-mediated 3-O-MG uptake shown in this study. However, modulating effects beyond the level of glucose phosphorylation also appear to operate.

The absolute values for 3-O-MG uptake were higher and the insulin-stimulated increment in 2-deoxy-D-glucose uptake was greater in intact P/O mice than in intact M/D mice. This may be associated with the higher titres of sex steroids in P/O mice.

The volume of extracellular fluid in each group was calculated and the results indicate that long term ovariectomy and treatment with sex steroids marginally increased the volume of extracellular fluid compared with intact mice, particularly in mice treated with a combined regimen of oestradiol and progesterone.

CHAPTER 6

PRELIMINARY STUDIES OF INSULIN RECEPTOR BINDING METHODOLOGY

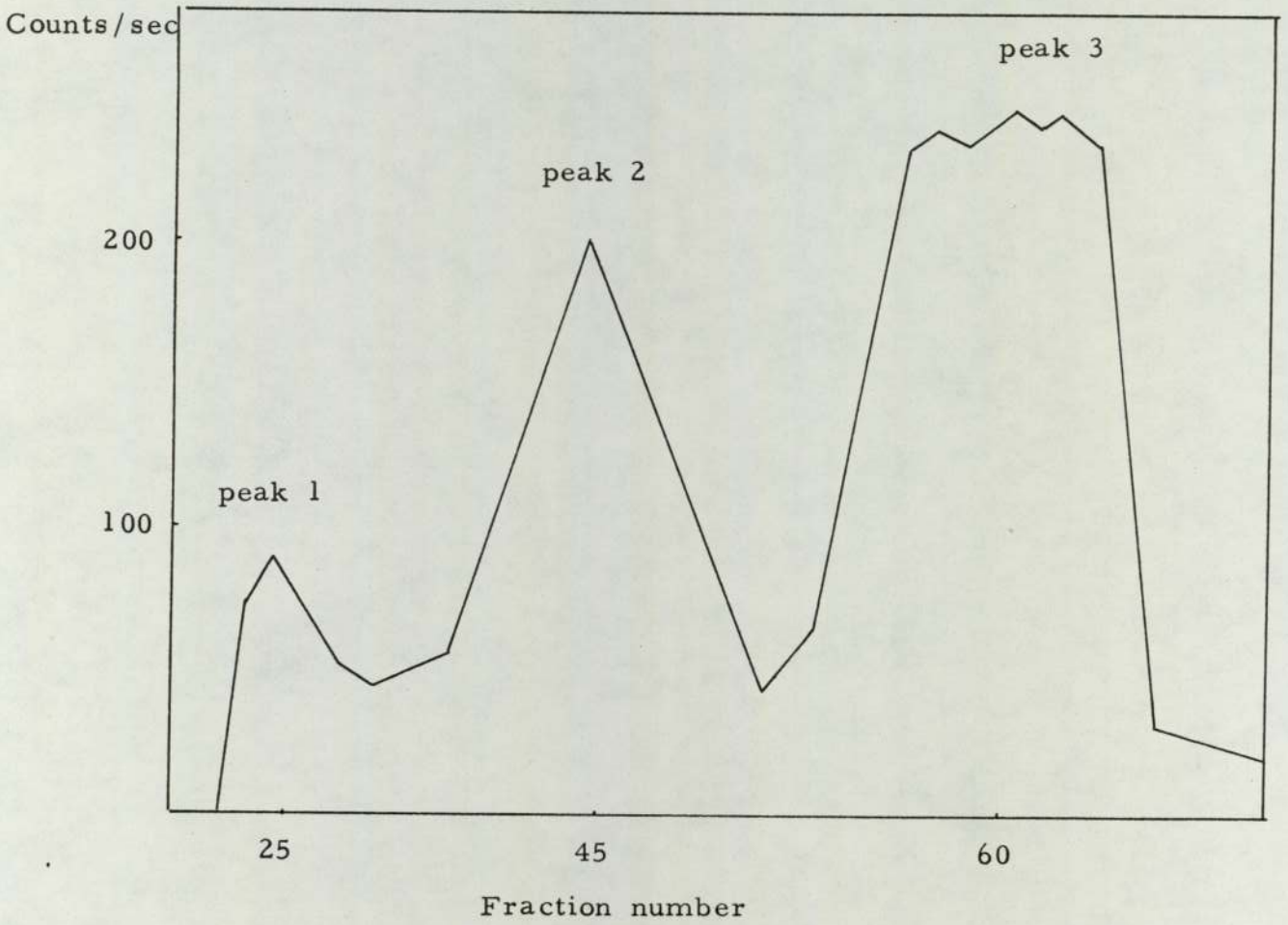
6.1 Radioiodination of insulin

The binding of insulin to its receptor in the membrane is recognised as the first step of insulin action (139). Early studies were hampered by the lack of a suitable tracer hormone, that is, one with a very high specific activity which retained identical biological and immunological properties to the native hormone. More recently, suitable labelling techniques have been developed (140, 141).

The method of labelling described here is based on that originally described by Hunter and Greenwood (103) which now serves as the 'blue-print' for all insulin iodinations. The technique yields mainly mono-iodinated hormone with minimal damage to the hormone. Briefly, Chloramine T is used to oxidise $Na^{125}I$ in the presence of insulin, with subsequent incorporation of [^{125}I]-insulin into the tyrosine residues of the hormone. The reaction is terminated with sodium metabisulphite. Excess Chloramine T and free iodide are separated from the labelled hormone by gel filtration. The method is considered in detail in Chapter 2, section 2.10.1.

Fig. 6.1 shows the elution profile of the radioactive tracer. There are three distinct peaks. The first peak to be eluted represents a high molecular weight fraction which is probably damaged and aggregated insulin. Peak 2 is the iodoinsulin and the third peak is the unreacted iodide. Radioimmunoassay of insulin in diluted aliquots of the fractions support this view (142). An analysis of the fractions from peak 2 shows that the tracer is greater than 95% precipitable with 10% TCA and greater than 90 % is bound onto charcoal. The specific activity ranged from 255 μCi/μg to 275 μCi/μg and the level of iodine incorporation was routinely 0.5-0.7 atoms of iodine per insulin molecule.

Fig. 6.1 Elution profile of Chloramine T iodination.

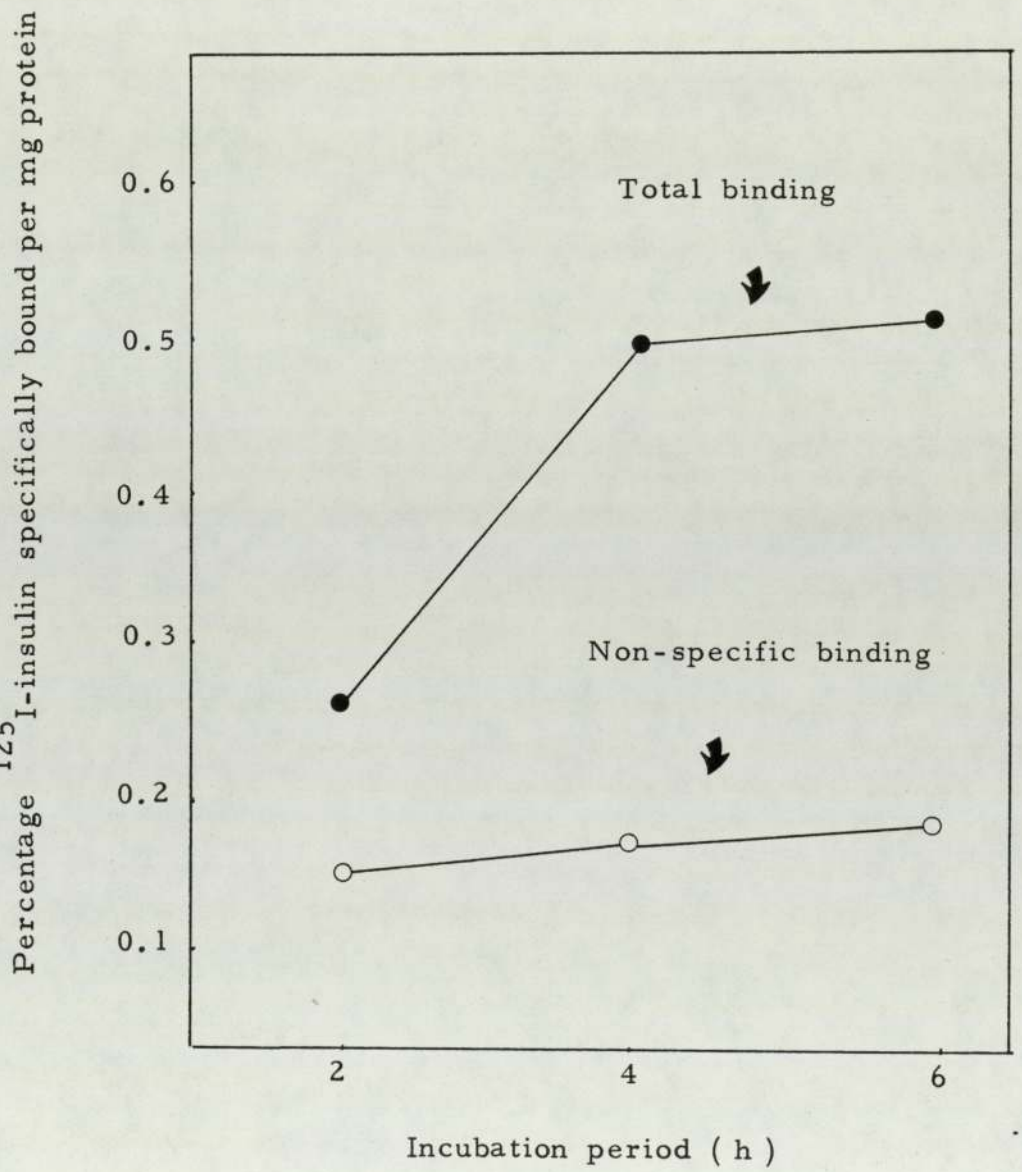


6.2 Time course of insulin receptor binding in mouse soleus muscle.

As reported in Chapter 4, oestradiol and progesterone treatments in ovariectomised female mice altered insulin-stimulated glucose uptake and metabolism in soleus muscle. Since the binding of insulin to a specific membranal receptor is the initial step in the biological action of this hormone (139), an alteration in insulin receptor binding activity represents one possible cellular location at which ovarian steroids might influence insulin action. Indeed, several recent reports in the literature have indicated that sex hormones may play a role in the control of insulin receptors (143-147). An influence of sex hormones on insulin binding has been indicated in monocytes and erythrocytes: in these cell models, insulin binding was increased during the follicular phase of the menstrual cycle (143, 148). Various changes in insulin binding to monocytes and red blood cells have also been observed during pregnancy and oral contraceptive treatment (145, 146). However, no studies of insulin binding in recognised insulin target tissues primed with ovarian sex steroids have been reported. This experiment was conducted to determine the time course of insulin binding to mouse soleus muscle, to enable selection of a suitable incubation period for subsequent studies.

Fig. 6.2 shows the time course of ^{125}I-insulin binding to mouse soleus muscle. When ^{125}I-insulin was incubated with soleus muscles, binding of the hormone increased with the duration of incubation. Binding appeared to reach a maximum at about 4 h, since a further increase in binding was not observed at 6 h. The figure shows that after a 4 h incubation at 20°C in the presence of 1 ng/ml ^{125}I-insulin, approximately 0.45 % of ^{125}I-insulin was bound per mg of muscle protein. Non-specific binding remained stable after 2 h within the range 19-33 % of total binding at all time intervals studied. Since a steady state of insulin binding was achieved at approximately 4 h, an incubation period of 4 h was selected for subsequent studies.

Fig.6.2 Time course of binding of ^{125}I-insulin to mouse soleus muscle. Vertical bars indicate the standard error of the mean. Each point is the mean of 3 determinations.



6.3 Discussion

The Hunter and Greenwood (103) technique for radiolabelling of insulin has been used successfully for the radioiodination of many peptide hormones and numerous variations of their original procedure have been reported (149). The present modification was designed to yield mono-iodinated insulin with a high specific activity (250-275 μCi/μg), and to retain the same insulin receptor binding properties as the native hormone. This was achieved with an average incorporation of 0.5-0.7 iodine atoms per molecule of insulin.

The ^{125}I is preferentially incorporated into the tyrosine residues of the hormone (149) particularly tyrosine residues on the A chain (Tyr A_{14} and Tyr A_{19}). Since residue A_{19} is important for the maintenance of the three dimensional structure of the insulin molecule (150), the position of iodination may influence the biological activity of the radioactive tracer. However, monoiodination of insulin usually occurs at residue A_{14}, except at extreme pH values. Since extreme pH values were not encountered in the present radiolabelling technique, the hormone conformation is not likely to be affected (151). The insulin iodinated in this manner was subsequently used in a preliminary experiment to determine the time course of insulin binding in mouse soleus muscle.

Recent reports in the literature have indicated that sex steroids might affect insulin action via an effect on the concentration of binding affinity of insulin receptors. An inverse relationship between the insulin receptor concentration and oestrogen level has been suggested on the basis of changes during the menstrual cycle and pregnancy (143). A decrease in insulin receptor concentration has been reported following administration of corticosteroids (152, 153), gestagens (154) and during the third trimester of pregnancy (148). This evidence has been derived from in vivo observations or in vitro work on rat adipocytes. To date, no published work has been carried on the effects of individual ovarian

sex steroids on the insulin binding capacity of skeletal muscle. It is proposed to conduct an experiment to investigate the effects of oestradiol and progesterone on the insulin binding capacity of the mouse soleus muscle, selecting the appropriate time course for the interaction between insulin and the receptor in the muscle preparation at 20°C. The present experiment suggests that a time course of 4 h is an appropriate incubation period, because maximum binding at 20°C is achieved within this time period.

Ovariectomised mice were used in this study to eliminate the effects of endogenous sex steroids on insulin binding. There is no evidence from the lietrature to suggest that the presence of sex steroids might alter the time required for equilibrium binding to be achieved. However, on the basis of experiments reported in Chapters 4 and 5, the level of binding after a 4h incubation period might be expected to vary in the presence of sex steroids. Mice used in the binding experiment were fasted for 40h to be consistent with previous studies on glucose uptake and metabolism (Chapters 4 and 5). In 1979, Le Marchand and Freychet (85) demonstrated that a 48h fast increased binding affinity in mouse soleus muscles compared with muscles from fed mice. This probably reflects the lifting of a down-regulatory effect of endogenous insulin, since endogenous insulin concentrations are lowered in fasting (155).

In conclusion, data presented in Fig. 6.2 indicate that at 20°C and 1 ng/ml ^{125}I-insulin, a steady state of insulin binding was achieved by 4 h. At an incubation period of 4 h, 0.45% of ^{125}I-insulin was bound per mg protein. Non-specific binding remained stable after 2 h incubation and did not exceed 33 % of total binding. Since steady state binding was achieved at approximately 4 h, an incubation period of 4 h has been selected for subsequent studies.

CHAPTER 7

EFFECT OF SEX HORMONES ON INSULIN RECEPTOR BINDING

7.1 Comparison of insulin binding capacity in soleus muscles from steroid treated mice in the fed and fasted state.

As mentioned in Chapter 1, most of the evidence that ovarian hormones influence carbohydrate metabolism has been derived by inference from in vivo observations of changes in plasma glucose and insulin homeostasis during different reproductive states and after administration of natural oestrogens and progestogens (25, 57, 131, 156). Considerable supporting evidence has been obtained from observations during pregnancy (70, 157-159) and oral contraceptive treatment (10, 11, 30, 160, 161). Several authors have reported an effect of ovarian steroids on glucose metabolism in isolated tissues (40, 42, 46, 54). In particular, certain of these studies (40, 42) and studies using a perfused rat hind limb (162) suggest a more direct effect of ovarian hormones on glucose metabolism in skeletal muscle. Indeed, results of experiments reported in Chapter 4 showed that physiological concentrations of oestradiol and progesterone have a stimulatory effect on insulin-mediated CO_2 production and glycogen formation in the isolated mouse soleus muscle.

The mechanism of action of sex hormones on glucose metabolism in skeletal muscle remains uncertain, but alterations at the level of transport, phosphorylation or enzyme induction are implicated (Chapters 4 and 5). As noted earlier, recent clinical observations have suggested that variations in sex hormone levels during the menstrual cycle (143, 144) and pregnancy (146, 148, 163) may be related to changes in insulin binding to erythrocytes and monocytes. Studies using erythrocytes from normal women in different phases of the menstrual cycle have been interpreted to suggest an inverse relationship between sex hormone levels and insulin binding : insulin binding is higher during the

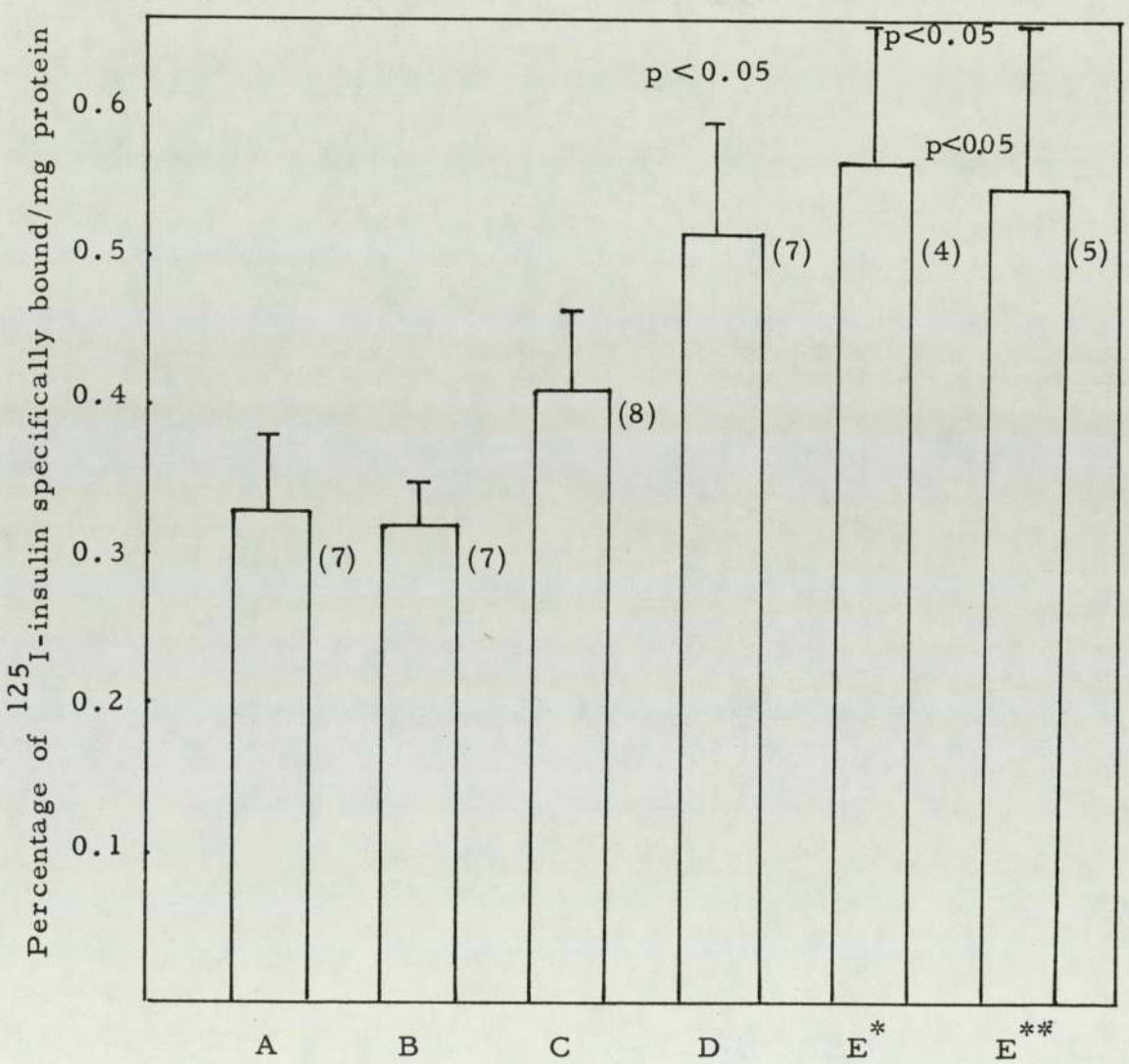
follicular than the luteal phase (143). During the third trimester of pregnancy however, insulin binding to erythrocyte insulin receptors are not decreased (146), as might be expected if the above interpretation is correct since there is an elevation in the levels of oestrogens (17) and progesterone (17) at this time. However, other hormones known to influence insulin binding such as cortisol (164,165) and human placental lactogen levels (166) are also very high at this time and might possibly alter the effects of sex hormones.

The entire picture relating insulin receptor status and sex hormones remains confused. A need clearly exists for an in vitro investigation of the effects of sex hormones on insulin receptor status to avoid interference from other factors which intervene in vivo. The present study was therefore undertaken to determine the effects of single and combined regimens of oestradiol and progesterone on insulin binding in the isolated mouse soleus muscle.

7.1.1 Percentage of ^{125}I-insulin specifically bound to soleus muscles of fed ovariectomised mice treated with ovarian sex steroids.

Fig.7.1 shows the effects of sex steroid treatment on insulin binding in soleus muscles from fed mice. Ovariectomy reduced (36 %) the amount of ^{125}I-insulin specifically bound compared with intact (P/O and M/D) female mice. This supports the contention that sex hormones affect insulin receptor status. Replacement doses of either oestradiol or progesterone however, did not significantly alter insulin binding. When the two hormones were administered together, the amount of ^{125}I-insulin specifically bound was increased by 60 %.

