Studies on the role of natural and synthetic sex steroids in the regulation of carbohydrate metabolism.

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A thesis submitted for the degree of Doctor of Philosophy at The University of Aston in Birmingham. October, 1980.

SUMMARY

This thesis examines the effects of natural and synthetic sex steroids on the regulation of glucose homeostasis in adult female mice.

Natural (estradiol-17β) and synthetic (ethynyl estradiol) estrogens elevate plasma insulin concentrations, glycogen deposition and peripheral glucose utilisation, and reduce plasma glucose concentrations.

Natural (progesterone) and synthetic (norethnodrel and norethisterone acetate) progestogens elevate plasma insulin concentrations and glycogen deposition, reduce peripheral glucose utilisation, and frequently elevate plasma glucose concentrations.

Since progestogens generally have deleterious effects on glucose homeostasis, their use is contra-indicated in those individuals with compromised β cell status.

Natural sex steroids have a greater hypoglycaemic and hyperinsulinaemic influence than synthetic sex steroids

Combined steroid preparations elevate glycogen deposition, increase peripheral glucose utilisation and lower plasma glucose without elevating plasma insulin.

Synthetic combined steroid preparations have a greater hypoglycaemic and hyperinsulinaemic influence than natural combined steroid preparations.

Combined sex steroid preparations have a beneficial influence on glucose homeostasis and do not appear to represent a diabetic threat in normal healthy individuals. Antagonism between the two steroid components may play a role in improving glucose homeostasis.

The subcutaneous route of administration potentiates, whereas the intramuscular and oral routes generally reduce the efficacy of the steriod preparations which benefit glucose homeostasis. Thus more frequent use of the subcutaneous route might be advantageous in hormonal contraceptive users.

The present study shows that female sex steroids influence carbohydrate homeostasis via changes in plasma insulin concentrations. Since these steroids bind to tissues involved in glucose metabolism, including liver, muscle, fat and pancreas, they may also act directly on these tissues. In addition, sex steroids might affect glucose homeostasis via changes in circulating adrenocorticoid levels.

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KEY WORDS— ESTROGEN

PROGESTOGEN

CARBOHYDRATE METABOLISM

Acknowledgements

I would like to extend my thanks to everyone who has contributed towards the preparation of this thesis. In particular my supervisor, Dr. C. J. Bailey, for his invaluable support and guidance, and Mr. J. G. Plant for his assistance and encouragement.

I would also like to thank the Science Research Council for financial support.

ABBREVIATIONS

cAMP	:	Adenosine 3':5'-cyclic monophosphate
ACTH	:	Adrenocorticotrophic hormone
Ab	:	Antibody
Ag	:	Antigen
CNS	:	Central Nervous System
CR	:	Channels Ratio
CCK	:	Cholyecystokinin
dpm/mg	:	Disintegrations per minute per mg
E_2	:	Estradiol-17 β
EE	:	Ethynyl estradiol
ESR	:	External standard ratio
GIP	:	Gastricinhibitory polypeptide
GTT	:	Glucose tolerance test
Im	:	Intramuscular
ip	:	Intraperitoneal
IUD	:	Intrauterine device
LSC	:	Liquid scintillation counting
$\bar{x}$	:	Mean
NA	:	Norethisterone acetate
N	:	Norethnodrel
P	:	Progesterone
RIA	:	Radioimmunoassay
SD	:	Standard deviation
SE	:	Standard error of the mean
Sc	:	Subcutaneous
VIP	:	Vasoinhibitory polypeptide
VLH	:	Ventro-lateral hypothalamus
VMH	:	Ventro-medial hypothalamus

The afore mentioned abbreviations were used in the present thesis.

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Chapter 1

INTRODUCTION

CHAPTER 1

GENERAL INTRODUCTION

There is considerable evidence to suggest an association between female sex steroids and intermediary metabolism (1). In particular there have been many reports in the literature that both natural ovarian steroids and their synthetic analogues influence carbohydrate metabolism (2 - 5). However, there is still a large measure of disagreement concerning the precise effects of these steroids in this respect. This thesis describes an investigation into the effects of natural and synthetic female sex steroids on aspects of carbohydrate metabolism in female mice. The work relates particularly to the influence of these steroids on the control of plasma glucose homeostasis and examines some of the endocrine and cellular mechanisms involved. This chapter presents a review of the specific literature pertaining to the effects of female sex steroids on carbohydrate metabolism, prefixed with a brief account of the nature of these steroids and a resumé of the aspects of carbohydrate metabolism examined in the present study.

THE DEVELOPMENT OF SYNTHETIC ESTROGENS & PROGESTOGENS

It has long been recognised that the ovaries produce steroid hormones which regulate reproductive physiology in the female. The principal natural estrogens (estradiol, estriol & estrone) were identified and purified in the 1930's(6 - 8) and the natural progestogens (progesterone & 17α-hydroxyprogesterone) were also purified around this time (9 - 11). As investigations proceeded, it became apparent that sex steroids might be used as contraceptive agents. Clinical trials reported the successful inhibition of ovulation by both estrogens and progestogens in women, but their use was impracticable due to the large dosage required, for example 300mg progesterone per day (12 & 13). It became apparent, therefore, that compounds were

needed which would resist liver metabolism and thus exert a higher contraceptive activity. Emphasis was placed on the selection of substances which could be administered orally since this route is easier to use for routine drug administration than other routes. Ethisterone was the first synthetic steroid produced (14) and it was rapidly followed by the synthesis of 19-nor steroidal compounds (15 - 17) and 17α-hydroxyprogesterone compounds (18 & 19). Ethynyl estradiol was the first synthetic estrogen produced (14) and a decade later mestranol was synthetised (20). Subsequent manipulation of these molecules has resulted in a large number of synthetic sex steroids becoming available. Clinical trials indicated that synthetic steroids offer effective control of ovulation in women (13, 21 & 22). Futhermore, they have proved to be the most efficient method for the control of human fertility yet available. A list of steroid contraceptive preparations available in the UK is given in table 1. The different preparations and regimes employed include: combined, sequential, depot, subcutaneous and vaginal preparations; mini-pills, pre-coital, post-coital and once-a-month pills (23). The most popular regime is an oral preparation containing a synthetic estrogen and a synthetic progestogen. In recent years, there has been a tendency to reduce the dose of oral contraceptive steroids. In particular the dose of the estrogen component of combined preparations has been reduced. This was prompted by reports that steroids, especially estrogens, produced a number of undesirable side-effects (see 24 - 26). Some of the more common side-effects include; an increased incidence of carcinoma, thrombo-embolism, secondary amenorrhoea post-pill infertility, gastro-intestinal complications, vaginal infections, raised blood pressure and elevated blood coagulability. Migraine, depression, breast changes, libido changes and weight changes are often reported by oral contraceptive users (27). More recent studies, however, have

Table 1 Contraceptive steroid preparations available in the UK.

(Prepared after Briggs, 1976).

NAME	PROGESTOGEN (mg)		ESTROGEN (mg)	
COMBINED PREPARATIONS				
Volidan-21	megestrol acetate	4.0	ethynyl estradiol	0.05
Anovular-21	norethisterone acetate	4.0	ethynyl estradiol	0.05
Gynovular-21	norethisterone acetate	3.0	ethynyl estradiol	0.05
Norlestrin	norethisterone acetate	2.5	ethynyl estradiol	0.05
Eugynon-30	d-l norgestrel	0.5	ethynyl estradiol	0.03
Ovaran	d-l norgestrel	0.5	ethynyl estradiol	0.05
Eugynon-50	d-l norgestrel	0.5	ethynyl estradiol	0.05
Lyndiol-2.5	lynoestrenol	2.5	mestranol	0.075
Ovulen-50	ethnodiol diacetate	1.0	ethynyl estradiol	0.05
Minilyn	lynoestrenol	2.5	ethynyl estradiol	0.05
Ortho-novin-2	norethisterone	2.0	mestranol	0.1
Ovulen-1mg	ethnodiol diacetate	1.0	mestranol	0.1
Conovid	norethnodrel	5.0	mestranol	0.075
Conovid-E	norethnodrel	2.5	mestranol	0.1
LESS POTENT PREPARATIONS				
Loestrin-20	norethisterone acetate	1.0	ethynyl estradiol	0.02
Microgynon-30	d-norgestrel	0.15	ethynyl estradiol	0.03
Ovranette	d-norgestrel	0.15	ethynyl estradiol	0.05
Demulen-50	ethnodiol diacetate	0.5	ethynyl estradiol	0.05
Norinyl-1	norethisterone	1.0	mestranol	0.05
Ortho-novin1/50	norethisterone	1.0	mestranol	0.05
Norinyl-1/28	norethisterone +7 placebo tablets	1.0	mestranol	0.05
Minovular	norethisterone acetate	1.0	ethynyl estradiol	0.05
Minovular-ED	norethisterone acetate +7 placebo tablets	1.0	ethynyl estradiol	0.05
Orlest-21	norethisterone acetate	1.0	ethynyl estradiol	0.05
Confer	norethisterone acetate	1.0	ethynyl estradiol	0.05
Ortho-novin-1/80	norethisterone	1.0	mestranol	0.08
Demulen	ethnodiol diacetate	0.5	mestranol	0.1
SEQUENTIAL PREPARATIONS				
Serial-28	megestrol acetate	1.0	ethynyl estradiol	0.1
Ovanon	lynoestrenol	2.5	mestranol	0.075
MINI-PILLS				
Femulen	ethnodiol diacetate	0.5		
Noriday	norethisterone	0.35		
Neogest	d-l norgestrel	0.075		
Micronor	norethisterone	0.35		
INJECTIBLE PREPARATIONS				
Depo-provera	medroxyprogesterone acetate	150.0		