Fig. 7.1 Insulin binding in soleus muscles from fed intact, ovariectomised and ovariectomised steroid treated mice. p denotes significance of difference compared with ovariectomised controls, determined by Student's ' t ' test. Figures in parenthesis denote the number of mice used. Values are expressed as percentage of ^{125}I-insulin specifically bound per mg protein, obtained by subtracting non-specific binding from total binding.



A = Ovx only B = Ovx + Oestradiol
C = Ovx + Progesterone D = Ovx + Oestradiol+Progesterone
E* = Intact proestrus/oestrus
E** = Intact metoestrus/dioestrus

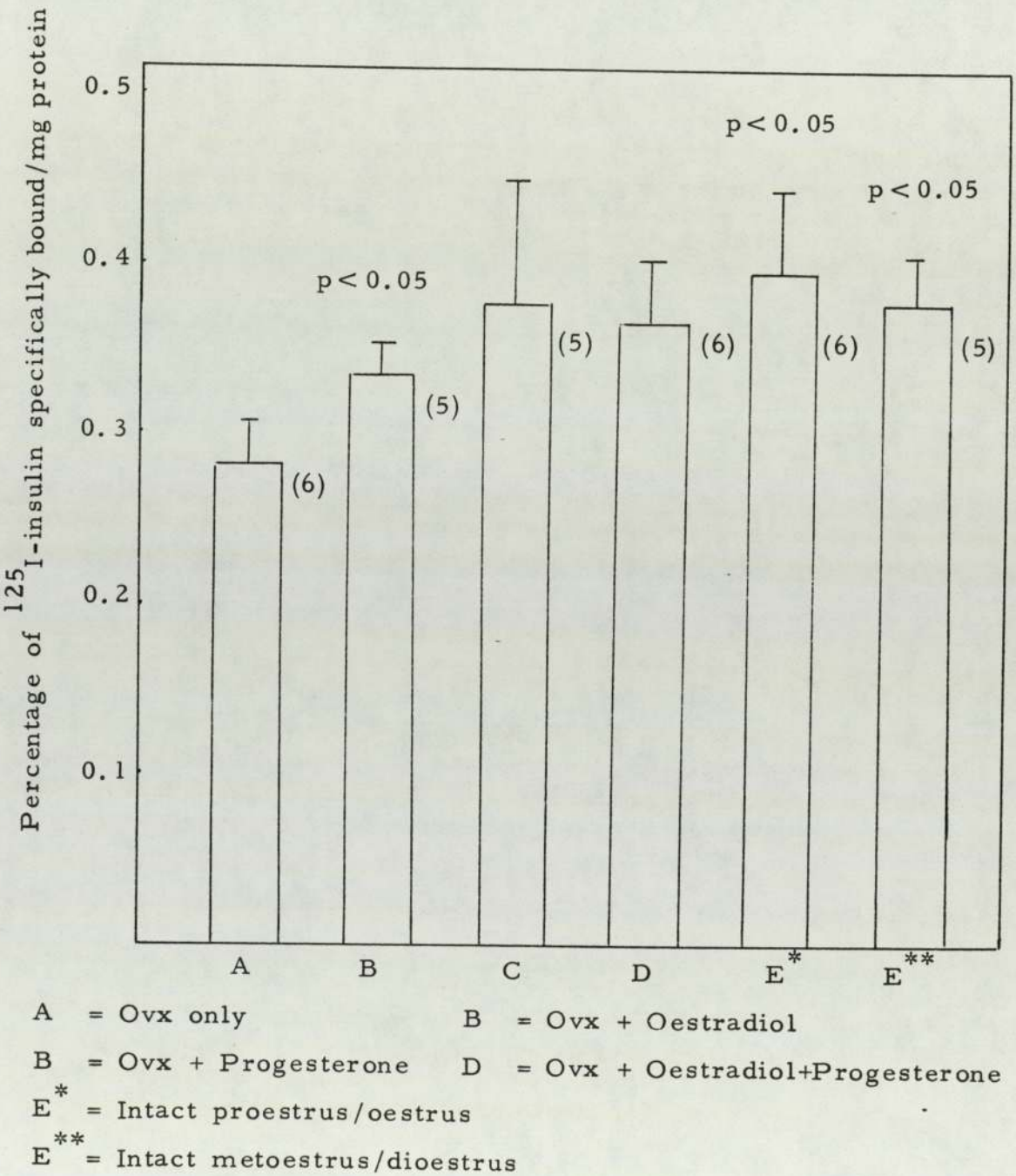
7.1.2 Percentage of ^{125}I-insulin specifically bound to soleus muscles of 40 h fasted ovariectomised mice treated with ovarian sex steroids.

Fig. 7.2 shows the percentage of ^{125}I-insulin specifically bound per mg protein in muscles from fasted intact, ovariectomised and ovariectomised steroid treated mice. The amount of ^{125}I-insulin specifically bound at 20°C ranged from 0.25 % to 0.45 % per mg protein and represents 40-60 % of total binding.

Ovariectomy reduced specific insulin binding by 20 % compared with intact P/O and M/D mice indicating a role of sex hormones in the control of insulin receptor function. Replacement with oestradiol (5 μg/kg/day) for 10 weeks increased binding by 19 % compared with ovariectomised controls. This is consistent with the increased biological responses to insulin observed in the soleus muscle (Chapter 4) and other tissues (40, 42, 46) after oestradiol treatment. The biological responses include an increase in both insulin-mediated glucose uptake and insulin-mediated glucose metabolism. When progesterone was administered alone or in combination with oestradiol, insulin binding was not significantly increased above ovariectomised controls.

Comparison of data presented in Figs 7.1 and 7.2 demonstrate the effects of nutritional status on specific binding of ^{125}I-insulin. In untreated ovariectomised mice and in ovariectomised mice treated with oestradiol or progesterone alone, there was no significant difference in specific binding of ^{125}I-insulin between fed and 40 h fasted states. Treatment of ovariectomised mice with the combined regimen increased binding in fed mice only while specific insulin binding was increased in both fed and 40 h fasted mice.

Fig.7.2 Insulin binding in soleus muscles from fasted intact, ovariectomised and ovariectomised steroid treated mice. p denotes significance of difference compared with ovariectomised controls, determined by Student's ' t ' test. Figures in parentheses denote the number of mice used.



A = Ovx only B = Ovx + Oestradiol

B = Ovx + Progesterone D = Ovx + Oestradiol+Progesterone

E* = Intact proestrus/oestrus

E** = Intact metoestrus/dioestrus

7.2 Discussion

Changes insulin binding to receptors are common in states of altered carbohydrate metabolism and altered insulin sensitivity. For example, reduced insulin receptor binding has been noted in obesity (167), diabetes mellitus (168-170) and increased binding has been reported in anorexia nervosa (171). Evidence suggesting an association between insulin receptor binding and ovarian hormones is derived mainly from changes in binding observed during different phases of the menstrual cycle (143, 144), pregnancy (146, 148, 163) and after oral contraceptive use (145, 172).

Although tentative correlations between sex hormones and insulin receptors have been proposed from these in vivo observations, an investigation of the effects of individual and combined sex hormone treatment on insulin receptor function has not been reported. This study was conducted to determine the effects of sex hormones on insulin binding in the isolated mouse soleus muscle. There is evidence to suggest that sex hormones can modify the action of insulin on carbohydrate metabolism in muscle and other tissues. As discussed in previous chapters, in vitro studies in particular, have shown that oestradiol has a stimulatory effect on glucose uptake and metabolism in mouse soleus muscle (Chapter 4), rat diaphragm (40, 42), liver (173) and uterine tissue (46, 174). Furthermore, oestrogens and progestogens have been shown to influence other membranal receptors : oestrogen pretreatment increases the number and affinity of oxytocin receptors in the uterus (175) and increases the uterine concentration of specific progesterone receptors (176). A recent report (177) has also shown that oestradiol can increase the number of progesterone receptors as well as stimulate the translocation of oestrogen receptors in rat pituitary and uterus.

The results of the present study show that sex hormones can affect the binding of insulin to its receptor. Ovariectomy reduced binding in both fed and fasted mice compared with intact

P/O and M/D mice. Replacement with physiological concentrations of oestradiol alone increased insulin binding to soleus muscles from fasted mice but not to soleus muscles from fed mice. The oestradiol induced increase in specific binding observed in fasted mice may be correlated with the increase in insulin-mediated CO_2 production and glycogen formation noted in previous experiments (Chapter 4). The apparent lack of effect of oestradiol treatment on insulin receptor binding in fed mice may be interpreted in the context of a recent report by Brady, Goodman, Kalish and Ruderman (178). These authors reported that compared with the fed state, fasting enhanced the rate of insulin-mediated glycogen synthesis, glycolysis and glucose uptake in the isolated rat soleus muscle. They suggested that this was due to both an increase in insulin binding and to increased responsiveness of the post-receptor effector sequence for insulin. In a similar study using the rat diaphragm, Shamoon and Felig (42) showed that short-term administration of oestradiol in vivo increased insulin-mediated glucose uptake but decreased basal glucose uptake. The authors attributed the decrease in basal uptake to the high intracellular stores of energy in the tissues isolated from fed animals, resulting in a decreased requirement for glucose. The lack of effect of oestradiol on specific insulin binding in fed mice noted in the present study may also reflect the condition of the muscle after prolonged treatment (10 weeks) with this hormone. Oestradiol treatment has been shown to increase muscle glycogen stores (179) which provide a reserve supply of energy. In the fed state, it may be advantageous for the muscle to operate a feed-back mechanism which inhibits any further increase in the rate of glucose uptake above normal rates, that is, above that which would result from normal physiological fluctuations in insulin concentrations. Control by feed-back inhibition is likely to be initiated at a post-receptor location and could suppress insulin receptor binding. This form of regulation might be related to the absolute amount of glycogen; relative activities of glycogenic

and glycogen degrading enzymes; the rate at which glycogen is mobilised to glucose-6-phosphate and metabolised via glycolysis or the overall energy status (ATP and NAD reserves) of the cell. Previous studies have shown that oestradiol affects the rates of glycogen formation and glycolysis (Chapter 4) suggesting that this hormone could influence the concentration and flux of metabolic intermediates, and so initiate a feed-back mechanism. During a 40 h fast, the cell's glycogen and ATP reserves are mobilised for expenditure of energy. Under these circumstances, the energy potential of the tissue is rapidly depleted. This might therefore remove the feed-back inhibition on insulin receptor binding, which is consistent with an increase in insulin binding after oestradiol treatment in 40 h fasted mice. The events can be depicted as shown in Fig. 7.3.

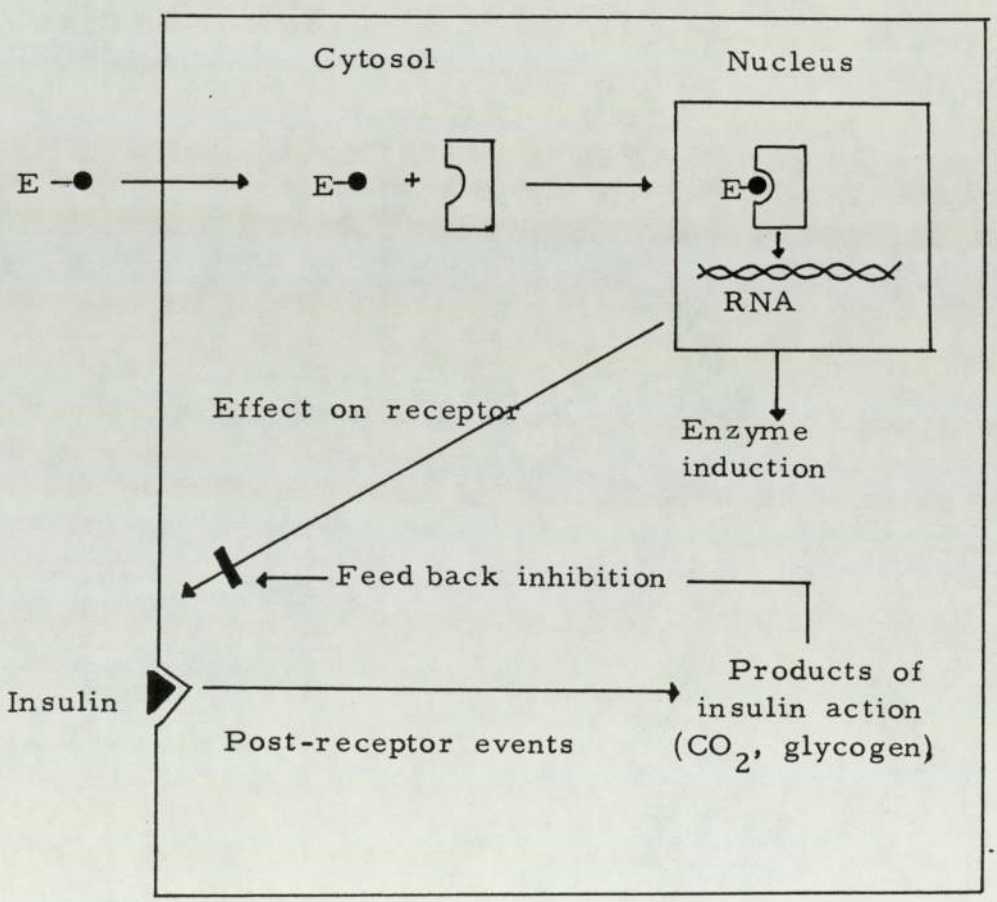


Fig. 7.3 Proposed mechanism of action of oestradiol on insulin-stimulated glucose metabolism.

Administration of replacement doses of progesterone alone to ovariectomised mice did not increase insulin receptor binding in either fed or 40 h fasted mice. This is consistent with results obtained in earlier experiments in which it was shown that progesterone did not significantly alter insulin-mediated glycogen deposition, 3-O-methyl-α-D-glucopyranose uptake or 2-deoxy-D-glucose uptake into mouse soleus muscle. This prompted speculation (Chapter 4) that progesterone may act at a post-receptor location.

When oestradiol and progesterone were administered together, binding was increased in muscles from fed but not from 40 h fasted mice. There is evidence to indicate that progesterone antagonises some effects of oestradiol on glucose metabolism (132,179) and results obtained from previous experiments with fasted mice support this view. It appears that alterations at the level of the insulin receptor may contribute to the antagonism between the two steroids. However, it is difficult to account for the increased insulin receptor binding in fed mice after the combined treatment, since it might be expected that the muscle, with its high energy reserves, would require less glucose. It is possible that the combined treatment does not accumulate glycogen stores in soleus muscle to the level produced by oestradiol alone. This has been observed in other skeletal muscles (179) but has not been examined in soleus muscle.

The levels of insulin binding achieved in this study are consistent with the in vitro biological responses of insulin in soleus muscles observed previously after steroid treatment. However, these observations are difficult to equate with clinical in vivo studies which have attempted to correlate the level of binding with the sex hormone titres during different reproductive states. Most of these studies conform with the interpretation that insulin binding is decreased in the presence of high sex hormone levels, based on observations during the luteal phase of the menstrual cycle (146), during the third trimester of pregnancy

(172) and oral contraceptive usage (145). It must be stressed that in these studies, insulin binding was assessed using monocytes and erythrocytes which may not be representative of the main insulin target tissue, namely skeletal muscle. Furthermore, evidence has been presented by Flint (163, 147) to contradict these reports. Flint reported an increase in insulin receptor numbers during pregnancy and lactation (147, 163). In contrast, Hendricks, Lippe, Kaplan, Hertz and Scott (180) found no difference in insulin binding in erythrocytes from adult females and children, although binding was increased in erythrocytes from men. This led Hendricks et al (180) to speculate that androgens may also influence insulin binding.

The results of this study indicate that sex hormones administered in vivo can affect insulin receptor status and this may be responsible in part for their effects on insulin-mediated glucose metabolism. Compared with their fed counterparts, 40 h fasted mice showed increased insulin binding in soleus muscles from intact mice and from mice treated with the combined oestradiol-progesterone regimen. However, no significant differences in binding were observed between fed and 40 h fasted mice after the individual hormone treatments. The results emphasise the importance of defining nutritional state when determining specific insulin binding.

CHAPTER 8

EFFECT OF SEX HORMONES ON GLUCO-REGULATORY ENZYMES

8.1 Ovarian steroid hormones and gluco-regulatory enzymes in mouse soleus muscle.

Previous chapters have presented evidence that the ovarian hormones oestradiol and progesterone contribute to the regulation of carbohydrate metabolism by modulating the effects of insulin on glucose metabolism. Although much of the available evidence from the literature has been derived from in vivo observations during oral contraceptive administration (26, 31), pregnancy (14) and the oestrous cycle (97), several authors have reported a direct effect of ovarian hormones on insulin-mediated glucose metabolism in vitro (40, 42, 46, 54). In general , it appears that naturally occurring oestrogens may improve the hypoglycaemic effect of insulin, although the effect is often offset when progesterone is present (162). Recently, clinical studies have sought a correlation between insulin receptor status and circulating sex hormone titres during different reproductive states in the female (143, 144, 146). However, the alterations of insulin receptor status in response to sex steroids have been obscured by concomitant changes in other hormones (145, 163, 180).

Nevertheless, the evidence clearly indicates that sex hormones influence carbohydrate metabolism by modulating insulin-mediated control of cellular glucose uptake and metabolism via effects at the level of the insulin receptor and at post-receptor sites of insulin action. Using an isolated mouse soleus muscle preparation, the effects of single and combined replacement doses of oestradiol and progesterone on glucose metabolism were determined, as described in Chapter 4. Subsequent studies . outlined in Chapter 5 indicated that the mechanism of action of sex hormones on glucose metabolism may be mediated via alterations in glucose transport and/or phosphorylation. This chapter describes experiments to evaluate the relationship between

ovarian steroid hormones and the activity of the enzymes hexokinase, glycogen synthase and 2-oxoglutarate dehydrogenase in mouse soleus muscle.

8.1.1 Hexokinase (Glucose ATP 6 phosphotransferase, EC 2.7.1.1)

The studies of Racker, Lowry and their co-workers (181-184) demonstrated the the mammalian hexokinases play a central role in glycolytic regulation. The majority of these studies were directed mainly towards the investigation of control sites in glycolysis ; consequently, little attention was given to the role of hormones in the modulation of hexokinase activity. Insulin is known to stimulate glycolysis (185, 186) but extensive studies designed to examine a possible relationship between hexokinase activity and the cellular action of insulin have provided conflicting and inconclusive results (187-190). Other observations indicate that the hexokinase from heart muscle, liver and other insulin sensitive tissues is subject to control by hormones such as cortisol and growth hormone (191, 192). Furthermore, addition of thyroxine increased glucokinase activity in livers from thyroidectomised rats (193).

The effect of ovarian hormone therapy on glucose uptake in mouse soleus muscles has been examined earlier (Chapter 4). The results suggest that replacement doses of oestradiol and progesterone to ovariectomised mice can modify the effect of insulin on glucose transport and glucose phosphorylation. In the absence of added insulin, membrane transport is known to be the major rate limiting step for glucose uptake (194-196). However, when glucose transport is stimulated by the addition of insulin, the intracellular concentration of glucose rises to measurable levels, and phosphorylation by hexokinase becomes a rate limiting step for glucose uptake (197-199). This rate limiting step therefore presents a possible site of action by oestradiol and progesterone. Indeed, studies on oestrogen control of carbohydrate metabolism in uterine tissue have indicated that

oestrogens exert an effect on carbohydrate metabolism by acting at a location prior to the intracellular formation of glucose-6-phosphate (174). There is also evidence to show that oestradiol monobenzoate may influence uterine carbohydrate metabolism by an action exerted at the level of hexokinase (200).

Fig. 8.1 shows the rate of hexokinase reaction in relation to time, quantitated by spectrophotometric measurement of NADPH formed at 340 nm. After an initial lag phase of 1 min in this experiment, the reaction is linear up to 11 minutes. The lag phase represents the time taken for the coupling intermediate glucose-6-phosphate to attain a concentration sufficient to maintain reaction C below.

A. $\text{ATP} + \text{creatine} \xleftarrow{\text{creatine kinase}} \text{creatine phosphate} +$

B. $\text{ATP} + \text{glucose} \xrightarrow{\text{hexokinase}} \text{glucose-6-phosphate} + \text{ADP}$

C. $\text{Glucose-6-phosphate} + \text{NADP}^{+} \xrightarrow{\text{glu-6-P dehydrogenase}} 6\ \text{phosphogluconolactone} + \text{NADPH} + \text{H}^{+}$

The 6 phosphogluconolactone formed in reaction C may act as a substrate for enzymes of the hexose monophosphate shunt such as lactonase and 6 phosphogluconate dehydrogenase, with further production of NADPH. However, since the hexose monophosphate shunt is negligible in skeletal muscle (95), correction for the presence of these two enzymes was not attempted.

Fig. 8.1 Rate of hexokinase reaction over a period of 10 minutes at 25 C.