shown that the issue is far more complex than one of dose alone. The precise chemical nature of the estrogen and progestogen, their relative potencies and the products of their metabolism appear to be equally critical (28 & 29). Interactions between the two components may also modify the side-effects produced by the preparation (30). Many preparations now contain an anti-estrogenic progestogen which counteracts the metabolic effects of the estrogen component without affecting the contraceptive effect (31). It is important to ensure that each individual receives the type of preparation that is most suitable to her because excesses and deficiencies of sex steroids can potentiate adverse effects (32).

A detailed account of the mechanisms through which synthetic sex steriods produce their contraceptive effects is beyond the scope of this thesis, and the interested reader is referred to:-(33 - 36). Briefly, the main actions involve interference with the endocrine axis between the hypothalamus, pituitary and ovary to prevent ovulation. In addition, alterations of the cilary, contractile activity and mucus secretions of the female reproductive tract may adversely affect sperm penetration.

Following the initial clinical trials with synthetic steriods (13, 21 & 22), this form of contraception was adopted widely despite the side-effects associated with their use. One particular aspect that has generated much interest is the effects of female sex steroids on carbohydrate metabolism. This subject forms the basis of this thesis, and a detailed review of the literature is presented later in this chapter.

CARBOHYDRATE METABOLISM

This section is concerned with an introductory discussion of carbohydrate metabolism with emphasis on the particular aspects studied in this thesis. Dietary carbohydrates represent a primary source of

energy which is made available to the cells in the form of glucose. The regulation of carbohydrate metabolism is crucial to the well being of the individual. The maintenance of the plasma glucose environment within strict limits is of primary importance (37 - 39). Insulin is intimately involved in controlling glycaemia as it is the main hypoglycaemic agent working against a host of hyperglycaemic hormones (40). This particular area is one of the key issues underlying the studies in this thesis. The maintenance of normal glycaemia is critical as both the central nervous system (CNS) and the red blood cells use glucose preferentially as an energy source. In addition, it is an important factor in preventing the development of various disease states associated with the disruption of normal glycaemia.

A very intricate regulatory system controls the availability of glucose for metabolic utilisation (38). Many hormones participate in this regulation including, glucocorticoids (41), adrenaline (42 & 43), glucagon (44 & 45) and insulin (40). The thyroid hormones, adrenocorticotrophic hormone (ACTH) and growth hormone may also influence carbohydrate metabolism (46). Glycogen is the primary storage form of carbohydrates in many animal tissues. The liver and muscle are recognised as the major storage depots in mammals. This particular area of glucose metabolism is also integrated with fatty acid and protein metabolism (39). Hence glycogen regulation is an important part of carbohydrate metabolism particularly with respect to the maintenance of plasma glucose levels. Glycogen metabolism has been reviewed in detail elsewhere (see 41, 47 & 48). Briefly, it appears that in times of excess glucose, glycogenesis ensures that glucose is stored in the body in a readily available form (glycogen), whereas in times of fasting, glycogenolysis ensures that glucose is released from glycogen depots. Hepatic glycogen is unique as it is released to maintain normal glycaemia which enables glucose utilisation

by other tissues.

INSULIN METABOLISM

In the earlier section, it was noted that the hypoglycaemic action of insulin is very important in the control of glucose homeostasis. Insulin counteracts the hyperglycaemic influence of dietary carbohydrates, a number of hormones and nervous pathways (49), as illustrated in table 2. Insulin also plays a major role in maintaining the balance between sugars, fatty acids, ketone bodies and amino acids as energy sources (39, 50 & 51). It has been shown that insulin stimulates glycogenesis *in vitro* (43 & 52) and *in vivo* (53). However, the mechanisms involved in this stimulation are still unresolved. For example, it has been proposed that insulin acts by increasing synthetase activity (53), inactivating phosphorylase (54), alteration of adenosine monophosphate (AMP) levels (55), alteration of cation levels (56), reduction of the glycogenolytic effect of glucagon (57) and counteraction of the glycogenolytic effect of catecholamines (58). However, it is still uncertain if insulin exerts a direct effect, as originally postulated by Miller (59), or an indirect effect by altering the metabolites perfusing the liver via changing extra-hepatic tissue metabolism (44, 47 & 60).

Insulin alters many aspects of metabolism in target tissues (61 - 63) and it has been suggested that a single primary action could not evoke all these changes. The molecular sequences manifested in a given tissue may be several steps removed from the initial molecular site of action. The insulin-receptor complex probably triggers a variety of events via several inter-dependent cascade mechanisms to alter the rate of metabolic pathways. The association between insulin and the adenyl cyclase system has been extensively studied (64 - 66). Although the data are often contradictory, it is generally believed that cAMP is not directly involved as a mediator for insulin in

Table 2 Hormones and nervous pathways which act to raise plasma glucose concentrations.

AGENT	ACTIONS
Glucagon	glycogenolysis & gluconeogenesis in liver
Adrenaline	glycogenolysis & gluconeogenesis in liver / lipolysis in adipose tissue
Glucocorticoids	glycogenesis in liver insulin antagonist
Growth hormone	insulin antagonist
CNS innervation	glycogenolysis in liver

carbohydrate metabolism.

Insulin is synthesised in the β cells of the pancreatic Islets of Langerhans via pre-proinsulin and proinsulin (62, 67 & 68). The latter is converted to insulin in the golgi apparatus (69). The hormone is stored in secretory granules attached to the β cell microtubular system (70) and released by emiocytosis (71 - 73). The kinetics of insulin release have been reviewed by Grill & Cerasi (74). Studies have revealed a biphasic release pattern (75 -77). The first phase derives from a small labile pool of insulin which is highly sensitive to secretagogues and the second phase is produced by release of a larger more stable insulin pool. The most important insulin secretagogue is glucose (78 & 79). The mechanism of the insulin secretory response to glucose is not entirely defined, although it has been extensively studied (80 -82). The data suggest that glucose produces a single initiating signal, whether this involves glucose or glucose metabolites is not resolved (70). The signal acts on both anion and cation fluxes at the plasma membrane of the β cell and between different ion pools within the β cell, eventually raising intracellular calcium levels which trigger insulin release (83 & 84).

Other factors besides glucose, play an important regulatory role. The opposing actions of insulin and glucagon are an important feature in the regulation of carbohydrate metabolism (82, 85 & 86). Both the sympathetic and the parasympathetic nervous systems may help co-ordinate the insulin response to secretagogues (87 -89). Various gastrointestinal hormones have been linked to insulin release, including gastricinhibitory polypeptide (GIP), vasoinhibitory polypeptide (VIP), chymodenin, cholecystokinin (CCK), secretin and pancreozymin (89 - 92).

Insulin is released from the pancreas into the hepatic portal system. It disappears rapidly from the circulation due to tissue

sequestration and degradation. The newly released hormone is immediately exposed to the liver where up to 60% is removed from the circulation. Thus this organ plays a crucial role in the regulation of insulin metabolism. Two insulin degrading enzyme systems have been identified. The major system is 'insulinase' and the second is the glutathione-protein disulphide oxidoreductase system (95). Both enzyme systems are associated with the plasma membrane (94 & 96).

Early experiments indicated that the presence of insulin within the cell is not required to initiate metabolic alterations (58 & 97) but this has recently been contested (98). It is generally accepted that insulin binds to specific receptors located on the plasma membranes of target cells as the initial step. Indeed it has been confirmed on many occasions that these receptors are an essential prerequisite for insulin action (98 & 99). Abnormalities of the insulin-receptor interaction may result in insulin resistance (99 - 101). The molecular mechanisms which produce this resistance are uncertain. Alterations in the number, structure and function of insulin-receptors may be involved (102 - 104). Certainly a relationship between insulin titre, receptor concentration and insulin resistance has been demonstrated in vivo (99) and in vitro (105). Hypoinsulinaemia for for example, is associated with elevations of insulin-receptors and insulin binding (106) whereas during hyperinsulinaemia, the reverse is observed (100 & 107). Insulin resistance may also result from disorders during the intracellular steps of insulin action (108). The clinical significance of these findings remains to be clarified.

CARBOHYDRATE METABOLISM & FEMALE REPRODUCTIVE STATUS

Female mammals have an estrous or menstrual cycle of reproductive activity which is controlled by a neuroendocrine axis between the hypothalamus, pituitary and ovaries (109 -111). A neural 'clock' localised within the hypothalamus appears to ensure the frequency of

each cycle subject to environmental influences and the competence of the hypothalamic-pituitary-ovarian system. For a detailed description of the estrous cycle readers are referred to the following reviews (112 & 115) and for the menstrual cycle (114 & 115).

There have been a number of accounts in the literature that carbohydrate metabolism is influenced by the reproductive state of the female. Many of these reports have suggested that changing titres of sex steroids may be responsible, at least in part, for changes in carbohydrate metabolism. This section presents an evaluation of this literature.