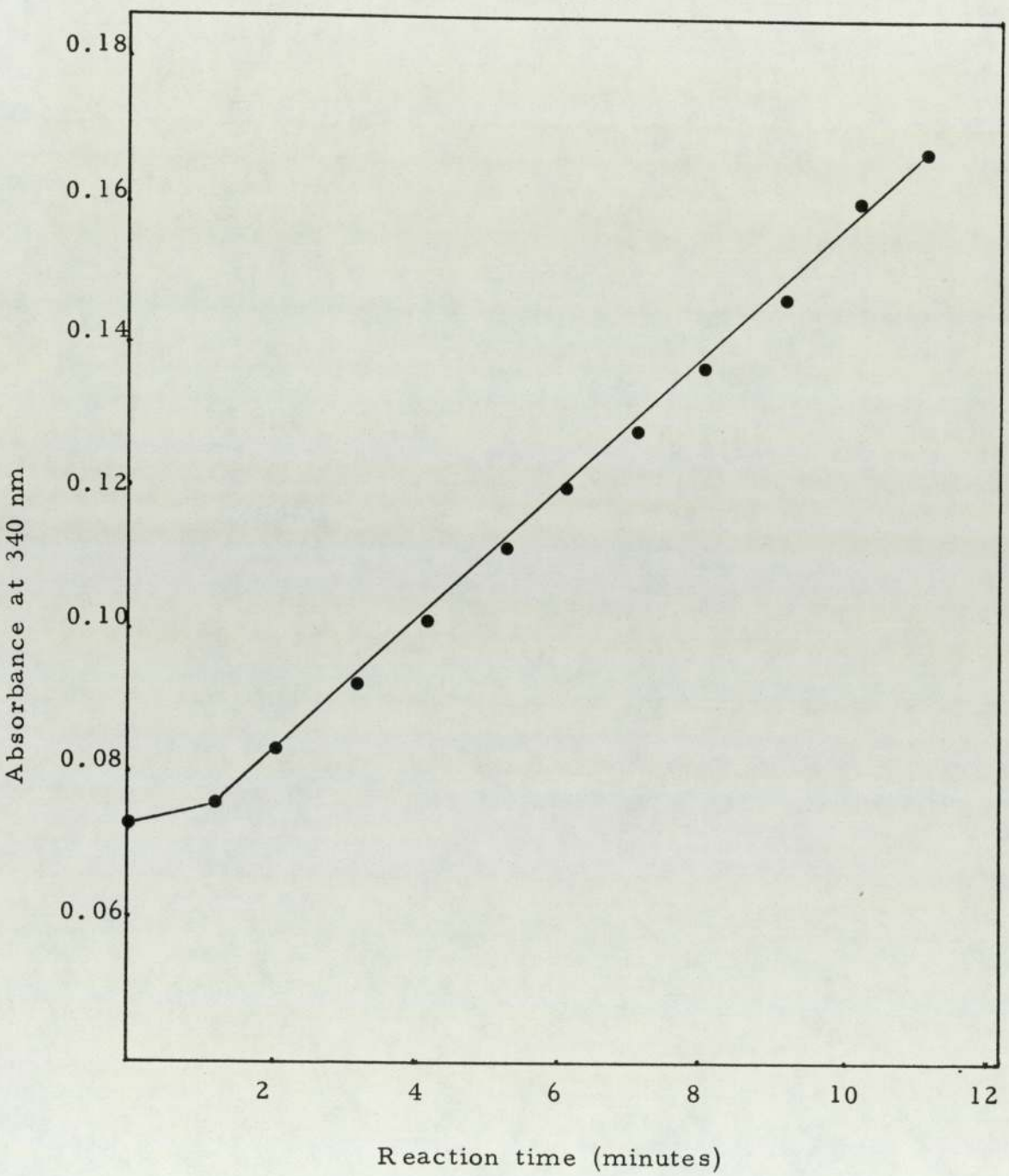
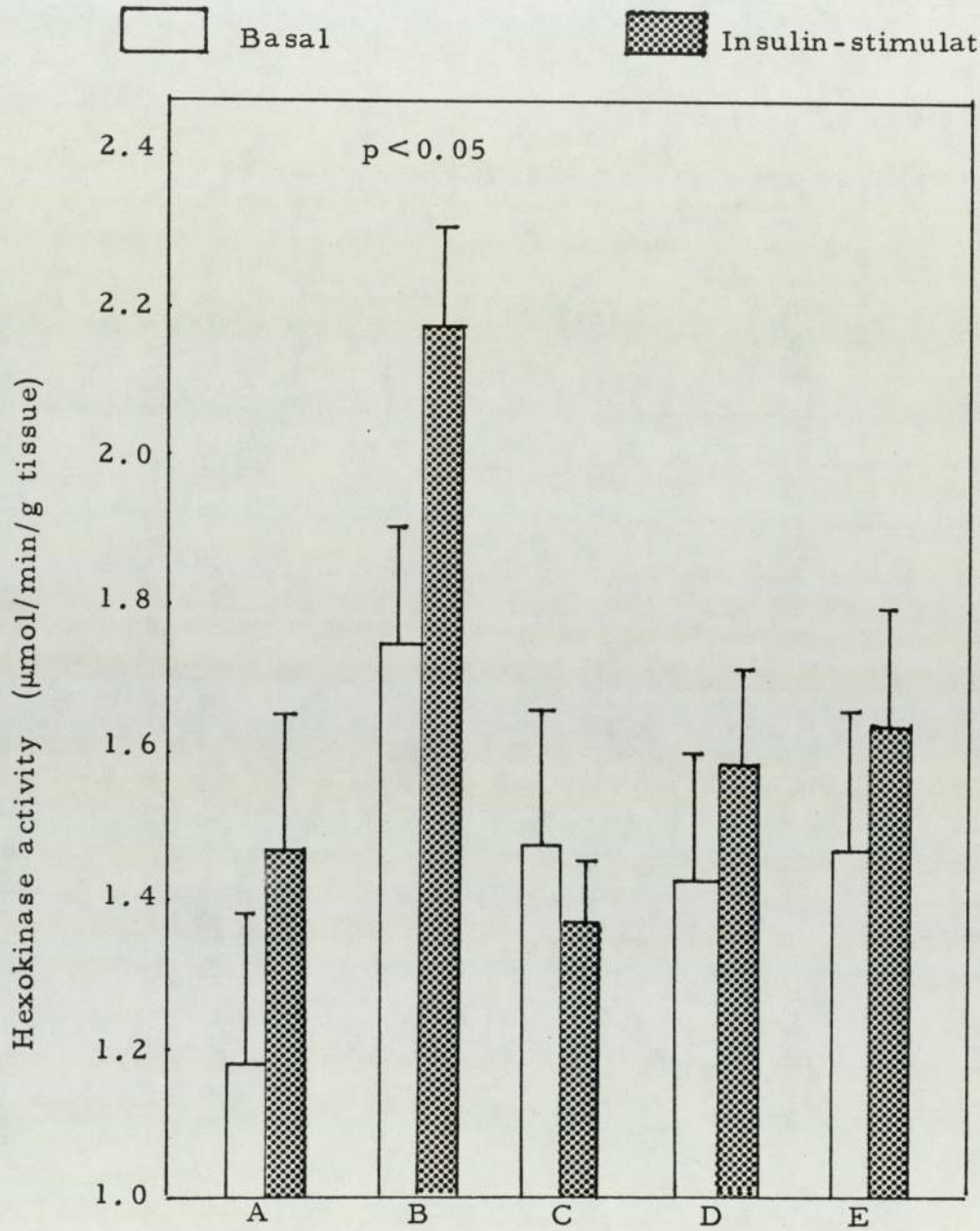


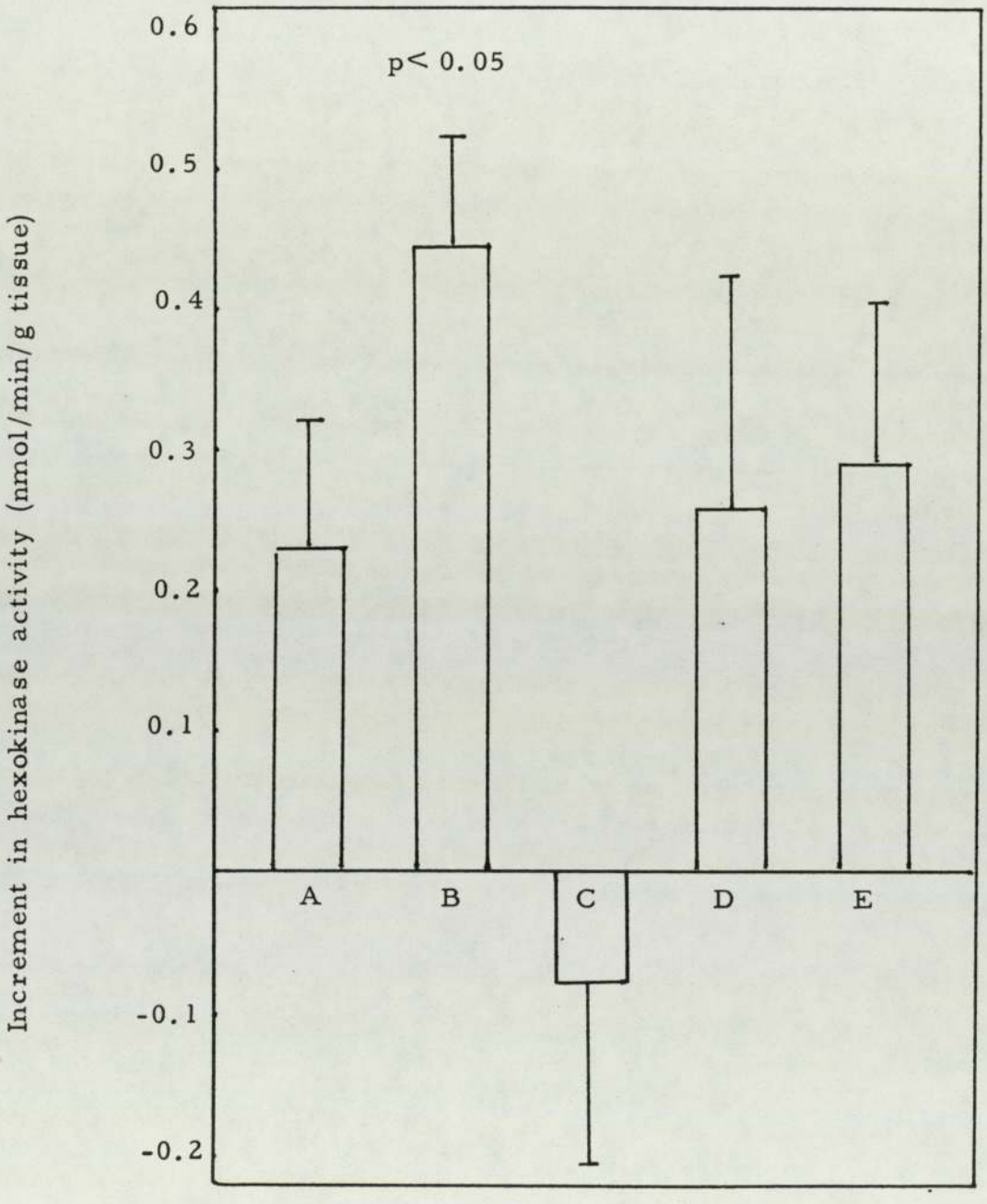
Fig. 8.2 shows the activity of hexokinase in soleus muscles from intact proestrus-oestrus mice, ovariectomised mice and ovariectomised mice treated with replacement doses of oestradiol and progesterone. Compared with intact proestrus-oestrus mice, hexokinase activity in the absence of insulin was not significantly altered by ovariectomy or treatment with progesterone alone or in combination with oestradiol. However, treatment with oestradiol alone increased ($p < 0.05$) hexokinase activity in the absence of insulin, compared with values observed in ovariectomised mice in the absence of insulin. The presence of insulin in the incubation medium did not significantly alter hexokinase activity in intact, ovariectomised or ovariectomised mice treated with progesterone alone or in combination with oestradiol. In contrast, mice treated with oestradiol alone showed a significant increase ($p < 0.05$) in hexokinase activity in the presence of insulin. The increment in hexokinase activity after insulin stimulation are compared in Fig. 8.3. The data indicate that oestradiol increased muscle hexokinase activity in the absence of insulin. Moreover, in the presence of insulin, hexokinase activity was significantly elevated above basal values. This may be explained by oestradiol acting synergistically with insulin to increase hexokinase activity in muscle.

Fig. 8.2 Hexokinase activity in soleus muscles from control and hormone treated mice, after incubation of muscles in the absence and presence of insulin (0.75 mU/ml). p denotes significance of difference between basal and insulin-stimulated hexokinase activity for each group, determined by Student's 't' test for paired data. All values are means ± SEM of 5 determinations.



A = Ovx only
B = Ovx + Oestradiol
C = Ovx + Progesterone
D = Ovx +Oestradiol+Progesterone
E = Intact proestrus/oestrus

Fig.8.3 Increment in insulin-stimulated hexokinase activity above basal in soleus muscles from intact proestrus-oestrus and ovariectomised hormone treated mice. p denotes significance of difference compared with ovariectomised mice, determined by Student's ' t ' test for unpaired data.



A = Ovx only

B = Ovx + Oestradiol

C = Ovx + Progesterone

D = Ovx +Oestradiol+Progesterone

E = Intact proestrus-oestrus

8.1.2 Glycogen synthase (uridine diphosphoglucose : glycogen α-4-glucosyltransferase, EC 2.4.1.11)

Since ovarian steroid hormones have been shown to alter the proportion of glucose directed towards glycogen synthesis, it was considered pertinent to examine the activity of glycogen synthase (UDPG : glycogen α-4-glucosyltransferase, EC 2.4.1.11), a rate limiting enzyme in the glycogenesis pathway (201,202). The enzyme can exist in either of two forms : a high activity dephosphorylated I form, the activity of which is not dependent on glucose-6-phosphate, or a low activity phosphorylated D form, the activity of which can be activated by glucose-6-phosphate (203-206). One important effect of insulin on skeletal muscle is to activate glycogen synthase by promoting the conversion of inactive D synthase to the active I form (207,208). This effect has been observed in the rat diaphragm (201,209), liver and heart (210) and in the mouse soleus muscle (85,211). The present study examines the hypothesis that the observed increase in insulin-mediated glycogen synthesis after oestradiol and combined oestradiol and progesterone treatments in soleus muscle of ovariectomised mice may involve a direct or indirect synergistic effect of sex steroids on insulin activation of glycogen synthase.

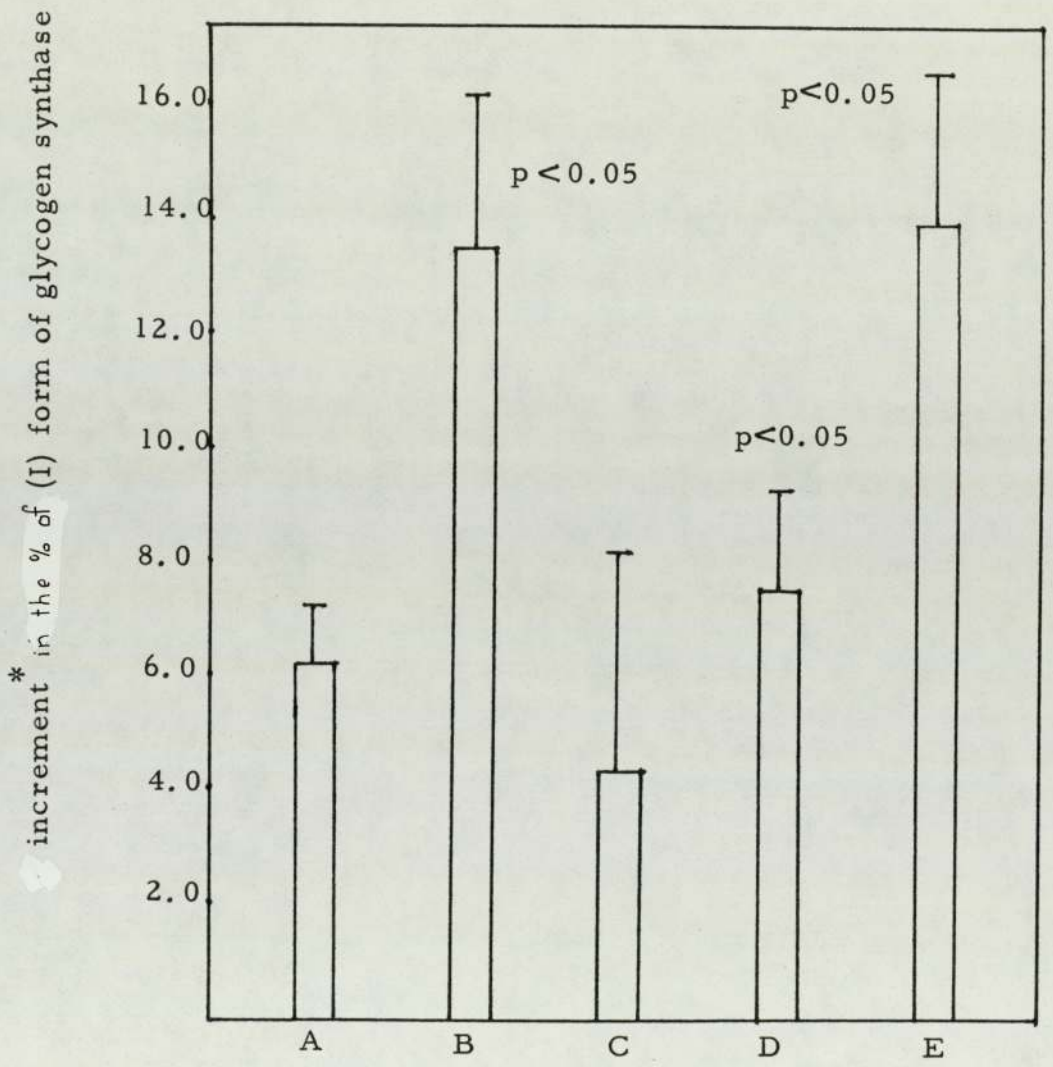
The results confirm the ability of insulin to activate glycogen synthase in mouse soleus muscle. In the presence of insulin (0.75 mU/ml), a higher percentage of the synthase was present in the active I form. This is illustrated in Fig.8.4 which shows the increment in the percentage of glycogen synthase I form produced by insulin stimulation.

Ovariectomy reduced basal and insulin-stimulated glycogen synthase activity (Table 8.2). Replacement with oestradiol alone and in combination with progesterone resulted in an increase in I form activity of the enzyme, consistent with the increase in glycogen synthesis observed previously. In contrast, progesterone treatment did not significantly alter glycogen synthase activity. This is again compatible with the earlier observation that

progesterone did not stimulate glycogen deposition in soleus muscle. The total (I + D) enzyme activities were not significantly different in all groups of mice. However, mice treated with oestradiol alone showed a higher mean value for total synthase activity than each of the other groups, and this observation will be considered in section 8.2.

Fig. 8.4 Percentage increment* in glycogen synthase active (I) form activity in soleus muscles from intact proestrus-oestrus and ovariectomised hormone treated mice. Values are means ± SEM of 4 or 5 determinations. p denotes significance of difference compared with ovariectomised mice, determined by Student's ' t ' test for unpaired data.

* Increment above basal



A = Ovx only

B = Ovx + Oestradiol

C = Ovx + Progesterone

D = Ovx + Oestradiol+Progesterone

E = Intact proestrus-oestrus

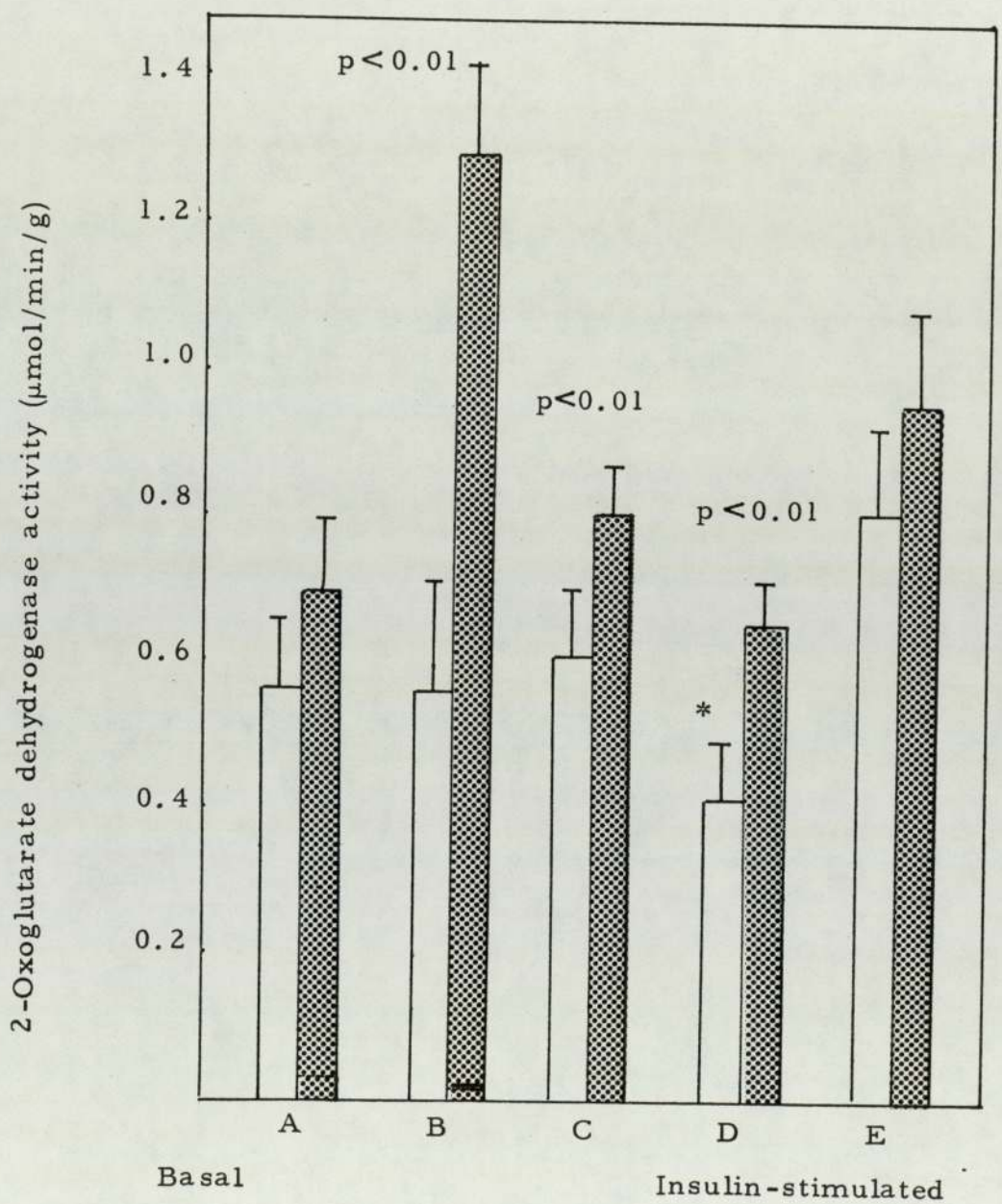
8.1.3 2-Oxoglutarate dehydrogenase (EC 1.2.4.2)

A previous study measuring the rate of production of $^{14}CO_2$ from U-^{14}C-glucose indicated that oestradiol increased the flux of glucose through the TCA cycle while progesterone antagonised this effect of oestradiol. This experiment was conducted to determine the maximum activity of 2-Oxoglutarate dehydrogenase, an enzyme complex catalysing the decarboxylation of 2-oxoglutarate to succinyl CoA. This enzyme was selected for the present study because it reflects the maximum flux of the TCA cycle in vivo (111). Consistent with the design of previous studies, the activity of 2-Oxoglutarate dehydrogenase was examined in soleus muscles isolated from intact female mice in the proestrus-oestrus stage of the oestrous cycle, and in ovariectomised female mice treated with single and combined replacement doses of oestradiol and progesterone.

Fig.8.5 shows the activity of 2-Oxoglutarate dehydrogenase in soleus muscles from intact mice, ovariectomised mice and ovariectomised mice treated with sex steroid replacement regimes. In groups of mice receiving sex hormone therapy, basal 2-oxoglutarate dehydrogenase activity was not significantly affected by hormone treatment compared with ovariectomised mice. In the presence of insulin, oestradiol significantly elevated 2-oxoglutarate dehydrogenase activity. It is noteworthy, however, that basal and insulin-stimulated 2-oxoglutarate dehydrogenase activity was significantly lower ($p < 0.05$) in ovariectomised mice treated with the combined regimen, compared with intact proestrus-oestrus mice.

The effect of sex steroid treatments is further illustrated in Fig.8.6, which shows the insulin-stimulated increment in 2-oxoglutarate dehydrogenase activity above basal levels. Administration of oestradiol increased 2-oxoglutarate dehydrogenase activity by 150% compared with ovariectomised mice. Insulin-stimulated enzyme activity was not significantly affected by progesterone alone or in combination with oestradiol.

Fig. 8.5 2-Oxoglutarate dehydrogenase activity in soleus muscles from intact proestrus-oestrus and ovariectomised hormone treated mice. Values are expressed as μmoles of product formed per minute per gram wet weight tissue. p denotes significance of difference between basal and insulin-stimulated (0.75 mU/ml) 2oxoglutarate dehydrogenase activity, determined by Student's 't' test for paired data. Values are means ± SEM of 5 determinations.



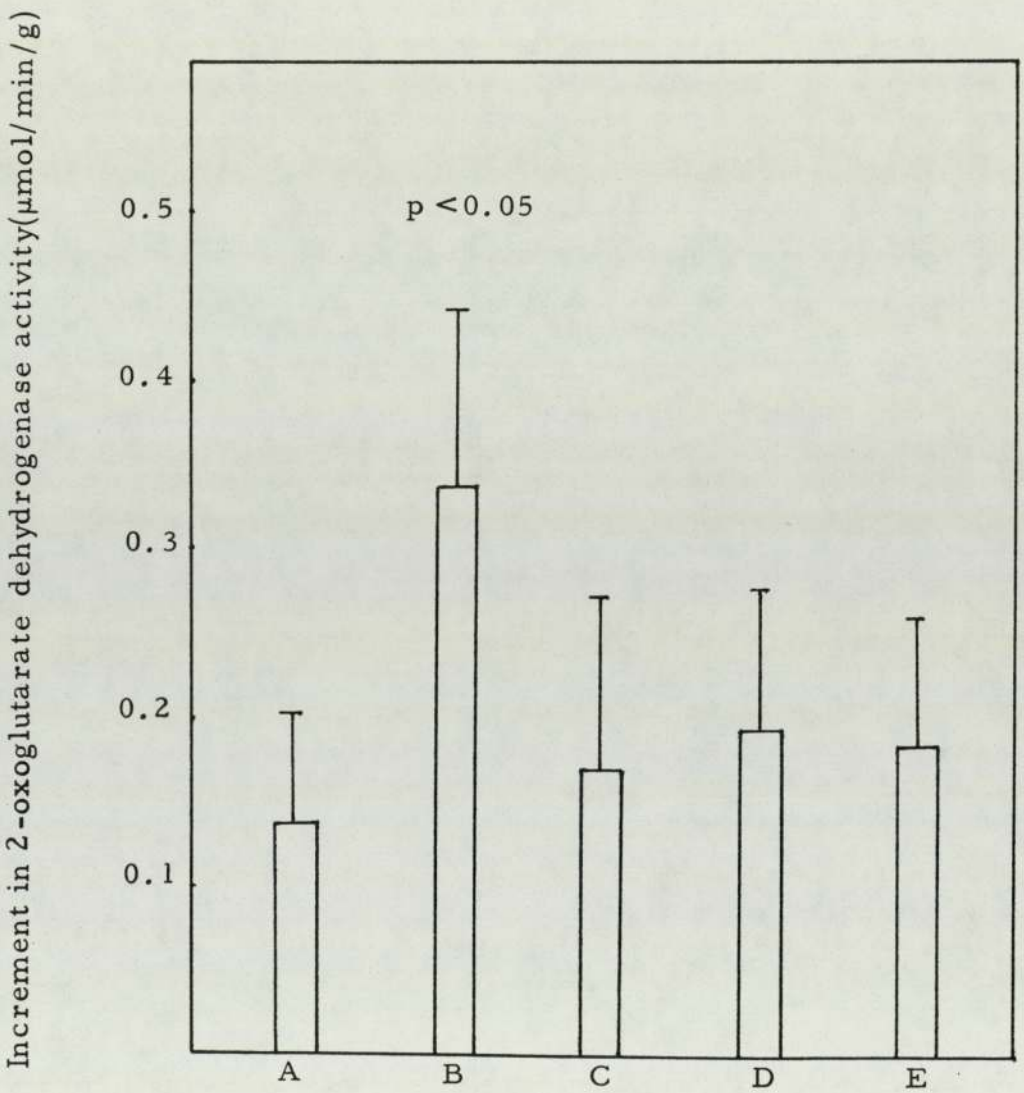
A = Ovx only B = Ovx + Oestradiol

C = Ovx + Progesterone D = Ovx +Oestradiol+Progesterone

E = Intact proestrus-oestrus

* = p 0.05 compared with intact proestrus-oestrus mice

Fig. 8.6 Increment in insulin-stimulated 2-oxoglutarate dehydrogenase activity above basal in soleus muscles from proestrus-oestrus and ovariectomised hormone treated mice. p denotes significance of difference compared with ovariectomised mice, determined by Student's ' t ' test for unpaired data.



A = Ovx only

B = Ovx + Oestradiol

C = Ovx + Progesterone

D = Ovx + Oestradiol+Progesterone

E = Intact proestrus-oestrus

8.2 Discussion

Ovarian steroid hormones have been shown to influence carbohydrate metabolism in many insulin sensitive tissues (25, 40, 41, 162). Indeed, in vivo studies reviewed elsewhere (21, 131) indicate that the effects of oestradiol and progesterone on carbohydrate metabolism are mainly differentiated at the level of peripheral tissue uptake of carbohydrates. After oestradiol administration, glucose conversion to glycogen or CO_2 is enhanced in muscle (42) and glycogenesis is increased in liver (40). Whilst these effects of natural oestrogens on carbohydrate metabolism are well substantiated in the literature (25, 40-42, 162), the relationship between progesterone and carbohydrate disposal remains controversial. There have been conflicting reports (44, 45, 135, 212, 213) describing both stimulatory and inhibitory effects of progesterone on insulin-mediated glucose uptake. This may be accounted for in part by differences in methodology, dose, route of progesterone administration, duration of treatment and the presence or absence of oestrogens. For example, some studies have measured glucose dissapearance from the blood while others have assessed glucose uptake into particular tissues. Progesterone has been administered to the intact animal for a duration of three weeks (45) in some studies while others have examined its effect in vitro after fifteen minutes of exposure to the tissue (214). Furthermore, some studies have used intact animals in which interference from circulating levels of other steroids was not controlled and others have used ovariectomised animals. However, the bulk of the literature appears to support the contention that the effects of progesterone are, in general antagonistic to those of oestradiol.

Results from the study described in Chapter 5 showed that the accumulation of the phosphorylated derivative of 2-deoxy-D-glucose within the tissue is increased after oestradiol treatment. Progesterone alone and in combination with oestradiol increased glucose metabolism to a lesser extent than oestradiol alone,

while uptake of 2-deoxy-D-glucose was not significantly altered by progesterone or the combined regimen.

It has been suggested that glucose phosphorylation may present a possible site for the action of hormones in the control of glucose metabolism (79,174,215). The phosphorylation process becomes rate-limiting in the presence of insulin (94,216). Since sex steroids alter the rate of glucose phosphorylation in the glycolytic pathway, it is tempting to speculate that this may be related to the activity of the glucose phosphorylating enzyme hexokinase. To accommodate changes in flux of glycolytic intermediates, the activity of hexokinase may be altered by sex hormones acting directly on the enzyme itself or as a consequence of stimulation of an earlier event such as insulin receptor binding. The concept of hormonal induction or repression of enzymes involved in metabolism is not new. Numerous experiments have validated the suggestion that the action of steroid hormones on target cells is mediated via nuclear gene transcription (217), preceeded by quantitative changes in the synthesis of cellular RNA (218-220).

In section 8.1.1 of the present study, an oppurtunity is provided to relate the activity of the glucose phosphorylating enzyme hexokinase with the rate of glycolysis observed in soleus muscles after sex hormone treatment in a previous experiment (Chapter 4). The results indicate that oestradiol can modify the activity of skeletal muscle hexokinase resulting in measurable alterations in the rate of glucose phosphorylation. Although ovariectomy did not significantly reduce hexokinase activity, the mean value for enzyme activity was lower in ovariectomised mice compared with intact female mice. This observation is in agreement with previous studies in which ovariectomy consistently impaired glucose metabolism(Chapters 4,5 and 7). It is noteworthy that considerable differences in activity between individual mice resulted in a broad distribution of enzyme activities in each group.

Oestradiol treatment resulted in a significant elevation

of hexokinase activity, under both basal and insulin-stimulated conditions. The observed increase in basal hexokinase activity was expected since oestrogenic hormones have been reported to increase the activity of hexokinase and other enzymes in uterine tissue (215,221-224) which does not appear to be dependent upon insulin to stimulate glucose metabolism. In the present study, the presence of insulin further increased the activity of hexokinase after oestradiol treatment. Whether the stimulation of enzyme activity is a consequence of oestradiol activation of pre-existing enzyme or de novo enzyme synthesis cannot be determined at this stage. Reports in the literature (225-227) favour the latter alternative. The relationship between insulin action and hexokinase activity is controversial (228-230). Sharma and his co-workers (188) reported a lack of effect of insulin on the hexokinase activity of heart muscle. However, this conflicted with earlier reports of Il'yin and co-workers (231) and Vester and Reino (232). Katzen (233) demonstrated an in vitro effect of insulin on hexokinase activity in the diabetic rat heart, and it has been shown that the stimulatory action of insulin on hepatic hexokinase is associated with stimulation of new enzyme synthesis (187,234). Hence, a concensus is accumulating to implicate insulin in the regulation of hexokinase activity. Indeed, various models of hexokinase-insulin interrelationships have been formulated in which the multiple hexokinases have been conceptualised as primary receptors of insulin in the membrane (228). In general, the hexokinases are visualised as 'carriers' within the cell membrane, the function of which is to actively transport glucose into the cell and to phosphorylate the sugar during the transport process. Insulin is thought to interact with the carrier system probably through a thiol-disulphide interchange reaction. Pilch and Czech (235) recently proposed that the insulin receptor is composed of two α and two β subunits with disulphide bridges linking these subunits. In the light of their evidence, it is possible that the hormone insulin, the insulin receptor and the hexokinases may

be functionally related within the membrane. If insulin is implicated in the control of hexokinase activity, it is possible that the increase in insulin-stimulated hexokinase activity after oestradiol treatment may represent a cumulative effect of synergism between insulin and oestradiol. Such a synergism has been implied for other actions of these hormones relating to glucose metabolism.(38, 42 57). In contrast to the effects of oestradiol alone, progesterone alone and in combination with oestradiol did not result in a detectable change in hexokinase activity. This is consistent with an earlier study on glucose phosphorylation (Chapter 5) in which 2-deoxy-D-glucose uptake was not significantly affected by either progesterone alone or in combination with oestradiol.

The results reported in section 8.1.1 are complementary to those of Singhal, Valadares and Ling (221). Singhal et al (221) examined the effects of single and combined doses of oestradiol and progesterone on the activity of phosphofructokinase in the uterus of ovariectomised rats. Oestradiol increased uterine phosphofructokinase activity and this effect was inhibited when progesterone was adminstered to oestradiol treated rats. However, progesterone alone did not produced any significant effect.

The studies of Smith and Gorski (174, 215) showed that oestrogen-induced stimulation of glucose phosphorylation is not the initial response effected by oestradiol. They postulated that increased glucose phosphorylation is a later effect of the hormone, consequent to increased synthesis of hexokinase. Their reports clearly indicate that the action of oestradiol, at least in the uterus is initiated at a site prior to glucose phosphorylation. Indeed, in the light of recent studies, it appears that the action of oestradiol on glucose metabolism is mediated via an effect on the initial step in the action of insulin. The study reported in Chapter 7 has shown that insulin receptor binding is increased in mouse soleus muscle after oestradiol administration.

The effect of sex hormone therapy on glycogen formation has been discussed in Chapter 4. In the presence of insulin, a

replacement dose of progesterone alone increased glucose transport but did not significantly alter glycogen deposition in soleus muscle. In contrast, administration of oestradiol alone and in combination with progesterone resulted in a marked stimulation of insulin-mediated glycogen accummulation. Although this may be accounted for in part by an enhanced capacity of the tissue for the uptake and storage of glucose, insulin is known to stimulate glycogen synthesis by activating the enzyme, glycogen synthase (UDPG-glycogen α-4 glucosyltransferase) (207,208,236). However, there is evidence that the effect of insulin on membrane glucose transport is completely dissociated from its intracellular effects on synthase conversion. This has been demonstrated in rat diaphragm (237), perfused rat heart (238) and more recently, in mouse soleus muscle (211). Thus, the present study was conducted in the absence of exogenous glucose to examine specifically the effect of ovarian sex steroids on insulin-mediated activation of glycogen synthase. Although insulin activation of glycogen synthase has been widely reported, the effect of glycogenic steroids and sex steroids on this enzyme remains uncertain. Evidence suggests that the early phase of the apparent stimulation of glycogen synthase by hydrocortisone is dependent on the presence of insulin (239). Other studies have shown that administration of progesterone to castrated rats rapidly increased glycogen levels in the levator ani muscle with a concomitant increase in 2-deoxy-D-glucose penetration and active I form synthase activity (240). It has been reported that oestrogens increased ^{14}C-glucose incorporation into glycogen, with a concomitant increase in synthase I activity (207). However, this has not been repeated and to our knowledge, no further surveys have been conducted to define the effects of sex steroids on insulin activation of glycogen synthase. This, together with evidence from recent experiments in which sex steroids were shown to modulate insulin-mediated glycogen synthesis (Chapter 4) prompted the present investigation into the effects of ovarian hormones on glycogen synthase activation

in mouse soleus muscle.

The results described in section 8.1.2 confirm a direct effect of insulin on synthase activation, independent of the effect on glucose transport. Ovariectomy impaired glycogen synthase activation compared with intact mice. The percentage of synthase I activity appeared to be quantitatively related to the accumulation of muscle glycogen from glucose reported in a previous study (Chapter 4). Synthase I activity was increased in soleus muscles from mice treated with oestradiol alone and from mice treated with the combined oestradiol-progesterone regimen. This observation is compatible with increased glycogen synthesis in these two groups of mice. Insulin did not significantly alter glycogen synthase activity in the presence of progesterone, consistent with the lack of effect of progesterone on glycogen synthesis observed previously (Chapter 4). This suggests that progesterone may antagonise insulin stimulation of glycogen formation.

The total (I + D) enzyme activities in each of the groups of mice were not significantly different. This constancy of the total enzyme activities has been interpreted as suggestive of an insulin-mediated interconversion between the two species of glycogen synthase (202). The higher mean total enzyme activity after oestradiol treatment is worthy of note. Although an interconversion between two species of the same enzyme cannot result in increased total activity, it is possible that oestradiol treatment might induce the intracellular synthesis of new synthase. Early work indicated that the initial effects of steroids to promote glycogen synthesis were independent of de novo enzyme synthesis (241). Since it is commonly onserved that many actions of sex steroids are mediated via nuclear gene transcription (55), it is quite reasonable to suggest that oestradiol might promote synthesis of new glycogen synthase (242-244). Indeed, female sex steroids have been reported to exert regulatory effects on the synthesis, activity and possibly even the degradation of other tissue enzymes and structural proteins (55). Furthermore,

Steiner, Rauda and Williams (245) reported an increase of total glycogen synthase activity in livers of alloxan diabetic rats. This effect occurred in the presence of added insulin and the authors postulated that the increase in maximal activity resulted from synthesis of new enzyme.

One important observation that has emerged from the present study concerns the non-hormonal control of synthase I activity by the tissue concentration of glycogen. To be consistent with earlier experiments in this series, mice were fasted for 40 h prior to study. After fasting, tissue glycogen is lowered by as much as 80% (85) and the overall capacity of the tissue for glucose utilisation is enhanced (246-248) to compensate for this deficiency. Shamoon and Felig (42) noted a lack of effect of oestradiol on basal glucose uptake in the diaphragm of fed rats. The authors attributed this to the fact that the fuel content of the tissue which had not been lowered by fasting presumably could not further increase glycogenesis. Thus, when the tissue concentration of glycogen is low, glycogen synthesis from glucose is stimulated as a result of an increase in synthase I activity. This relationship between synthase I and glycogen deposition was first observed by Danforth (209) in mouse muscle in vivo and in the isolated rat diaphragm (209). Danforth concluded that glycogen partly controls its own synthesis by affecting the interconversion of glycogen synthase I $\longrightarrow$ D in a manner which favours glycogen synthesis when tissue concentrations of glycogen are low, and which reduces synthesis when tissue glycogen concentrations are high. The results from the present study suggest that oestradiol alone and in combination with progesterone may synergise the action of insulin in shifting the control of glycogen synthase to favour glycogen synthesis in mouse soleus muscle. Hence, in the presence of insulin, oestradiol has been shown to produce a greater increase in the activity of hexokinase and to produce a greater shift to the active I form from the less active D form of glycogen synthase. These effects will favour activation of the

synthase.

Oestradiol has also been shown to stimulate CO_2 production from glucose in soleus muscles (Chapter 4). The increased flux through the TCA cycle may reflect stimulation of 2-oxoglutarate dehydrogenase activity by the steroid. This enzyme was selected as an index of the flux through the TCA cycle since it is the only enzyme of the cycle which exhibits maximum activity that is similar to the maximum flux through the TCA cycle in vivo (111). Furthermore, the reaction catalysed by 2-oxoglutarate dehydrogenase is strongly exergonic and is therefore likely to be irreversible in vivo.

The results from section 8.1.3 show that oestradiol and progesterone treatment of ovariectomised mice resulted in a stimulation of the maximum activity of 2-oxoglutarate dehydrogenase in the presence of insulin. However, this finding by itself does not exclude the possibility that the observed stimulation of 2-oxoglutarate dehydrogenase activity after oestradiol administration may be an indirect consequence of an increased availability of substrates for the enzymes of TCA cycle, thereby increasing its overall activity. Nevertheless, this observation serves to emphasise the role of oestradiol and progesterone as potentiators of insulin-mediated glucose utilisation in skeletal muscle.

The observed increase in maximum activity of a TCA cycle enzyme in mouse soleus muscle after treatment with oestradiol is in contrast to a report by Eckstein and Villee (224) who failed to observe any effect of oestradiol on several TCA cycle enzymes in rat uterine tissue. This could reflect differences between species or between tissues. In the study by Eckstein and Villee, the data obtained with ovariectomised oestradiol-treated rats were not compared with ovariectomised controls, but with intact animals. This could account for the reported lack of effect of oestradiol on TCA cycle enzyme activities. Indeed, maximum activities of aconitase, isocitrate dehydrogenase and fumarase were reported to be either decreased or unchanged

after oestradiol administration. These results may be criticised further due to the non-physiological dose of oestradiol used by these authors- 150 μg/kg body weight, compared with a replacement dose of 5 μg/kg body weight used in the present study.