(i) THE ESTROUS CYCLE

The cyclical alterations of female sex hormone titres during the estrous cycles of small laboratory mammals are well established (116 - 118). When circulating estrogen and progestogen titres are highest, during proestrus and estrus, glucose tolerance is improved and plasma insulin is elevated (119 -121). Hepatic glycogen content is also greatest at these times (122). Conversely during diestrus, when ovarian steroid hormone production is lowest, glucose tolerance is impaired and plasma insulin levels are reduced (119, 120 & 123).

(ii) OVARIECTOMY

Following ovariectomy, circulating sex steroid levels fall below the normal diestrus values (124 & 125). Extrapolating from the estrous cycle data, one might expect glucose tolerance to be impaired after ovariectomy and this has been confirmed (126 & 127). Reduced hepatic glycogen levels have also been reported in ovariectomised rodents (122). Long term ovariectomy results in a decrease in the number of β cells and an impaired insulin response to secretagogues ie. glucose, glucagon and arginine (126). These data suggest that normal circulating ovarian hormone concentrations are important in the maintenance of normal β cell competence. It has been

suggested that insulin resistance may develop in ovariectomised animals that are deprived of ovarian steroids for prolonged periods and that this contributes towards the impairment of glucose tolerance (128). The incidence and severity of experimental diabetes is aggravated by ovariectomy, further supporting the view that ovarian steroids may facilitate glucose homeostais (129).

(iii) THE MENSTRUAL CYCLE

There is no concensus of aggreement concerning the effects of endogenous sex steroids on carbohydrate metabolism in women. Alterations of glucose metabolism during the menstrual cycle have been reported by several investigators but no consistent conclusions can be drawn (130 & 131). The majority of studies suggest that tolerance was improved during the follicular phase and impaired during menstruation. These data corroborate evidence from animal studies that glucose homeostasis deteriorates when circulating ovarian steroid titres are reduced. The clinical significance of these variations is uncertain. Several studies have failed to show any significant fluctuations of carbohydrate metabolism during the menstrual cycle (132 & 133). However studies in diabetic women indicate that difficulties may arise in controlling the disease at the time of menstruation (132). These reports confirm the association between impaired glucose tolerance and low endogenous sex steroid titres.

(iv) PREGNANCY

Pregnancy is accompanied by gross alterations in the endocrine status. Elevations of estrogens, progestogens, adrenal corticoids, placental lactogen and human chorionic gonadotrophin and a reduction of growth hormone are all seen in the pregnant woman (134 & 135). It is recognised that pregnancy presents a major diabetic stress (136 & 137). Indeed,the incidence of impaired tolerance increases with each successive pregnancy (138) and 'gestational diabetes' can develop in

women who are without any signs of abnormal carbohydrate metabolism in the non-gravid state (140). 'Gestational diabetes' is particularly prevalent in potential and latent diabetic women (2 & 136). During pregnancy, the insulin requirement is raised in these women (138).

Normal pregnancy is accompanied by elevated plasma insulin titres (141 & 142). Islet hyperplasia is seen (143 & 144), and the β cell may show an increased response to secretagogues (145). However, glucose tolerance is impaired, probably due to the development of insulin resistance in these women. Resistance has been attributed to the elevations of placental lactogen and glucocorticoids (144 & 146).

The endocrine changes associated with pregnancy in animals are similar to those of human pregnancy. Increased secretion of estrogens, progestogens, adrenal corticoids and placental lactogen are seen (118, 147 & 148), while growth hormone levels are reduced (149). Hypoglycaemia is frequently observed in pregnant animals (150 & 151) and in contrast to the clinical data, glucose tolerance is rarely significantly altered (151 & 152). Insulin secretion is elevated in pregnant animals (145, 152 & 153) and the islets develop a greater sensitivity to insulin secretagogues (108 & 145). A worsening of the diabetic condition during pregnancy in rats, as in human subjects, has been observed (154).

(v) MENOPAUSE

The menopause is essentially a condition of ovarian hormone insufficiency (155 & 156). The post-menopausal ovary produces neither estrogens nor progestogens (157), but small amounts of circulating sex steroids do arise from the adrenal cortex and from peripheral conversion of adreno-cortical andostenedione to estrogen (158 & 159). Glucose tolerance is impaired and the insulin response to secretagogues is reduced in post-menopausal women (160).

CONCLUSIONS

Thus, there is strong evidence to link carbohydrate metabolism and circulating levels of naturally occurring sex steroids in female animals and women. Any impairment of glucose tolerance is of particular interest with respect to the development of disease states such as Diabetes Mellitus. Thus, it is important that we should acquire a full understanding of the role of female sex hormones in this respect.