Although no change in enzyme activity was observed after progesterone administration as shown in section 8.1.3, the possibility exists that progesterone may alter the Km of the enzyme without affecting the V_{max}. This would not have been detected in the present study, since the assay was conducted by substrate saturation. However, in the light of the similarities between oestradiol and progesterone both structurally and physiologically, this appears unlikely. Nevertheless, the issue does highlight the problem of employing crude homogenates in enzyme assays, particularly when only small differences are involved (108).

CHAPTER 9

EFFECT OF SEX HORMONES ON STREPTOZOTOCIN-INDUCED DIABETES

The incidence of type I (insulin-dependent) diabetes has been shown to be gender related. Mortality studies before the insulin era demonstrated a male preponderance to this disease (249). This has been substantiated recently by surveys conducted among newly diagnosed diabetic children in which a higher percentage of boys was observed to develop type I diabetes than girls (250, 251).

These observations have been extended to the evolution of experimental diabetes induced by subtotal pancreatectomy, alloxan and streptozotocin in laboratory animals. Male rats become diabetic more readily than female rats after subtotal pancreatectomy (57). Removal of the ovaries after subtotal pancreatectomy increased the incidence and severity of diabetes in female rats while castration of male rats had the converse effect (22, 57, 58). Moreover, males are less resistant than females to the diabetogenic effects of the encephalomyocarditis virus (252 , 253).

Similar experiments with streptozotocin and alloxan alos suggest a greater male susceptibility to experimental diabetes. Streptozotocin, a methylnitrosourea derivative of 2-deoxy-D-glucose is frequently used to induce diabetes in laboratory rodents because of its selective toxicity to pancreatic β cells (112, 254-256). Multiple low dose injection of streptozotocin induce diabetes in male mic e but not in females or in castrated males (257). Furthermore, a single injection of the same drug can produce severe hyperglycaemia in male mice while female mice remain euglycaemic (258). Chronic treatment of castrated female rats with oestradiol 17β was found to augment plasma insulin levels (17, 132, 259) and to increase pancreatic insulin content (260). These effects may in part be mediated by hypertrophy

and hyeprplasia of the islets of Langerhans (261-264). In vivo observations indicate that oestrogen therapy prior to streptozotocin injection inhibits the induction of experimental diabetes in male rats (265). Furthermore, oestradiol administration to non-diabetic ovariectomised female rats lowered blood glucose concentrations and induced hyperinsulinaemia after a two week period (266). It was suggested that this effect may be mediated in part by increased production of corticosteroids, although this is not fully consistent with the known antagonistic properties of corticosteroids (267). Indirect evidence from other studies has indicated that glucose tolerance is greatest and plasma insulin levels are highest at proestrus when circulating titres of sex hormones are high (97). More recent investigations revealed that sex hormones may directly modulate β cell activity : streptozotocin toxicity in vitro was shown to be greater in islets from adult male rats compared to islets from female animals (268).

Several lines of evidence indicate that sex hormones may influence glucose homeostasis in humans and in animals acting via mechanisms that are dependent on insulin, but mediated at the site of insulin action. As early as 1940, Spiegelman noted a decreased requirement for insulin by a group of post-menopausal insulin-dependent diabetic women who were receiving oestrogen therapy (38). Other investigators showed that oestradiol administration to alloxan diabetic rats diminished glycosuria and hyperglycaemia in these animals (39, 57). Earlier chapters of this thesis have reviewed in detail the effects of ovarian hormones on insulin-mediated glucose uptake and metabolism in muscle and adipose tissue (See also 269-272). It is evident that natural oestrogens promote insulin-mediated glucose metabolism in these tissues and progestogens may partly antagonise this effect.

In general , these observations are consistent with the view that female sex hormones have a protective function in the aetiology of both human and experimental diabetes. In spite of the numerous reports dealing with the effects of sex hormones

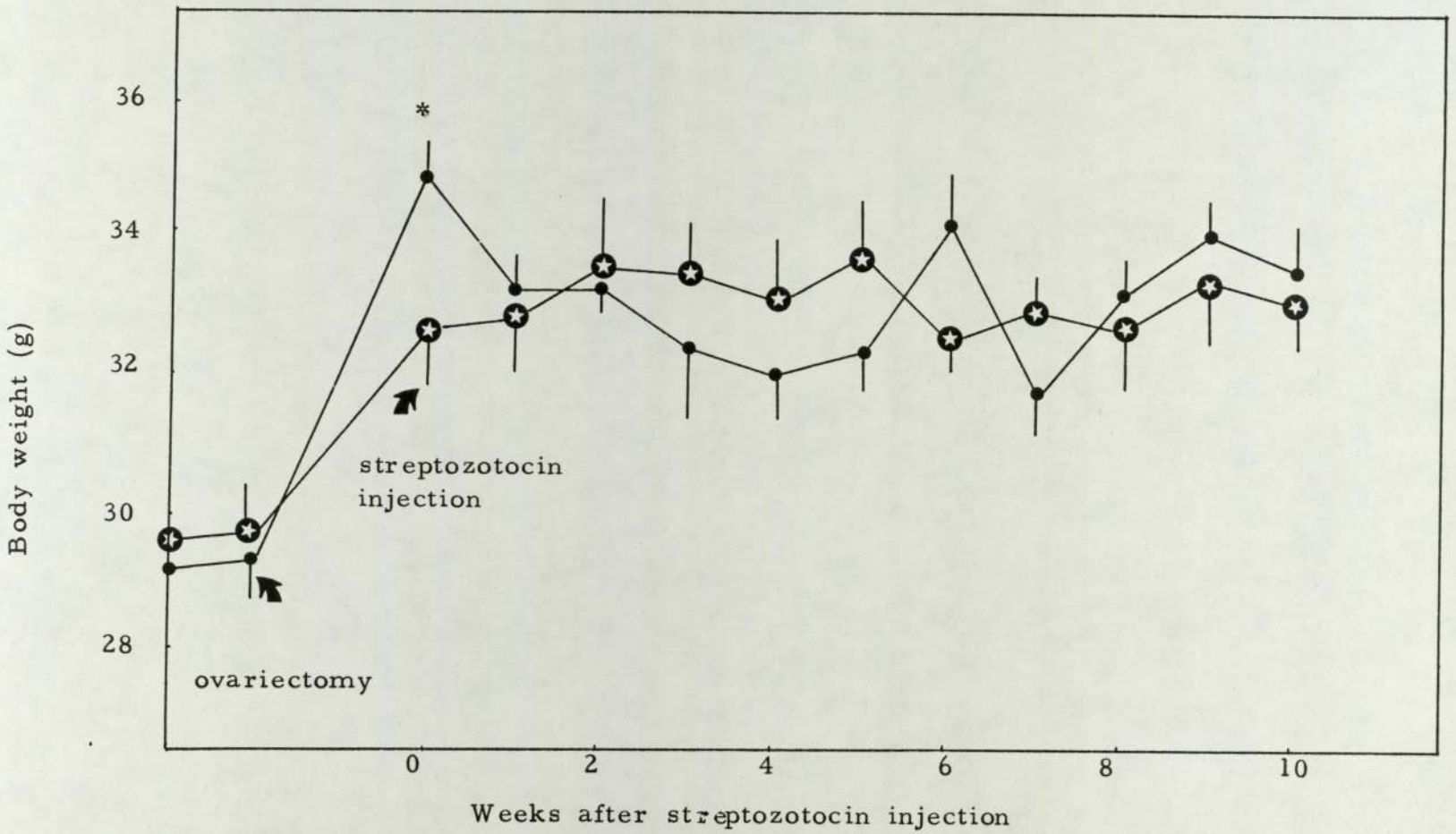
on glucose homeostasis, the cellular mechanisms by which sex hormones act remains uncertain. Available evidence suggests there may be alterations at the level of the insulin receptor (143-145), subsequently influencing cellular and metabolic events located at post-receptor sites (17, 42, 212). Studies reported herein (269, 270) demonstrate a stimulatory effect of sex hormones on insulin-mediated glucose metabolism in the isolated skeletal muscle of non-diabetic ovariectomised female mice. Although there are reports of studies conducted in alloxan diabetic and partially pancreatectomised diabetic animals (22, 39, 60), no invest--igation to date has examined the effect of long term oral administration of ovarian sex hormones in streptozotocin diabetic mice. This model of diabetes is now preferred to the alloxan diabetic and partial pancreatectomy models because it provides a more specific destruction of islet β cells (254). Thus, the present study was undertaken to examine the effect of endogenous and exogenously administered oestradiol and progesterone on plasma glucose, plasma insulin and pancreatic insulin content using the streptozotocin diabetic mouse.

9.1 Comparison of intact and ovariectomised mice.

9.1.1 Body weight

Two weeks after surgery, ovariectomised mice showed an increase in body weight of 2.3 ± 0.01g compared with an increase of 1.1 ± 0.05g ($p < 0.05$) in intact mice over the same period (Fig. 9.1). Following the administration of streptozotocin, the body weight of ovariectomised mice decreased by 2.2% compared with a 0.7% decrease in intact mice, so that throughout the 10 week course of the study, the body weights of the two groups of mice were similar. There was no significant difference in food intake in the two groups of mice.

Fig. 9.1 Body weight of intact (✪) and ovariectomised (●) mice prior to and after streptozotocin injection. Vertical bars represent means ± SEM (n= 12). * $p < 0.05$ compared with intact mice.



9.1.2 Plasma glucose and insulin.

Ovariectomised and intact mice developed a marked hyperglycaemia within two weeks of the streptozotocin injection (Fig. 9.2). Although plasma glucose in the two groups did not differ significantly at this stage, the level was higher in ovariectomised diabetic mice (18,6 ± 2.25 mmol/l) than intact diabetic mice (17.4 ± 2.40 mmol/l). In subsequent weeks, the hyperglycaemia increased in both groups of mice and significantly higher plasma glucose concentrations were observed in the ovariectomised mice ($p < 0.05$ at weeks 5, 6, 8 and 10).

Following streptozotocin injection, plasma insulin concentrations declined in both groups of mice, but intact mice maintained higher insulin concentrations than ovariectomised mice ($p < 0.01$ at week 4 and $p < 0.05$ at week 10) (Fig. 9.3).

9.1.3 Total pancreatic insulin

Ten weeks after streptozotocin injection, the total pancreatic insulin content of intact mice was higher than that of ovariectomised mice (Table 9(a)). These results are compatible with the higher levels of circulating insulin in the intact mice.

	Ovx. placebo	Intact placebo
Wet wt pancreas(g)	0.31 ± 0.09	0.36 ± 0.11
Pancreatic insulin content (µg/g wet wt)	13.4 ± 3.1	22.6 ± 5.7
Total pancreatic insulin (µg/pancreas)	4.15 ± 1.9	8.20 ± 2.8

Table 9(a). Wet weight of pancreas, pancreatic insulin content and total pancreatic insulin in ovariectomised and intact placebo mice.

Fig. 9.2 Plasma glucose of intact (✪) and ovariectomised (●) diabetic mice over a 10 week period. Vertical bars represent means ± SEM (n=12).
* $p < 0.05$ compared with intact mice.

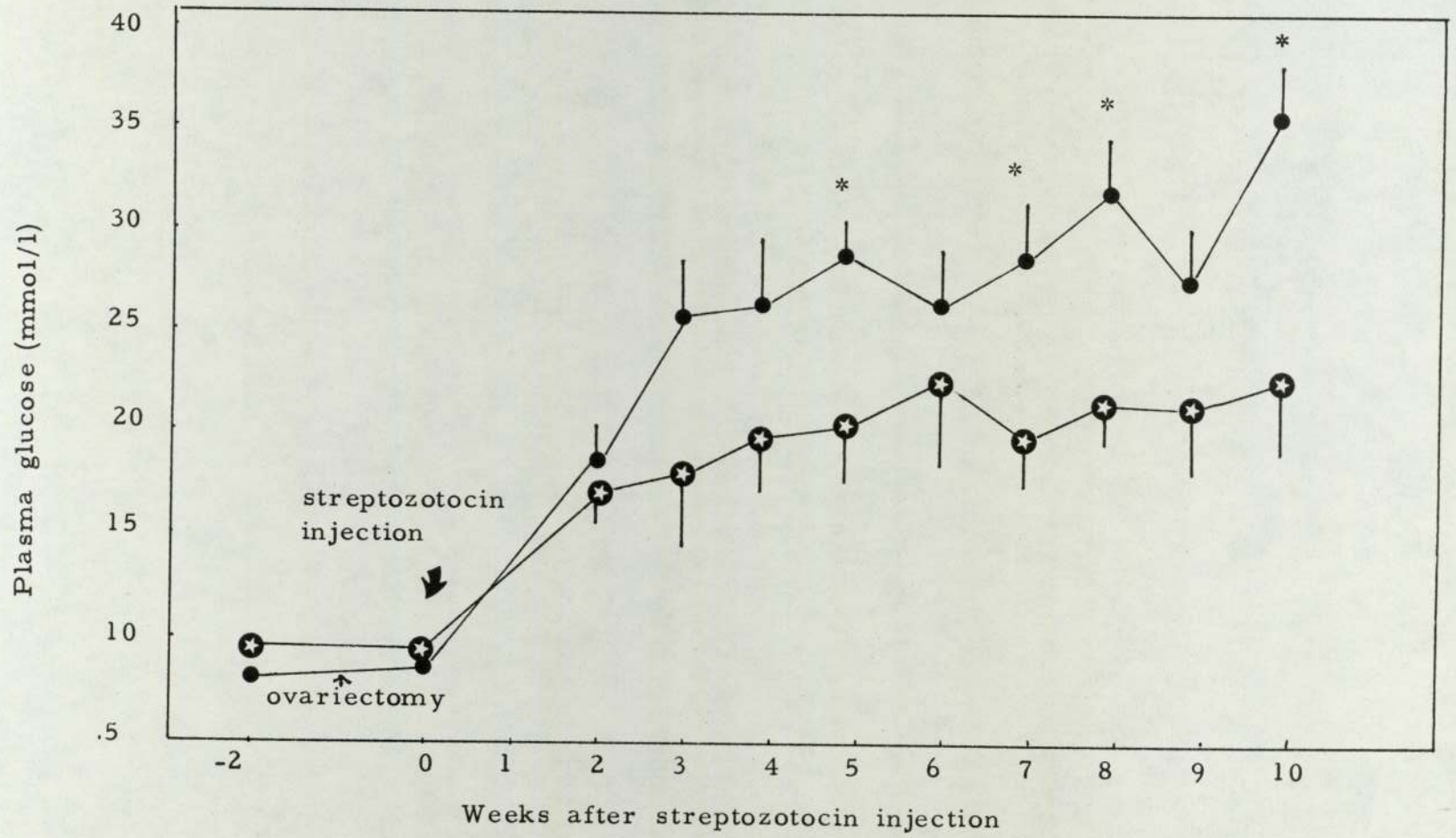
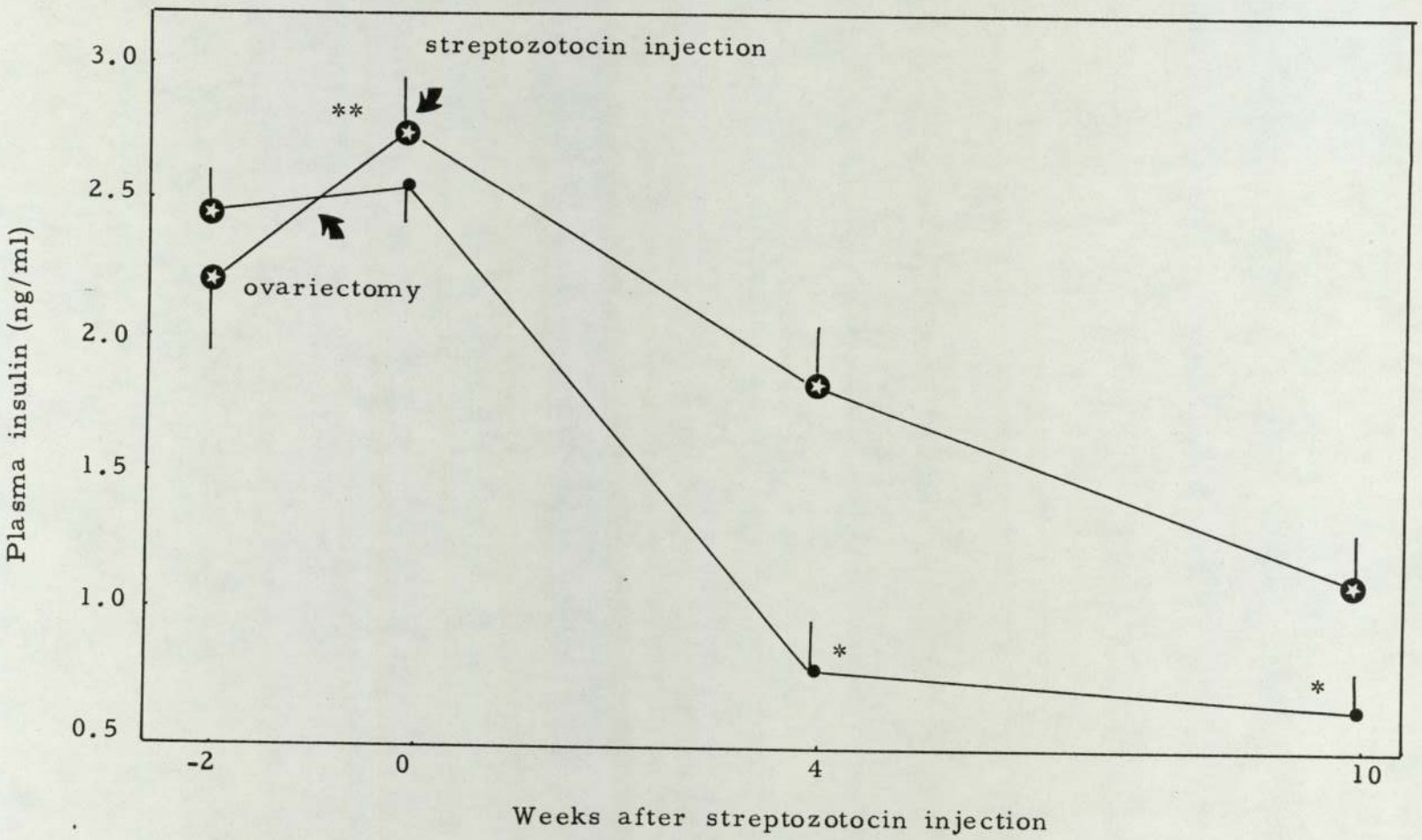


Fig. 9.3 Plasma insulin of intact (✪) and ovariectomised (●) mice prior to and after streptozotocin injection. Vertical bars represent means ± SEM (n=12).

* $p < 0.05$ compared with intact mice.

** p 0.05 compared with week -2



9.2 Comparison of different regimens of sex hormone therapy.

9.2.1 Body weight.

Before the sex hormone treatments were begun, there were no significant differences between the body weights of the groups of mice (Fig.9.4). Streptozotocin diabetes impaired the normal age-related gain in weight. Ovariectomised diabetic mice showed only a 4.4% increase in body weight during the ten week period of the study, compared with a 15% increase in age-matched non-diabetic ovariectomised female mice (noted in previous experiments). After two weeks of hormone therapy, body weight was significantly higher ($p < 0.05$) in HE (high dose of oestradiol, 500μg/kg/day) treated mice than in ovariectomised diabetic mice on placebo. This increase in body weight in the HE treated mice persisted until the end of treatment. At the end of the 10 weeks, the body weight of this group of mice was 20% greater than at the start of treatment. There were also increases in body weight after 10 weeks in mice treated with progesterone (17%), a low dose of oestradiol (12%) and progesterone and oestradiol combined (9%). The body weight increments in mice treated with progesterone were significantly greater than in ovariectomised controls.

9.2.2 Plasma glucose and insulin

Plasma glucose concentrations of all mice were normal (8.0 ± 0.01 mmol/l) at the start of the experiment. Two weeks after the injection of streptozotocin, the mice developed hyperglycaemia. (Fig.9.5). Streptozotocin-induced hyperglycaemia was suppressed by oestradiol in a dose dependent manner. The suppressive effect was clearly evident in mice treated with thelower dose of oestradiol (5 μg/kg/day) and was more pronounced in mice treated with the higher dose of oestradiol (500 μg/kg/day). In the latter group, the plasma glucose concentration was markedly lower than that in ovariectomised control mice. Progesterone also reduced the extent of hyperglycaemia, and the combined oestradiol-progesterone

regimen produced a smaller effect which was only significant at 2, 3, 4 and 8 weeks.

Two weeks after streptozotocin injection, plasma insulin concentrations were decreased to 0.5 ng/ml. Treatment with the two different doses of oestradiol as well as progesterone increased the concentration of plasma insulin. The combined oestradiol-progesterone regimen did not alter plasma insulin concentration. (fig. 9.6)

9.2.3 Total pancreatic insulin

Hormone therapy increased total pancreatic insulin content. Oestradiol at the higher dose (500 μg/kg/day) and progesterone produced particularly large increases. The lower dose of oestradiol (5 μg/kg/day) also increased total pancreatic insulin but to a lesser extent than the higher dose of this hormone [Table 9(b)].

	Wet wt pancreas (g)	Pancreatic insulin content (μg/g wet wt)	Total pancreatic insulin μg/pancreas)
Ovx placebo	0.31±0.09	13.4 ± 3.1	4.15 ± 1.9
Oestradiol (5 μg/kg)	0.33±0.08	23.9 ± 4.8	7.59 ± 2.1
Oestradiol (500 μg/kg)	0.29±0.10	30.0 ± 7.1	8.70 ± 1.6
Progesterone (1 mg/kg)	0.26±0.13	26.9 ± 4.6	6.81 ± 2.5
Oestradiol-Progesterone (5μg/kg + (1 mg/kg)	0.35±0.13	19.6 ± 8.3	6.90 ± 2.2

Table 9(b). Wet weight pancreas, pancreatic insulin content and total pancreatic insulin in ovariectomised placebo, oestradiol (5μg/kg and 500 μg/kg), progesterone and oestradiol-progesterone treated mice.

Fig. 9.4 Body weights of ovariectomised diabetic mice during treatment with placebo (●), 5 μg/kg oestradiol (★), 500 μg/kg oestradiol (○), 1 mg/kg progesterone (☆) and 5 μg/kg oestradiol + 1 mg/kg progesterone (□). Vertical bars represent means ± SEM.

a, b, c, d $p < 0.05$ compared with [a] oestradiol (5 μg/kg); [b] oestradiol (500 μg/kg); [c] progesterone (1 mg/kg); [d] oestradiol+ progesterone (5 μg/kg + 1 mg/kg), for each week. All statistics refer to ovariectomised mice on placebo.

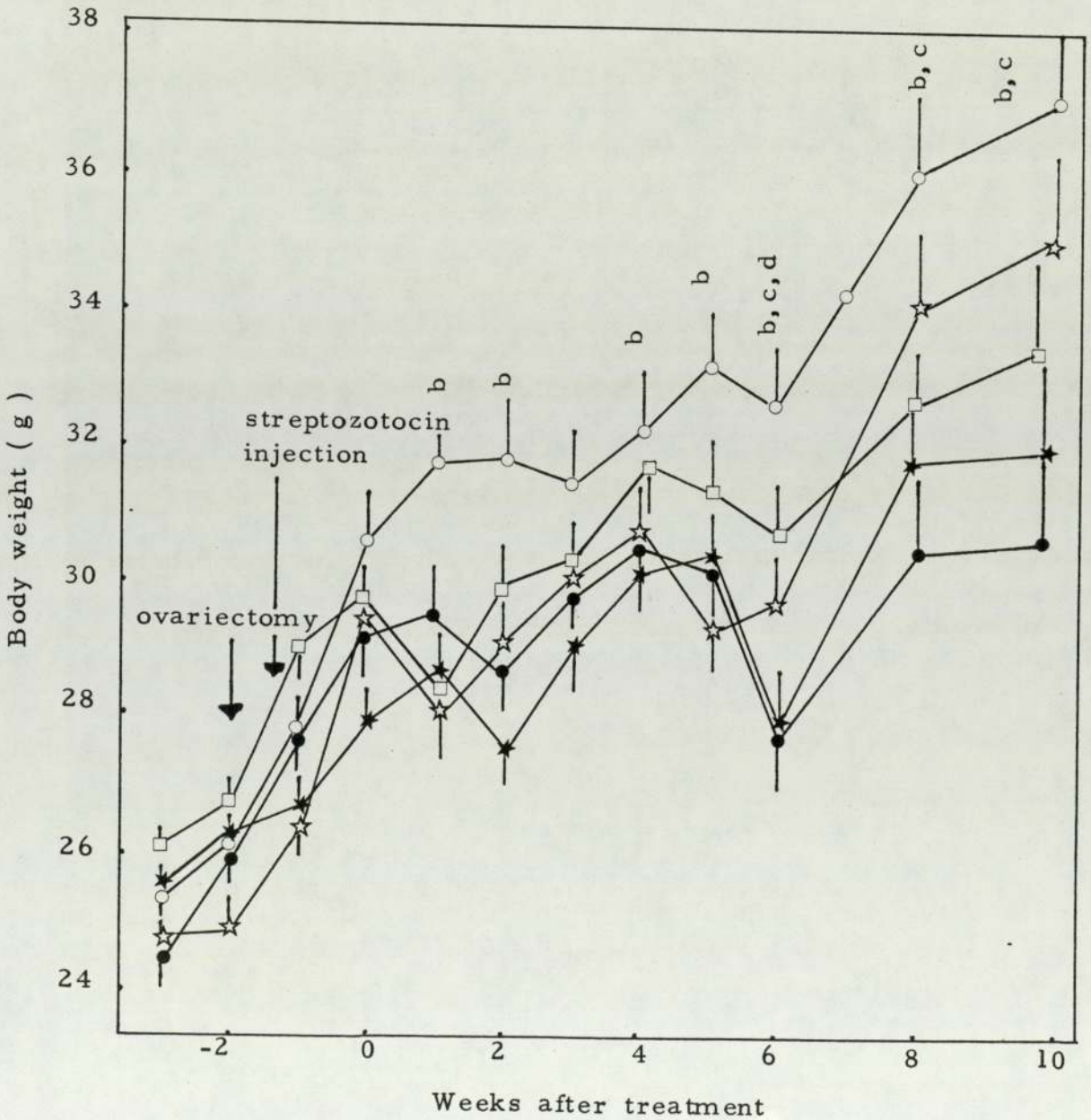
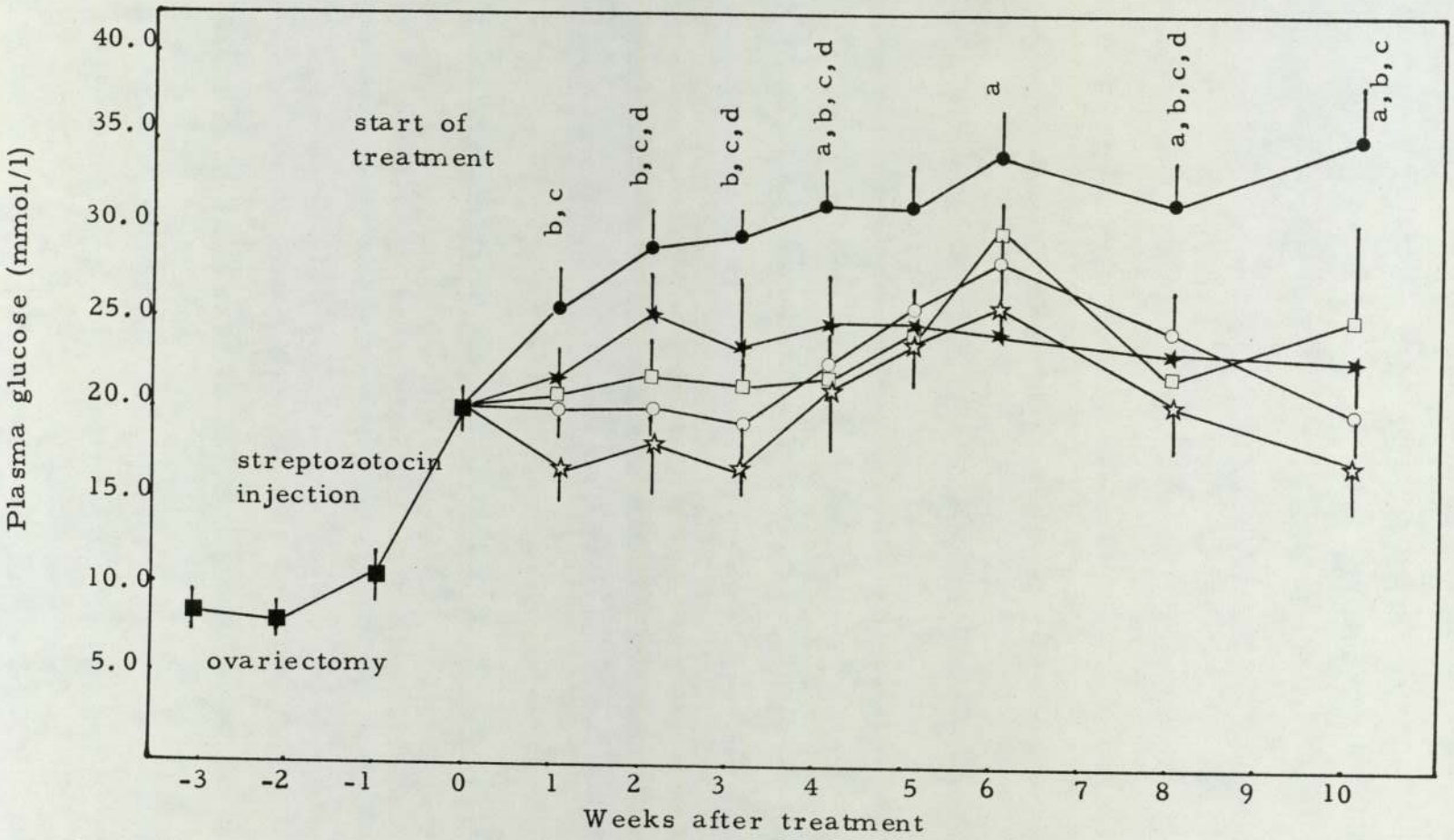
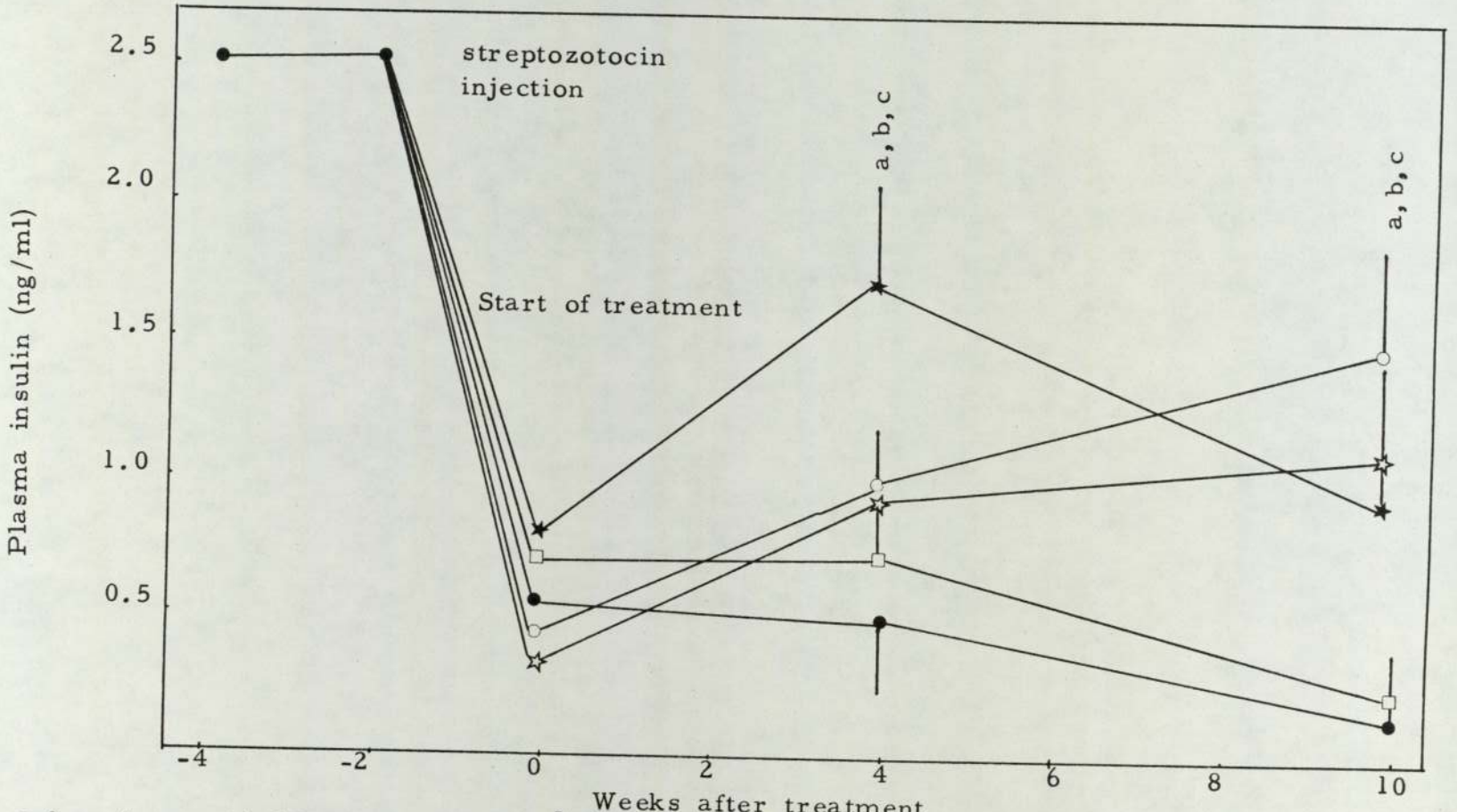


Fig. 9.5 Plasma glucose of ovariectomised diabetic mice during treatment with placebo (●), 5 μg/kg oestradiol (★), 500μg/kg oestradiol (○), 1 mg/kg progesterone (☆) and 5 μg/kg oestradiol+1 mg/kg progesterone. □ Vertical bars represent means ± SEM.



a, b, c, d $p < 0.05$ compared with [a] oestradiol(5μg/kg); [b] oestradiol(500μg/kg); [c] progesterone(1 mg/kg); [d] oestradiol+progesterone(5μg/kg+1 mg/kg), for each week. Statistics refer to ovariectomised mice.

Fig. 9.6 Plasma insulin of ovariectomised diabetic mice during treatment with placebo (●), 5 μg/kg oestradiol (★), 500 μg/kg oestradiol (○), 1 mg/kg progesterone (☆) and 5 μg/kg oestradiol+1 mg/kg progesterone (□). Vertical bars represent means ± SEM.



a, b, c, d $p < 0.05$ compared with [a] oestradiol(5 μg/kg); [b] oestradiol(500 μg/kg); [c] progesterone(1 mg/kg); [d] oestradiol+progesterone (5 μg/kg+1 mg/kg), for each week. Statistics refer to ovariectomised mice.

9.3 Discussion

Ovarian steroid hormones are among many factors known to have a modulatory effect on insulin regulation of glucose homeostasis (10, 11, 21, 131). Indeed, recent reports indicate that susceptibility to insulin-dependent diabetes in both humans and experimental animals is influenced by common aetiological steps that are under the control of sex hormones (251, 257, 258, 265). A greater incidence and severity of diabetes has been noted in male laboratory rodents after induction of experimental diabetes by subtotal pancreatectomy (57), alloxan (39) and streptozotocin injection (257). This sex difference may be accounted for in part at the level of the islet β cells as indicated by sex differences in cellular toxicity to streptozotocin in vitro (268, 273). Islet cells from male rat donors were more susceptible to the toxic effect of a high dose of streptozotocin than those from female rats. Thus, it appears that there is a genetic predisposition to human and experimental diabetes which is related in some way to gender.

Data obtained in this study are consistent with the view that female sex hormones have a beneficial role in the evolution of experimental diabetes. After a single diabetogenic dose of streptozotocin, ovariectomised female mice developed a more severe hyperglycaemia than intact female counterparts. This was coincident with lower concentrations of plasma insulin in ovariectomised female mice after the onset of diabetes. Ovariectomy has been reported to reduce glucose tolerance (132), lower fasting plasma insulin concentrations (266) and impair the secretory response to glucose stimulation from isolated pancreatic islets (274) and the perfused pancreas of rats (275). Taken together, the evidence suggests that ovariectomy alters the glucose-homeostatic mechanism, resulting in insulin resistance. This interpretation is not consistent with a recent report that the secretory capacity of pancreatic β cells was enhanced after ovariectomy (276). However, the authors stressed that this

controversial observation might have been caused by the short duration of their study (3 days) and by the fact that the animals used in the study did not have normal β cell function at the start of the experiment. Although a deficiency in endogenous ovarian sex hormones play a large part in increasing the sensitivity of ovariectomised female mice to streptozotocin, recent reports suggest that the elevation of androgen bioactivity may also contribute to the severity of experimental diabetes in these mice (258,277,278).

Data presented in this study demonstrate a beneficial role of long term exogenous sex hormone therapy on streptozotocin induced diabetes. The streptozotocin-induced hyperglycaemia in ovariectomised mice was ameliorated by oestradiol treatment. The degree of protection conferred by oestradiol appeared to be dose-dependent. The hyperglycaemic response to streptozotocin was partially suppressed by oestradiol at a dose of 5 μg/kg/day and almost completely prevented by a high dose of oestradiol (500 μg/kg/day). This effect appeared to be mediated in part through an effect on the endocrine pancreas since plasma insulin concentrations and total pancreatic insulin content were elevated after oestradiol administration. Other investigators have reported similar effects following oestrogen treatment (17,261,264) and this has been substantiated by studies on the pancreatic β cells after exposure to oestradiol. Islet diameter and protein content are increased (17) and there is greater numerical density of the light β granules and swollen mitochondria in animals treated with oestradiol (261). In general, the trophic effect of oestrogens on islets has been likened to hyperinsulinism and mild islet hypertrophy and β cell hyperplasia, similar to the third trimester of pregnancy when circulating titres of sex steroids are high (20). The insulinotropic effect of oestrogens on the pancreatic β cells may be mediated directly (134). In addition, the effect may be mediated indirectly via corticosteroids, since the effects of oestrogens are reduced after adrenalectomy (22,131,280).

Following oestrogen administration, growth hormone and endogenous corticosteroids are increased (164, 281, 282) and they have been shown to raise plasma insulin concentrations (116, 283, 284) through β cell hyperplasia (285). Thus, it appears that the action of glucocorticoids may in part be a prerequisite for the effect of oestrogens on β cells.

Equally important when considering the mechanism of action of oestrogens are reports that glucagon, an insulin antagonistic hormone from the A cells is increased after ovariectomy (266) and decreased after oestradiol treatment (266). Consistently, glucagon suppression by glucose is more pronounced in pregnant than in non-pregnant women (286).

Attempts to define the relative effects of progesterone and other progestogens on glucose homeostasis have to date produced discrepant and often conflicting reports. This may be related to the dose of progesterone, the route of administration and the prevailing oestrogen enviroment, as determined by the use of either ovariectomised or intact animals (287). In this study, streptozotocin-induced hyperglycaemia was suppressed by progesterone more strongly at 3 weeks and 10 weeks after the onset of treatment than during the interim period. Progesterone increased plasma insulin concentrations, consistent with other reports that progesterone increases insulin secretion in laboratory rodents (132, 135). An enhanced plasma insulin response to intravenous glucose following progesterone treatment is well documented in the lietrature (44, 45, 120, 134). However, these augmented insulin levels have frequently been interpreted to represent a physiological response necessary to overcome peripheral resistance to insulin after progesterone treatment (24, 45, 135, 288).

In general, the literature supports the view that progesterone, in the presence of endogenous oestrogens, impairs glucose tolerance, causing insulin resistance and increase plasma insulin concentrations. It must be emphasised however, that this view is derived from studies conducted after

relatively short periods : for example 3 weeks gestation in pregnant rats (135), 3-5 days treatment in women (120), 21 days in female rats (40) and monkeys (45). Furthermore, there is increasing evidence that the diabetogenic stress observed in human pregnancy could be caused not by progesterone, but by human placental lactogen (289). The present study was conducted over a period of 11 weeks and it is possible that progesterone may require a prolonged period of action to generate a positive effect on β cell division and insulin biosynthesis (132,290,291).

Although a contribution of progesterone to changes in plasma insulin and pancreatic islet secretion of insulin is recognised, the mechanism is not well understood. Progesterone may increase the insulin secretory response to glucose through an effect on β cell adenyl cyclase activity (44). Progesterone may also affect both the β cell and peripheral sites of glucose metabolism via alterations at the level of the insulin receptor (154).

Treatment with the combined oestradiol-progesterone regimen did not consistently improve the hypoglycaemic response to streptozotocin, compatible with several reports that the beneficial effects of oestradiol and progesterone alone are offset when the two hormones are present in combination (132,162). The antagonistic effect of progesterone has been reported earlier in this thesis(Chapters 4 and 5) and during oestradiol-induced synthesis of uterine glucose-6-phosphate dehydrogenase (292) and oestradiol stimulation of uterine growth (293).

The influence of ovarian sex hormones on food consumption and body weight regulation is well documented in the literature (294,295). Indirect evidence obtained during the oestrous cycle correlates with the peak levels of oestrogens during the afternoon of proestrus, showing a depression of food intake (296). The results from the present study demonstrate an increase in body weight following ovariectomy in the female mouse, confirming reports by other investigators (297-299). The post-

ovariectomy increase in body weight was subsequently masked by the effect of streptozotocin in the present study. Streptozotocin is known to have a catabolic effect. The loss of body weight induced by the drug is associated with a rapid decrease in the abdominal fat depots (112). Indeed, lack of weight gain is one of the criteria used to confirm the induction of severe hyperglycaemia by sterptozotocin (112).

Oestrogen treatment has been reported to reduce the weight increase seen after ovariectomy in normal (non-diabetic) animals (297-299). In streptozotocin diabetic mice, which increased in body weight after ovariectomy, there was a transient decrease in body weight observed with the lower dose of oestradiol. The high dose of oestradiol resulted in a significant increase in body weight which persisted throughout the study. The difference between non-diabetic and diabetic mice is readily accounted for by the improved insulin status and improved anabolic capacity of oestradiol treated diabetics to store and consolidate ingested nutrients such as fat, glycogen and muscle proteins. A further explanation could lie in the water-retaining properties of oestradiol. It is well known that one of the anabolic effects of oestrogen is to retain sodium, leading to water retention (300, 301). Several reports have shown that despite its mild catabolic effect (133), progesterone often has a weight promoting effect in the presence of intact ovaries (302, 303). This may be partly accounted for by increased food consumption and decreased exercise. However, Bogart (304) has demonstrated an increase in body weight in ovariectomised rats after progesterone administration, and the results in this study are consistent with this report.