CARBOHYDRATE METABOLISM - EXOGENOUS SEX STEROIDS

This section examines the association between carbohydrate metabolism and the administration of exogenous natural sex hormones. Data from animal and clinical studies, including both normal and diabetic individuals are discussed. It is important to identify the alterations produced by these exogenous steroids due to the increasing use of these preparations for contraceptive purposes, and for the treatment of the climacteric symptoms in post-menopausal women.

(i) ANIMAL - EXOGENOUS ESTRADIOL-17 β

Estradiol lowers plasma glucose titres (161) and improves glucose tolerance (162 & 163) in many animal species. These changes appear to be dose-dependent, within the physiological and lower pharmacological range ie. up to 20μg/kg (164). Some studies, however, have failed to demonstrate any significant alterations of carbohydrate metabolism following estradiol administration (165) and it has been suggested that glucose tolerance may be impaired in some species (192). These anomalies may be due to the time-factor involved in estradiol-induced alterations. It appears that although initially plasma glucose levels are elevated, in the long-term estradiol exerts a protective influence which extends for a considerable time after the cessation of treatment (166). Treatment length, therefore, is an important factor to consider when comparing different studies. Elevations of tissue glycogen and reductions in hepatic glucose output

have been reported in estradiol treated animals (126, 167 & 168) and they may contribute towards the improvement of glucose tolerance. Futhermore, several studies have demonstrated that natural estrogens increase insulin-induced glucose uptake in skeletal muscle (169 & 170) and uterine muscle (171 & 172) thus suggesting that synergism between estradiol and insulin is involved. Indeed, this type of relationship has been observed in the adipose tissue (173).

Female animals treated with estradiol exhibit hyperplasia and hypertrophy of the pancreatic islets (152, 154 & 161), elevated pancreatic insulin content (126 & 174) and a reduction in the ratio of α to β cells (174 & 175). It is unclear whether estradiol brings about these alterations directly or indirectly, but it has been suggested that estradiol has a direct effect on the β cell (175). There are a few studies that have failed to demonstrate the insulinogenic effects of estradiol (176), but they may be attributed to the short treatment length used in these experiments. Clinically, it appears that 3 - 6 months of treatment are required before the insulinogenic changes are manifested, although 2 - 4 weeks may be adequate in laboratory rodents (119 & 152). The effect of exogenous estrogen on the pancreas accounts for the hyperinsulinaemia and the elevated insulin response to secretagogues frequently observed in estradiol treated animals (177 & 178).

(ii) ANIMAL - EXOGENOUS PROGESTERONE

The effects of natural progestogens on glucose metabolism are discussed in this section. It is believed that physiological doses of progesterone do not alter glucose metabolism significantly (152 & 179), or may slightly improve glucose metabolism (120, 162 & 180). High doses, however, exert an adverse influence (181 & 182). The data regarding the effects of progesterone on glycogen metabolism are contradictory. Glycogen levels have been reported as unaltered (122)

and raised (162) following progesterone administration. It appears that progesterone may have differing effects in different tissues, in addition, the effects may be dependent on the prevailing estradiol titre.

There are isolated reports that have failed to demonstrate changes of insulin metabolism following progesterone treatment (178 & 183) for which there are no obvious explanations. More generally, progesterone treatment is associated with hypertrophy and hyperplasia of the pancreatic β cells (152 & 174) and an elevation of pancreatic insulin content (176 & 180). Consistent with these alterations, the insulin response to secretagogues is often raised (180 & 184) and basal insulin levels may be elevated (120 & 143). There are numerous reports that suggest progesterone antagonises the hypoglycaemic action of insulin (178 & 179). It has also been suggested that progesterone treatment may increase the incidence of diabetes in partially pancreatectomised castrated female rats (183). Thus it appears that progesterone produces hyperinsulinaemia by affecting the β cell as a result of the antagonism with insulin. In this metabolic environment, glucose tolerance is maintained within normal limits (152 & 184). Many of the anomalies reported in the literature may be attributed to the belief that alterations of carbohydrate metabolism are only manifested following the administration of high doses of progesterone over a long period (174 & 180). Furthermore, the effects of progesterone may be highly dependent on the prevailing hormonal environment. A recent study has suggested that progesterone may not impair the hypoglycaemic action of insulin when acting in the absence of circulating estrogens (126). This study also suggests that progesterone antagonises the facilitation by estrogens of insulin-induced hypoglycaemia. Thus the extent to which progesterone impairs metabolism will depend on the relative concentrations of both

progesterone and estradiol.

(iii) ANIMAL - EXOGENOUS ESTRADIOL & PROGESTERONE

The effect of a preparation containing estradiol and progesterone on carbohydrate metabolism has not been extensively studied. This combination has been reported to improve glucose tolerance in animals, although fasting glucose may be elevated (126 & 185). Progesterone does not appear to completely counteract the overall beneficial influence of estradiol on glucose metabolism, in spite of the apparent antagonism of the insulin-mediated glucose uptake produced by estrogen treatment (152).

A preparation containing estradiol and progesterone has been reported to increase the pancreatic insulin content in mice (126), however, other reports have failed to find any significant changes (186). The insulin response to secretagogues appears to be elevated (126 & 186) although this has not been confirmed by Beck & Hoff (185). It has been suggested that the combination of estradiol and progesterone increases the insulin response to glucose to a greater extent than either of the two hormones achieved alone (152). This would account for the overall improvement of glucose tolerance inspite of the insulin antagonism by progesterone. Thus it appears that interactions between these two steroids are likely to modify the alterations of carbohydrate homeostasis, but the degree of modification is probably also dependent on the time-course, and dosage as well as the ratio of the two steroids in the preparation. Clarification of these issues might help to explain some of the contradictory data found in the literature.

(iv) EXPERIMENTAL DIABETES - EXOGENOUS STEROIDS

The majority of experiments on the influence of exogenous sex steroids on the incidence and severity of experimental diabetes have been performed in partially pancreatectomised rats. These studies have

been reviewed elsewhere (129, 166 & 183). An association between the incidence and severity of diabetes and gonadal steroids was initially reported by Foglia (154), who demonstrated that estrogens exerted a protective effect against the onset and degree of hyperglycaemia. These observations were corroborated in ovariectomised rats treated with estradiol (166 & 183) and in other animal species (187 & 188). The amelioration of the diabetic syndrome was attributed to islet hypertrophy and increased insulin biosynthesis and secretion (161, 176 & 186). It was pointed out by Lewis (186) that initially the diabetes may be aggravated by estrogens, but this is transient, and marked improvements occur in the long-term. This time-dependency may explain why some investigators have failed to find any improvements in experimental animals treated with estradiol (189 & 190). The time-dependent changes might also account for the isolated reports of a worsening of the diabetes (191). Since the protective influence of estrogens is effective in both adrenalectomised and hypophysectomised animals, it has been suggested that neither the adrenal nor the pituitary glands are important for the development of this protective influence (129 & 154). In conclusion, long-term administration of estradiol ameliorates rather than aggravates diabetes in partially pancreatectomised animals. In contrast, progesterone does not appear to have any significant effect on experimental diabetes (183 & 166).

(v) CLINICAL - EXOGENOUS ESTRADIOL-17 β

Preparations of naturally occurring gonadal steroids do not appear to have a detrimental effect on carbohydrate homeostasis in healthy women of child-bearing age. Since the number of studies performed is limited, there is insufficient evidence to define precisely the alterations of either glucose or insulin metabolism following the use of particular preparations.

Estrogen therapy frequently improves glucose tolerance in women

(192 & 193). There have been some reports, however, of a slight deterioration of glucose tolerance in estrogen treated post—menopausal women (194 & 195) but there are no instances of impairments in pre-menopausal healthy women. Elevations of hepatic glycogen and peripheral tissue glucose utilisation (193) may contribute towards the improvement of glucose homeostasis seen in estrogen treated women.

Natural estrogens appear to enhance insulin sensitivity and elevate plasma insulin titres in women (193). There are studies in post-menopausal women that have failed to show hyperinsulinaemia following estradiol administration (194 & 195), but they may reflect the reduced capacity of the β cells to adapt in older individuals (37).

(vi) CLINICAL - EXOGENOUS PROGESTERONE

It is evident from the literature that progesterone does not significantly influence glucose homeostasis in either post-menopausal (196 & 197) or menopausal (143 & 198) women when given in physiological doses. Indeed, in women with endometrial carcinoma, glucose tolerance was improved following progesterone treatment (199).

Insulin metabolism, however, is altered by progesterone therapy. The basal insulin titre and the insulin response to glucose are raised above the pre-treatment values (196 - 198). Circulating plasma insulin may also be increased by progesterone therapy (143), but this was not observed in other studies (198). Since plasma glucose is frequently unaltered in the presence of hyperinsulinaemia in these women, it appears that the hypoglycaemic action of insulin is reduced. Alterations in peripheral tissue sensitivity are implicated similar to those described in progesterone treated animals.

(vii) CLINICAL - DIABETES & EXOGENOUS STEROIDS

Naturally occurring estrogens appear to improve glucose tolerance

and reduce hyperglycaemia in diabetic women (2 & 3). Data also suggest that the insulin requirement of diabetics is reduced during estradiol therapy (2 & 200).There is only a single report concerning the influence of a progestogen on carbohydrate metabolism in diabetic women. 17-α-hydroxyprogesterone caproate was reported to produce a deterioration of diabetes (198).