In summary, the present study indicate that long-term oestradiol administration ameliorates the severity of streptozotocin diabetes in ovariectomised mice. The protection conferred by oestradiol is dose related. Progesterone alone also ameliorated the severity of experimental diabetes in ovariectomised mice, but progesterone antagonised the effect of oestradiol when the

two steroids were administered in combination. The modulatory effects of sex hormones on streptozotocin diabetes appear to be mediated partly via increased pancreatic insulin production and increased plasma insulin concentrations.

GENERAL DISCUSSION

In recent years, our comprehension of the metabolic and biochemical functions of the ovaries has been expanding rapidly (305). The chemical nature of sex hormones and their reactions at the cellular level have been prominent in this advance. Indeed, there is increasing evidence that the endocrine functions of the pituitary, gonads and adrenal glands can markedly affect the metabolic interrelationships of glucose and insulin (10, 306). However, studies to determine the mode of action of sex hormones at the cellular level and the relationship to the biological actions of insulin have not as yet provided clear-cut answers.

An association between ovarian steroid hormones and the endocrine pancreas has long been suspected, early support for this view arising from reports of sex differences in the prevalence of diabetes (307). Since the early nineteen hundreds, most investigators have focused their attention on the effects of sex hormone administration on islet secretory function (10, 131, 308). Few attempts were made to delineate the action of ovarian steroid hormones on carbohydrate metabolism until the use of oral contraceptive agents became widespread. Then, reports began to appear, suggesting that the synthetic steroidal agents used for contraceptive purposes had a deleterious effect on carbohydrate metabolism (9, 21). Subsequent studies showed that oral contraceptive agents can cause glucose intolerance, particularly in gestational diabetics (24) and in women with a recognised genetic predisposition to diabetes for example, family history of diabetes(10) and in subclinical diabetics and women with unexplained pregnancy wastage or a history of large babies (9, 21). Following oral contraceptive administration, fasting insulin levels were increased (30, 161, 309, 310) in the presence of normal or elevated glucose concentrations, indicating insulin antagonism. Although glucose metabolism may be altered in these women, the changes in glucose that are observed vary considerably,

depending upon the combination of steroids employed, the duration of treatment, route of administration and to the ability of the individual to increase insulin production in the face of a hyperglycaemic challenge (11,21). However, in normal healthy women, these changes in glucose tolerance are usually small and their interpretation has proved to be a difficult task, particularly when the great variety of contraceptive preparations in clinical use and the frequent changes in dosage are taken into account. Indeed, there has been a tendency to ascribe the observed metabolic changes to the 'pill' in general without any attempt to identify the metabolic effect contributed by each steroid component. Recent reports (23,27) suggest that the synthetic oestrogen component is responsible for the impaired glucose tolerance, particularly in women who are subclinically diabetic. Oral progestins alone do not appear to have any effect (30,311,312).

Numerous reports have indicated the presence of a diabetogenic stress during the third trimester of pregnancy, manifested as a progressive biological resistance to endogenous insulin(313-315). Normal women are able to compensate by increasing plasma insulin levels with little change in carbohydrate tolerance. Subclinical and overtly diabetic subjects, however, frequently demonstrate a marked deterioration of carbohydrate tolerance during late gestation, despite hyperinsulinaemia (315). In recent yaers, reports in the literature have suggested that progesterone (135), human placental lactogen (166,289) or growth hormone (316) may contribute both to the insulin resistance and the increased plasma insulin responses observed in the gravid state.

The balance of evidence clearly suggests that there is an association between carbohydrate metabolism and sex steroids. Whether the effect is due to the oestrogen or the progestogen component and the extent to which the individual effects of the steroids are altered when administered in combination remains uncertain. Moreover, as discussed in Chapter 1, studies with natural sex hormones which have been conducted mainly

in animals, conflict with data from the clinical studies which use the synthetic derivatives of sex steroids in oral contraceptive preparations. The situation is further complicated by evidence that the different synthetic oestrogens differ in the extent to which they impair glucose tolerance : for example, reported abnormalities of glucose tolerance are more common with mestranol (26) than with ethinyl oestradiol (317, 318). Nevertheless, a pattern does appear to be emerging. In general , animal studies show that natural oestrogens improve the action of insulin, whilst the synthetic oestrogens alone can cause insulin resistance in patients with compromised pancreatic adaptive ability. This thesis has focused on the mechanism of action of the natural sex hormones, with a view to identifying the cellular and metabolic sites at which sex hormones act.

The laboratory mouse was selected as a suitable animal model for these studies because it is relatively cheap, readily available commercially and easily maintained. Furthermore, inbred strains eliminate the problems of genetic variations (319). The value of the laboratory mouse as a model for the study of human ovarian hormone deficiency has been described elsewhere (320).

Throughout the present study, mice were ovariectomised to reduce interference from endogenous sex hormones. Ovariectomy and replacement with physiological concentrations of sex hormones is one of the most common methods of studying ovarian hormone deficiency. Although other techniques for substitution therapy have been documented, particularly in women (321-324), the oral route of administration of sex steroids was selected for these studies since it was deemed more practical for long-term animal studies and is the most common route in clinical studies (325). One disadvantage of the oral route is the variable dimunition in circulating levels of the steroid due to hepatic deactivation od sex steroids. However, Vallette, Verine, Vares and Boyer (326) have conducted a bioavailability test following

oral administration of ethinyl oestradiol and progesterone in the rat and established that the circulating concentrations of these steroids in the plasma after long-term administration, was similar or very close to the calculated doses administered.

After ovariectomy, concentrations of oestrogens and progestogens are decreased, although a baseline level of sex steroids is maintained by aromatisation of androgens in the adrenals (327). The principle of negative feedback control of gonadotrophin (Luteinising hormone [LH] and follicle stimulating hormone [FSH]) release in the female dictates that a lowering of oestrogens in the blood leads to increased secretion rates of serum LH and FSH (328, 329). Indeed, levels of LH and FSH are elevated 5 h after ovariectomy (127) but are inhibited following administration of oestradiol (329). Recent reports have shown that pituitary gonadotrophin releasing hormone receptors are increased after ovariectomy (330) and decreased after oestrogen treatment of ovariectomised rats (331). This raises the question of whether a comparison between ovariectomised mice and ovariectomised oestrogen-treated mice is juctified, since gonadotrophin levels are elevated in ovariectomised mice and suppressed in mice treated with oestradiol. However, a study with human menopausal gonadotrophin has shown that very high doses of pituitary gonadotrophins are required to improve glucose tolerance in castrated female rats (97). Hence, gonadotrophins are not considered to cause an important effect on carbohydrate metabolism under physiological conditions.

All of the in vitro studies reported in this thesis have been conducted on the isolated soleus muscle. Apart from the many advantageous features offered by this particular tissue preparation (Chapter 2), the skeletal mass in general represents 40 % of body weight in the mouse (332, 333) and contributes to the disposal of at least 35 % of an intravenously administered glucose load (334). Furthermore, muscle is known to be one of the most important target tissues of insulin action. One of the

preliminary experiments in this thesis was an insulin-dose response atudy to select a suitable dose of insulin. A sub-maximal insulin concentration of 0.75 mU/ml was selected to allow for detection of any synergistic effects of sex hormones with insulin. This dose of insulin has been confirmed in a recent report by Chiasson, Dietz, Shikama, Wooten and Exton (335). The authors showed that ^{14}C-glucose incorporation into muscle glycogen was near maximal at an insulin concentration of 1.0 mU/ml and that the effect of added insulin on muscle glucose metabolism achieved significance only after 30 minutes or more of tissue exposure to the hormone. From our preliminary time-course study, an incubation period of 120 minutes was selected.

In the soleus muscle, as in most mammalian tissues, glucose metabolism is mainly a function of both plasma glucose and plasma insulin concentrations (336), although the nutritional state of the animal and the influence of counter-regulatory hormones and nervous influences are also important. Marked differences in responsiveness between fed and fasted mice have been observed in insulin receptor binding (85) and in the secretory response of islets to glucose (337). Glucose utilisation by incubated adipose tissue has been reported to decrease during starvation (338-340). In contrast, glucose utilisation in preparations of skeletal muscles such as the perfused rat hindquarter, the incubated rat soleus and extensor digitorum longus is not depressed in starved rats. In fact, at physiological concentrations of insulin, glucose utilisation is enhanced when muscles of starved rats are used (248, 341). Consequently, mice used for the experiments reported in this thesis were fasted for 40 h. Approximately 35 % of muscle glycogen is lost after a fast of 40 h (246), resulting in increased requirement for exogenous glucose. Thus, under circumstances when tissue energy stores are decreased, insulin acts to stimulate the generation of storage materials from available precursors such as glucose.

The experiments reported in this thesis have showed

that sex hormones modulate the action of insulin on glucose metabolism. Ovariectomy reduced insulin receptor binding, suggesting a decreased capacity of insulin to initiate the post-receptor and effector mechanisms within the cell. Whether this is due to a decrease in the number of receptors or the binding affinity of the receptors is uncertain. There is also speculation as to whether the decrease in insulin receptor binding is associated with a genomic effect of sex steroids on insulin receptor biosynthesis or an effect on the rate of insulin receptor degradation and recycling. However, it appears that normal circulating concentrations of ovarian hormones contribute to the regulation of insulin receptors - a function which is likely to influence the metabolic effects of insulin, particularly at submaximally stimulating concentrations of insulin, as discussed in Chapter 7.

The impairment of insulin action in ovariectomised mice is evidenced by the reduction in insulin-stimulated glucose uptake and CO_2 production from glucose. This is supported by the observation made by other researchers that long-term ovariectomy may decrease plasma insulin concentrations in normal mice (132) and in the present studies using streptozotocin diabetic mice (Chapter 9). A decreased pancreatic insulin content, which is likely to reflect at least in part, a decrease in insulin biosynthesis, appears to be associated with the decreased plasma insulin concentrations after ovariectomy. This suggests a chronic trophic effect of ovarian sex hormones on β cell growth and differentiation (342). Also relevant to the reduced plasma insulin concentration after long-term ovariectomy is the reduced responsiveness of isolated islets to glucose stimulation (275), suggesting that the trophic effect of sex steroids on β cells is also neccesary for maximal responsiveness to nutrient secretagogues.

In addition to the effects observed in the presence of insulin, ovariectomy also reduced glucose uptake and metabolism in the absence of insulin. This may be interpreted as an indication that normal physiological concentrations of sex hormones

contribute to the maintainance of basal glucose uptake and metabolism.

The effects of ovariectomy were also examined in the context of the activities of the gluco-regulatory enzymes involved. Ovariectomy reduced the insulin-stimulated increment of the active (I) form of glycogen synthase, indicating an impairment of insulin activation of glycogen synthase from the D to the I form. The activities of hexokinase and 2-oxoglutarate dehydrogenase were lowered in ovariectomised mice, though not significantly reduced, reflecting the variability in enzyme activities when crude homogenates are employed.

The action of insulin on glucose metabolism was improved after long-term (10 weeks) treatment of ovariectomised mice with oestradiol 17β and to a lesser extent with progesterone. These effects appeared to be mediated both at receptor and post-receptor levels. After oestradiol administration, insulin receptor binding was increased and insulin-mediated glucose oxidation and glycogenesis was enhanced. At this point, it may be useful to compare oestradiol effects and circulating plasma insulin concentrations. Reports in the lietrature and our own studies (Chapter 9) showed that plasma insulin concentrations were raised following sex hormone treatment. This is frequently associated with hypertrophy and hyperplasia of the islets of Langerhans (18-20). The principle of down-regulation implies that an elevation of plasma insulin concentrations will lead to decreased binding and impaired tissue responsiveness to insulin (343) resulting from a loss of insulin receptor binding sites. This is not the case in the studies with oestradiol. Kobayashi and Olefsky (344) however, recently showed that glucose oxidation was increased in adipocytes in the face of prevailing hyperinsulinaemia, lending support to our observations. Thus, a stimulatory effect of sex steroids on insulin receptor binding sites appears to overide a down-regulatory effect of the raised plasma insulin concentrations. It seems likely that the effect of sex hormones may be exerted most

strongly at sites diatal to receptor binding. The effects of sex hormones therefore appear to generate post-receptor effects which lead to induction of key glycolytic enzymes, thereby enhancing intracellular glucose metabolism. These sites of action at which sex steroids overide the effect of small changes in plasma insulin are not known. However, as reviewed elsewhere (155, 345), it is the positive effects of insulin action which exert the major feed-back control on insulin receptor status. It is known that factors in addition to plasma insulin concentrations participate in the regulation of insulin receptor numbers. Factors such as growth status (346) and dietary fat intake (347) have been shown to modify insulin receptors independent of changes in plasma insulin concentrations. It is therefore feasible to postulate that while plasma insulin concentration is an important regulator of membrane insulin receptors, additional factors such as sex hormones can also modulate this process.

The effects of progesterone on carbohydrate disposal are particularly difficult to interpret. This steroid appears to direct its action mainly at post-receptor sites of insulin action with little alteration of insulin receptor status. When present in combination with oestradiol however, progesterone appeared to antagonise the effect of oestradiol on selected aspects of glucose metabolism. Aerobic glucose utilisation and glucose phosphorylation was decreased with no change in the activities of hexokinase and 2-oxoglutarate dehydrogenase. The combined regimen enhanced insulin receptor binding and appeared to direct a greater proportion of exogenous glucose towards glycogen synthesis. The increase in glycogenesis was associated with an increased percentage of the active (I) form of glycogen synthase. On the basis of presently available evidence, it appears that progesterone promotes selected aspects of insulin-stimulated glucose metabolism in the absence of oestrogens. However, the antagonistic effect of progesterone on certain of the gluco-regulatory effects of oestradiol may account for the conflicting reports in the literature concerning the effect

the effect of this steroid (progesterone).

The effect of ovarian steroid hormones was examined in streptozotocin diabetic mice. Ovariectomy increased the rate of onset and severity of hyperglycaemia. These effects were opposed by oestradiol in a dose dependent manner. Although progesterone also reduced the extent of hyperglycaemia, this hormone partly antagonised the effect of oestradiol when the two hormones were present in combination. A possible explanation for this situation is mentioned above and considered in detail in Chapter 9. The protective effect of ovarian steroids against streptozotocin diabetes correlated with the extent to which the steroids opposed the fall in plasma insulin and pancreatic insulin content after streptozotocin administration. These in vivo effects also correlate with the capacity of the steroids to increase insulin-stimulated glucose uptake into soleus muscles. Taken together, the evidence suggests that the ovarian steroid hormones, oestradiol and progesterone can reduce the severity of streptozotocin diabetes in female mice through effects on both insulin production and insulin action.

APPENDIX I

Chapter 3

Table 3.1 Characteristics of experimental animals.

Body wt (g) Mean ± SEM	Right soleus muscle Weight (mg) Mean ± SEM	Left soleus muscle Weight (mg) Mean ± SEM
27.1 ± 1.0	5.0 ± 0.1	5.3 ± 0.1
25.4 ± 0.5	5.9 ± 0.9	6.0 ± 0.5
22.7 ± 0.4	4.7 ± 0.5	5.0 ± 0.5
23.4 ± 1.6	5.9 ± 0.4	6.1 ± 0.3
24.2 ± 1.8	5.5 ± 0.6	6.0 ± 0.3

Table 3.2 CO_2 production by mouse soleus muscles incubated for increasing duration in the absence and presence of insulin (1.0 mU/ml). Values are expressed as nmoles of glucose converted to CO_2 per mg protein. p denotes significance of difference between basal and insulin-stimulated CO_2 production at each incubation time.

Incubation time (mins)	CO_2 production nmol/mg protein		
	No insulin Mean ± SEM	With insulin Mean ± SEM	p
30	8.42 ± 0.40	9.88 ± 0.31	< 0.05
60	12.42 ± 1.16	16.95 ± 0.88	< 0.05
90	26.18 ± 2.09	31.77 ± 1.22	< 0.05
120	30.63 ± 1.39	38.47 ± 4.66	< 0.05
150	34.71 ± 3.38	47.11 ± 5.11	< 0.05

Table 3.3 Glycogen formation by mouse soleus muscles incubated for increasing duration in the absence and presence of insulin (1.0 mU/ml). Values are expressed as nmoles of glucose converted to glycogen per mg protein. p denotes significance of difference between basal and insulin-stimulated glycogen formation at each incubation time.

Incubation time (mins)	Glycogen formation nmol/mg protein		
	No insulin Mean ± SEM	With insulin Mean ± SEM	p
30	3.5 ± 1.0	6.7 ± 1.3	NS
60	4.6 ± 1.2	12.2 ± 0.9	<0.05
90	9.3 ± 3.7	16.5 ± 3.0	<0.05
120	11.1 ± 1.2	17.8 ± 3.4	<0.05
150	10.4 ± 1.1	18.5 ± 3.2	<0.05

Table 3.4 CO_2 production during incubation of mouse soleus muscles in the absence and presence of insulin (0.1, 0.5, 1.0, 5.0 and 10.0 mU/ml). Values are expressed as nmoles of glucose converted to CO_2 per minute per mg protein. p denotes significance of difference between basal and insulin-stimulated CO_2 production at each insulin concentration.

Insulin (mU/ml)	Insulin-stimulated CO_2 production nmol/min/mg	Basal CO_2 production nmol/min/mg	p
0.1	0.09 ± 0.04	0.06 ± 0.03	NS
0.5	0.37 ± 0.16	0.07 ± 0.04	NS
1.0	0.72 ± 0.32	0.09 ± 0.03	<0.05
5.0	0.79 ± 0.31	0.06 ± 0.02	<0.05
10.0	0.83 ± 0.39	0.08 ± 0.03	<0.05

Table 4.1 Characteristics of experimental animals.

	Body wt (g) Mean ± SEM	Right soleus Muscle wt(mg) Mean ± SEM	Left soleus Muscle wt(mg) Mean ± SEM
Group A Ovx only	36 ± 1.5	12.9 ± 2.1	13.8 ± 2.6
Group B Ovx + E	33 ± 0.9	13.8 ± 2.2	12.0 ± 1.3
Group C Ovx + P	35 ± 2.2	14.0 ± 2.0	13.1 ± 2.1
Group D Ovx + E + P	32 ± 2.4	12.9 ± 2.3	12.8 ± 2.2
Group E Intact P/O	36 ± 2.1	11.3 ± 3.1	12.0 ± 4.0
Intact M/D	33 ± 1.0	11.1 ± 1.2	11.8 ± 0.8

Ovx = Ovariectomised
E = Oestradiol
P = Progesterone
P/O = Proestrus/oestrus
M/D = Metoestrus/dioestrus

Table 4.2 CO_2 production by mouse soleus muscles from control and hormone treated mice. Values for CO_2 production are expressed as nmoles of glucose converted to CO_2 per minute per mg muscle protein. p denotes significance of difference between basal and insulin-stimulated CO_2 production, determined by Student's 't' test for paired values.

	With insulin CO_2 production (nmol/min/mg)	No insulin CO_2 production (nmol/min/mg)	p
Group A Ovx only	0.093 ± 0.003	0.035 ± 0.018	<0.05
Group B Ovx + E	0.158 ± 0.026	0.066 ± 0.011	<0.01
Group C Ovx + P	0.125 ± 0.014	0.062 ± 0.005	<0.01
Group D Ovx + E + P	0.068 ± 0.012	0.059 ± 0.007	<0.05
Group E Intact P/O	0.149 ± 0.001	0.103 ± 0.014	<0.05
Intact M/D	0.120 ± 0.013	0.093 ± 0.011	<0.05

Table 4.3 Insulin-stimulated increment in CO_2 production above basal values. p denotes significance of difference compared with ovariectomised controls.

	Increment in CO_2 production (nmol/min/mg protein)	p
Group A Ovx only	0.048 ± 0.008	
Group B Ovx + E	0.100 ± 0.033	<0.05
Group C Ovx + P	0.081 ± 0.016	<0.05
Group D Ovx + E + P	0.011 ± 0.004	NS
Group E Intact P/O	0.060 ± 0.011	NS
Intact M/D	0.019 ± 0.020	NS

NS = not significant

Table 4.4 Glycogen formation by mouse soleus muscles from control and hormone treated mice. Values for glycogen formation are expressed as nmoles of glucose converted to glycogen per minute per mg protein. p denotes significance of difference between basal and insulin-stimulated glycogen formation, determined by Student's 't' test for paired data.

	With insulin Glycogen formation (nmol/min/mg)	No insulin Glycogen formation (nmol/min/mg)	p
Group A Ovx only	0.083 ± 0.011	0.025 ± 0.020	<0.05
Group B Ovx + E	0.123 ± 0.023	0.037 ± 0.025	<0.05
Group C Ovx + P	0.085 ± 0.003	0.051 ± 0.011	<0.05
Group D Ovx + E+P	0.164 ± 0.027	0.113 ± 0.019	NS
Group E Intact P/O	0.090 ± 0.011	0.031 ± 0.009	<0.05
Intact M/D	0.078 ± 0.025	0.037 ± 0.024	NS

Table 4.5 Insulin-stimulated increment in glycogen formation above basal values. p denotes significance of difference compared with ovariectomised controls.

	Increment in glycogen formation (nmol/min/mg protein) Mean ± SEM	
Group A Ovx only	0.042 ± 0.009	
Group B Ovx + E	0.098 ± 0.019	< 0.05
Group C Ovx + P	0.037 ± 0.011	NS
Group D Ovx + E+P	0.079 ± 0.020	< 0.05
Group E Intact P/O	0.080 ± 0.005	< 0.05
Intact M/D	0.041 ± 0.001	NS

Table 5.1 Characteristics of experimental animals.

	Body wt(g) Mean± SEM	Muscle wt (mg) Right soleus Mean ± SEM	Muscle wt (mg) Left soleus Mean ± SEM
Group A Ovx only	31 ± 2.5	10.9 ± 1.0	11.0 ± 1.1
Group B Ovx + E	34 ± 2.4	11.1 ± 1.0	11.9 ± 1.0
Group C Ovx + P	29 ± 0.5	11.0 ± 1.2	10.8 ±1.0
Group D Ovx + E+P	36 ± 2.7	12.0 ± 1.3	12.1 ±1.1
Group E Intact P/O	30 ± 1.9	9.8 ± 1.0	10.1 ± 0.8
Intact M/D	32 ± 2.5	10.1 ± 0.9	11.2 ± 1.1

Ovx = Ovariectomised only

E = Oestradiol 17β

P = Progesterone

P/O = Proestrus/oestrus

M/D = Metoestrus/dioestrus

Table 5.2 3-O-methyl-α-D-glucopyranose (3-O-MG) uptake by soleus muscles from control and hormone treated mice, after incubation of muscles in the absence and presence of insulin (0.75 mU/ml). p denotes significance of difference between basal and insulin-stimulated 3-O-MG uptake, determined by Student's 't' test for paired data. Values in each group are means ± SEM of 5 or 6 determinations.

	With insulin 3-O-MG uptake nmol/min/mg	No insulin 3-O-MG uptake nmol/min/mg	p
Group A Ovx only	0.197 ± 0.025	0.185 ± 0.023	NS
Group B Ovx + E	0.255 ± 0.010	0.208 ± 0.009	<0.05
Group C Ovx + P	0.138 ± 0.032	0.113 ± 0.021	NS
Group D Ovx + E+P	0.367 ± 0.013	0.265 ± 0.044	<0.05
Group E			
Intact P/O	0.291 ± 0.011	0.258 ± 0.001	<0.05
Intact M/D	0.260 ± 0.008	0.214 ± 0.022	<0.05

NS = not significant

Table 5.3 Insulin-stimulated increment in 3-O-MG uptake above basal values. p denotes significance of difference compared with ovariectomised controls.

	Increment in 3-O-MG uptake nmol/min/mg protein Mean ± SEM	p
Ovx only	0.019 ± 0.008	
Ovx + E	0.039 ± 0.017	NS
Ovx + P	0.018 ± 0.006	NS
Ovx + E+P	0.091 ± 0.026	< 0.01
Intact P/O	0.041 ± 0.016	< 0.05
Intact M/D	0.046 ± 0.011	< 0.01

Table 5.4 Volume of extracellular fluid in mouse soleus muscles from control and hormone treated mice.

	Right soleus ECF space (µl/g) Mean ± SEM	Left soleus ECF space (µl/g) Mean ± SEM
Ovx only	448 ± 26	401 ± 14
Ovx + E	538 ± 49	506 ± 56
Ovx + P	496 ± 27	437 ± 24
Ovx + E+P	472 ± 27	454 ± 47
Intact P/O	498 ± 29	431 ± 25
Intact M/D	537 ± 51	456 ± 55

ECF = extracellular fluid

Table 5.5 Volume of extracellular fluid (ECF) space in muscles from control and hormone treated mice.

	Right soleus muscle ECF space (μl/g)	Left soleus muscle ECF space (μl/g)
Ovx only	560 ± 64	467 ± 36
Ovx + E	577 ± 53	655 ± 59
Ovx + P	487 ± 48	419 ± 34
Ovx + E+P	724 ± 51	610 ± 64
Intact P/O	426 ± 43	350 ± 30
Intact M/D	459 ± 32	394 ± 18

Table 5.6 Characteristics of experimental animals (2-deoxy-D-glucose study)

	Body wt (g) Mean ± SEM	Right soleus Muscle wt (mg) Mean ± SEM	Left soleus Muscle wt (mg) Mean ± SEM
Ovx	33.9 ± 2.7	11.3 ± 1.3	12.8 ± 1.2
Ovx +E	37.7 ± 3.5	12.0 ± 1.2	13.0 ± 1.1
Ovx +P	31.1 ± 2.2	9.1 ± 1.0	10.0 ± 1.2
Ovx +E+P	36.0 ± 2.8	8.9 ± 1.2	8.9 ± 1.1
Intact P/O	32.5 ± 3.8	10.0 ± 1.1	11.4 ± 1.3
Intact M/D	28.5 ± 3.1	8.1 ± 1.2	8.2 ± 1.3

Table 5.7 2-deoxy-D-glucose uptake by soleus muscles from control and hormone treated mice, after incubation of muscles in the absence and presence of insulin (0.75 mU/ml). p denotes significance of difference between basal and insulin-stimulated 2-deoxy-D-glucose uptake, determined by Student's 't' test for paired data. Values are means ± SEM of 6 or 7 determinations.

	With insulin 2-deoxy-D-glucose nmol/min/mg	No insulin 2-deoxy-D-glucose nmol/min/mg	p
Group A Ovx only	0.639 ± 0.040	0.370 ± 0.045	< 0.05
Group B Ovx + E	1.040 ± 0.046	0.420 ± 0.035	< 0.001
Group C Ovx + P	0.754 ± 0.067	0.479 ± 0.072	NS
Group D Ovx + E+P	0.891 ± 0.065	0.588 ± 0.045	< 0.01
Group E Intact P/O	0.861 ± 0.023	0.482 ± 0.020	< 0.01
Intact M/D	0.856 ± 0.027	0.546 ± 0.022	< 0.01

Table 5.8 Insulin-stimulated increment in 2-deoxy-D-glucose uptake above basal values. p denotes significance of difference compared with ovariectomised controls.

	Increment in 2-deoxy-D-glucose Mean ± SEM	p
Ovx only	0.286 ± 0.020	
Ovx + E	0.617 ± 0.031	<0.001
Ovx + P	0.380 ± 0.023	NS
Ovx + E+P	0.297 ± 0.019	NS
Intact P/O	0.434 ± 0.024	<0.05
Intact M/D	0.293 ± 0.009	NS

Table 6.1 Characteristics of experimental animals.

Incubation period (h)	Body wt (g) Mean ± SEM	Right soleus Muscle wt (mg) Mean ± SEM	Left soleus Muscle wt (mg) Mean ± SEM
2	21.1 ± 0.1	7.5 ± 0.6	7.4 ± 0.3
4	21.6 2.1	6.7 ± 0.6	7.3 ± 0.9
6	21.5 ± 0.7	7.1 ± 0.5	6.9 ± 0.2

Table 6.2 Time course of insulin binding to soleus muscles from 40 h fasted ovariectomised mice.

Incubation period (h)	Total binding Percentage ^{125}I-insulin bound/mg protein	Non-specific binding Percentage ^{125}I-insulin bound/mg protein
2	0.18 ± 0.02	0.06 ± 0.01
4	0.42 ± 0.03	0.08 ± 0.01
6	0.46 ± 0.02	0.09 ± 0.02

Table 7.1 Characteristics of experimental animals.

	Body wt (g) Mean ± SEM	Right soleus Muscle wt (mg) Mean ± SEM	Left soleus muscle wt(mg) Mean ± SEM
A. Fasted animals			
Group A Ovx only	32.8 ± 1.1	9.9 ± 0.7	10.1 ± 0.7
Group B Ovx + E	30.5 ± 0.9	9.0 ± 0.4	9.4 ± 0.5
Group C Ovx + P	43.3 ± 3.7	12.8 ± 1.3	12.9 ± 1.2
Group D Ovx + E+P	31.2 ± 2.6	9.0 ± 0.3	9.3 ± 0.8
Group E Intact P/O	33.4 ± 1.8	8.6 ± 1.2	9.4 ± 1.4
Intact M/D	32.6 0.9	9.3 0.8	9.7 ± 0.9
B. Fed animals			
Group A Ovx only	40.5 ± 2.9	11.5 ± 0.6	11.9 ± 0.7
Group B Ovx + E	42.6 ± 1.3	12.3 ± 0.7	12.2 ± 0.3
Group C Ovx + P	37.5 ± 1.7	10.5 ± 0.5	11.4 ± 0.9
Group D Ovx + E+P	36.9 ± 1.7	8.9 ± 0.9	9.6 ± 0.4
Group E Intact P/O	37.4 ± 1.4	9.0 ± 0.3	11.4 ± 1.6
Intact M/D	35.8 ± 0.9	11.3 ± 0.8	12.5 ± 1.2

Table 7.2 Insulin binding in soleus muscles from fasted intact, ovariectomised and ovariectomised steroid treated mice. Values are expressed as percentage of ^{125}I-insulin specifically bound per mg protein and are means ± SEM of 5 determinations in each group. p denotes significance of difference compared with ovariectomised mice.

Treatment	Percentage ^{125}I-insulin specifically bound per mg protein	p
Ovx only	0.291 ± 0.032	
Ovx + E	0.345 ± 0.020	< 0.05
Ovx + P	0.387 ± 0.078	NS
Ovx + E+P	0.372 ± 0.058	NS
Intact P/O	0.394 ± 0.053	< 0.05
Intact M/D	0.378 ± 0.048	< 0.05

Table 7.3 Insulin binding in soleus muscles from fed intact, ovariectomised and ovariectomised steroid treated mice. Values are expressed as percentage of ^{125}I-insulin specifically bound per mg protein and are means ± SEM of 5, 7 or 8 determinations in each group. p denotes significance of difference compared with ovariectomised mice.

Treatment	Percentage ^{125}I-insulin specifically bound per mg protein	p
Ovx only	0.338 ± 0.048	
Ovx + E	0.302 ± 0.031	NS
Ovx + P	0.404 ± 0.048	NS
Ovx + E+P	0.516 ± 0.076	< 0.05
Intact P/O	0.566 ± 0.081	< 0.05
Intact M/D	0.543 ± 0.102	< 0.05

NS = not significant

Table 8.1 Hexokinase activity in soleus muscles from intact proestrus/oestrus mice and ovariectomised hormone treated mice. p denotes significance of difference between basal and insulin-stimulated hexokinase activity using Student's ' t ' test for paired data. Values are means ± SEM of 5 determinations.

Treatment	Hexokinase activity (μmol/min/g) Basal	Insulin-stimulated	p
Ovx only	1.16 ± 0.20	1.48 ± 0.18	NS
Ovx + E	1.72 ± 0.21	2.14 ± 0.19	0.05
Ovx + P	1.44 ± 0.19	1.32 ± 0.08	NS
Ovx +E+P	1.38 ± 0.16	1.49 ± 0.09	NS
Intact P/O	1.45 ± 0.18	1.58 ± 0.17	NS

E = Oestradiol

P = Progesterone

P/O = Proestrus/oestrus

NS = not significant

Table 8.2 Glycogen synthase activity in soleus muscles from intact proestrus-oestrus and ovariectomised hormone treated mice. △ is the increase in the percentage of glycogen synthase active (I) from brought about by insulin (0.75 mU/ml). Values are means ± SEM of 5 determinations.

Treatment	% of active (I) form		△	Total(I + D) activity
	Basal	Insulin		nmol/min/mg
Group A Ovx only	26.4 ± 4.3	31.1 ± 4.5	6.1 ± 0.6	16.6 ± 1.7
Group B Ovx + E	35.6 ± 3.9	48.6 ± 4.7	13.0 ± 3.2	19.7 ± 2.0
Group C Ovx + P	12.6 ± 2.5	17.6 ± 5.1	4.1 ± 3.8	14.6 ± 3.9
Group D Ovx +E+P	22.1 ± 1.7	29.7 ± 2.6	7.8 ± 1.3	12.0 ± 3.3
Group E Intact P/O	36.2 ± 1.8	49.7 ± 2.9	13.5 ± 3.4	13.9 ± 2.8

Ovx = Ovariectomised

E = Oestradiol

P = Progesterone

P/O = Proestrus/oestrus

Table 8.3 2-oxoglutarate dehydrogenase activity in soleus muscles from intact proestrus-oestrus and ovariectomised hormone treated mice. Values are expressed as μmoles of product formed per minute per gram wet weight of muscle and are means ± SEM of 5 determinations. p denotes significance of difference between basal and insulin-stimulated 2-oxoglutarate dehydrogenase activity for each group, determined by Student's 't' test for paired data.

Treatment	2-oxoglutarate dehydrogenase activity (μmol/min/g)		
	Basal	Insulin	p
Group A Ovx only	0.59 ± 0.09	0.68 ± 0.12	NS
Group B Ovx + E	0.60 ± 0.15	1.28 ± 0.18	<0.01
Group C Ovx + P	0.61 ± 0.06	0.84 ± 0.10	<0.01
Group D Ovx + E+P	0.41 ± 0.09	0.69 ± 0.06	<0.01
Group E Intact P/O	0.89 ± 0.11	1.11 ± 0.15	NS

Table 8.4 Insulin-stimulated increment in 2oxoglutarate dehydrogenase activity above basal expressed as μmol/min/g wet weight. p denotes significance of difference compared with ovariectomised mice.

Ovx	Ovx + E	Ovx +P	Ovx +E+P	Intact P/O
0.13±0.07	0.34±0.09	0.15±0.08	0.18±0.05	0.16±0.05
p	0.05	NS	NS	NS

Ovx = Ovariectomised
E = Oestradiol
P = Progesterone
P/O = Proestrus-oestrus
NS = not significant

Table 9.1 Body weight, plasma glucose and plasma insulin of ovariectomised mice prior to and after streptozotocin injection. Each value represents the mean ± SEM of 10 determinations.

	Body wt(g) Mean ± SEM	Plasma glucose (mmol/l) Mean ± SEM	Plasma insulin (ng/ml) Mean ± SEM
Before ovx.	28.0 ± 0.56	8.9 ± 0.31	2.6 ± 0.12
After ovx.	29.2 ± 0.59	8.3 ± 0.27	2.6 ± 0.16
Weeks after streptozotocin			
0	35.1 ± 0.74	-	-
1	33.6 ± 0.72	-	-
2	33.9 ± 1.27	18.6 ± 2.25	-
3	32.4 ± 1.33	25.0 ± 2.08	-
4	32.0 ± 0.98	26.2 ± 2.35	0.78 ± 0.19
5	32.3 ± 0.68	28.7 ± 1.92	-
6	34.5 ± 1.33	26.3 ± 2.10	-
7	31.9 ± 1.16	27.3 ± 2.12	-
8	33.0 ± 0.92	31.0 ± 3.11	-
9	34.8 ± 0.91	25.0 ± 1.84	-
10	33.8 ± 0.99	35.1 ± 1.97	0.76 ± 0.10
11	34.6 ± 1.12	37.1 ± 2.50	-

Table 9.2 Body weight, plasma glucose and plasma insulin of intact mice prior to and after streptozotocin injection. Each value represents the mean ± SEM of 10 determinations.

	Body wt (g) Mean ± SEM	Plasma glucose (mmol/l) Mean ± SEM	Plasma insulin (ng/ml) Mean ± SEM
Before streptozotocin	29.7 ± 0.59	9.48 ± 0.22	2.40 ± 0.11
	29.8 ± 0.48	8.85 ± 0.41	-
Weeks after streptozotocin			
0	32.3 ± 0.91	-	2.81 ± 0.38
1	32.9 ± 1.20	-	-
2	33.5 ± 1.14	20.7 ± 2.30	-
3	33.4 ± 1.28	19,3 ± 3.22	-
4	33.0 ± 1.48	20.7 ± 2.30	2.07 ± 0.19
5	33.9 ± 0.98	21.9 ± 2.52	-
6	32.4 ± 1.24	24.3 ± 2.66	-
7	33.1 ± 0.87	19.9 ± 2.75	-
8	33.0 ± 0.96	22.3 ± 2.41	-
9	33.8 ± 1.01	22.1 ± 2.79	-
10	33.6 ± 1.23	26.6 ± 1.29	1.48 ± 0.17

Table 9.3 Body weights of ovariectomised diabetic mice during treatment for 10 weeks with placebo, oestradiol (5 μg/kg/day), oestradiol (500μg/kg/day), progesterone (1mg/kg/day) and oestradiol + progesterone(5μg/kg/day + 1 mg/kg/day). Each value represents the mean ± SEM of 10 determinations.

Weeks after treatment	Body weights (g)				
	Ovx.	E (5 μg/kg)	E (500μg/kg)	P (1mg/kg)	E + P (5μg/kg+ 1mg/kg)
0	29.2±1.58	28.0±0.79	27.8±0.89	29.3± 0.88	29.9± 1.07
1	29.5±1.17	28.7±1.18	30.6±0.87	28.0± 1.16	28.3± 1.05
2	28.7±1.05	27.5±1.02	31.8±0.79	29.1± 1.17	29.9± 1.16
3	29.8±1.23	29.2±1.12	31.9±1.03	30.0±1.09	30.2± 1.13
4	30.5±1.14	30.1 ±1.11	32.3±1.01	30.8±1.14	31.6± 1.55
5	30.2±1.16	30.3±1.09	33.3±1.16	29.3± 1.09	30.7± 1.14
6	27.6±0.83	27.8±1.31	32.6±1.17	29.8± 1.11	32.8± 1.13
7	-	-	-	-	-
8	30.5±1.35	31.9±1.15	36.0±1.17	34.1± 1.15	31.9± 1.14
9	-	-	-	-	-
10	30.8±1.17	32.0±1.18	36.9±1.12	35.3±1.08	33.7± 1.28

Ovx = Ovariectomised

E = Oestradiol

P = Progesterone

Table 9.4 Plasma glucose of ovariectomised diabetic mice during treatment for 10 weeks with placebo, oestradiol (5 μg/kg/day), oestradiol (500 μg/kg/day), progesterone (1 mg/kg/day) and oestradiol + progesterone (5 μg/kg/day + 1 mg/kg/day). Each value represents the mean ± SEM of 10 determinations.

Weeks after treatment	Plasma glucose (mmol/l)				
	Ovx.	E (5 μg/kg)	E (500μg/kg)	P (1 mg/kg)	E + P (5 μg/kg+ 1mg/kg)
0	20.0±1.23	19.9±0.39	19.2±1.20	20.8±0.78	18.8±1.28
1	25.4±1.75	21.6±1,03	20.5±1.40	17.2±1.73	21.1±2.61
2	28.6±1.72	25.9±1.63	20.5±1.50	18.4±3.03	22.1±1.54
3	29.5±0.57	23.6±2.24	19.0±2.26	16.3±1.39	21.1±2.55
4	31.8±1.36	25.2±2.24	21.8±1.45	21.0±2.70	21.2±1.79
5	31.9±1.87	25.8±1.73	25.8±1.00	24.9±2.64	25.5±1.47
6	33.4±1.97	25.4±1.88	28.6±1.64	27.1±3.60	29.7±1.81
7	-	-	-	-	-
8	30.5±1.35	23.2±2.19	24.2±2.37	21.2±2.61	22.8±1.99
9	-	-	-	-	-
10	33.5±2.20	23.6±1.52	20.0±2.38	17.5±0.98	25.5±2.38

Ovx = Ovariectomised

E = Oestradiol

P = Progesterone

APPENDIX II

The calculation for extracellular fluid volume and 3-O-methy-α-D-glucopyranose accumulation in soleus muscle is similar to that for 2-deoxy-D-glucose accumulation as shown below:

Total activity in the incubation medium was:

196,746 dpm/μl of ^{3}H-inulin

376,386 dpm/μl of 2-deoxy-D-[1-^{14}C] glucose

11.6 μl of ^{3}H-inulin and 10 μl of 2-deoxy-D-[1-^{14}C] glucose were present in 20 ml of incubation medium. Hence, the concentration of ^{3}H-inulin in the incubation medium is 114 dpm/μl and the concentration of 2-deoxy-D-[1-^{14}C] glucose in the medium is 188 dpm/μl.

An example of the counts from a soleus muscle is:

339 dpm ^{3}H-inulin

3297 dpm 2-deoxy-D-[1-^{14}C] glucose

Assuming the concentration of inulin in the extracellular fluid space is the same as that in the incubation medium, the volume of ^{3}H-inulin in the muscle is $\frac{339}{114}$ = 2.974 μl

Since the muscle weighs 0.0085 g, the volume of extracellular fluid is 349 μl/g.

The number of ^{14}C counts that is present in the extracellular fluid is 2.974 x 188 = 559 dpm

Therefore, the 2-deoxy-D-[1-^{14}C] glucose that was present inside the cells : 3297 - 559 = 2738 dpm.

The specific activity of 2-deoxy-D-[1-^{14}C] glucose in the medium is 0.0998×10^{-3} μCi/nmole and since 1 μCi = 2.22×10^{6} dpm, the specific activity will be 222 dpm/nmole.

Hence, 2738 dpm (from earlier) will be equal to 12.33 nmoles for an incubation period of 15 minutes.

The number of nmoles of 2-deoxy-D-glucose present per minute is 0.822.

The protein content of the muscle is 0.731 mg.

Therefore, the rate of 2-deoxy-D-glucose uptake into the muscle per minute per mg muscle protein is 1.054 nmol/min/mg protein.

Calculation of hexokinase activity in soleus muscle

An example of the calculation of enzyme activity in μmol/min/g tissue is shown below.:

Change in optical density per minute = 0.0094

The optical density/min is divided by 6.22, which is the millimolar absorptivity of NADPH at 340 nm.

$$\frac{0.0094}{6.22} = 0.00151$$

The weight of muscle used in this example is 0.0148 g

The volume of homogenisation is 200 μl.

Therefore, the correction factor for the dilution of homogenisation = 13.51

Total volume in the cuvette = 2.0 ml

Correction factor for sample volume(25 μl) = 40

Hence, hexokinase activity = 0.00151 x 2 13.51 x 40

= 1.632 μmol/min/g tissue

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