(viii) CONCLUSIONS

It appears that natural estrogens exert a beneficial influence on carbohydrate metabolism in healthy individuals and in individuals with compromised carbohydrate metabolism. These alterations include an increase in insulin production and release, and an increase in glucose uptake by peripheral tissues. Natural progestogens, however, do not generally alter glucose tolerance beyond the normal limits in healthy individuals. Nevertheless, they often cause hyperinsulinaemia associated with a reduction of insulin sensitivity. In diabetic individuals, progesterone treatment may cause a deterioration in the diabetic condition.

CARBOHYDRATE METABOLISM - EXOGENOUS SEX STEROIDS (SYNTHETIC)

It is nearly 30 years since the first clinical trials on oral hormonal contraceptives were conducted (22) and these drugs are used by an estimated 80-100 million women throughout the world. 20-40% of all fecund women have taken oral contraceptives in developed countries in recent years. Considerable concern has been raised over the possible hazards incurred during and following contraceptive steroid use. Waine & Co. (201) first drew attention to the apparent diabetogenic influence of these agents. Following this report, many papers and several reviews have appeared (1 - 3, 5, 119, 165, 202 & 203). Inspite of this attention, the influence of contraceptive steroids on carbohydrate metabolism remains to be completely elucidated.

Although many of the studies are contadictory, there is general agreement that these drugs do produce alterations of carbohydrate metabolism. Many of the studies have been principally concerned with determining the number of individuals that show a deterioration of carbohydrate homeostasis and have examined the possibility that the degree of deterioration could precipitate a condition of diabetes.

A striking feature of the literature is the extensive spread of results with regard to any particular preparation and particular regime. Many of these anomalies may be attributed to inherent variables, particularly in clinical studies. For example, the heterogeneity of a group of individuals which may include those with all manner of pathological conditions who may be receiving other medications. These problems are difficult to control and are often ignored when investigators interpret their results. In addition, the criteria adopted to assess the effects of the treatment are widely different. The influence of sex steroids on carbohydrate metabolism will be affected by differences in methodology, dose, treatment duration, administration route, individual differences, chemical compositon of the steroid and the type of regime utilised. Since these factors are important they will be discussed in detail in the following sections.

(i) METHODOLOGY.

One of the major difficulties encounted when attempting to compare different studies is the use of the control group, particularly in clinical investigations. Often a separate control group and test group are employed. In clinical trials it is difficult to match individuals in two such groups to the degree that is required in metabolic studies. Therefore, the validity of some data must be questioned in studies using this design. It is more meaningful to compare pre-test and post-test readings in the same individual. The situation is further

complicated by the fact that individual differences are wide and that it is difficult to accurately define what is normal and what is abnormal with respect to plasma insulin and glucose levels. Thus, different criteria have been used by different investigators to define abnormalities in carbohydrate metabolism and it is not always possible, therefore, to compare between studies.

Alterations of carbohydrate metabolism during the artificial menstrual cycle of women taking oral contraceptives have been reported (204 & 205). However, most studies do not take these cyclical variations into account and futhermore, do not provide information about the time in the cycle that sampling was performed. Hence, inaccuracies may arise in this manner.

The glucose tolerance test (GTT) is frequently used to assess carbohydrate metabolism. The validity of this type of test and some of the inherent difficulties involved are discussed in Chapter 2. Clinical studies use either an oral or an intravenous test, but intraperitoneal tests are also used in animal studies. It is important to distinguish between the type of test used when comparing data because the outcome may be affected. For example, the incidence of impaired glucose tolerance in women and animals receiving synthetic sex steroids is higher following oral than following intravenous GTTs (206 - 211). This information has led to the suggestion that synthetic sex steriods alter carbohydrate metabolism by changing intestinal glucose absorption in addition to changing the metabolism of glucose after it has entered the circulation (206). The oral GTT is used more frequently than the intravenous test in clinical studies for reasons of convenience The oral test, however, does not accurately distinguish non-diabetics from subclinical diabetics who usually only manifest carbohydrate abnormalities during periods of metabolic stress such as pregnancy or contraceptive steroid therapy (138 & 146).

(ii) DOSAGE

It is apparent from the literature that steroid-induced alterations of carbohydrate homeostasis are influenced by the dose administered. The incidence of abnormalities increases as the dosage increases (207, 212 - 214). Changes of plasma glucose and insulin titres will probably be seen at some dosage level for most contraceptive steroids. Since present day preparations contain steroid concentrations that are much lower than those used in earlier studies, inaccuracies may arise when the two are compared. Indeed, 15-40% of women in the earlier studies developed diabetes, whereas only 4-12% develop diabetes in more recent trials with lower dose preparations (2 & 3).

(iii) TREATMENT DURATION

Treatment duration is an important factor to be considered when comparing data from different studies. The frequency of abnormalities has been shown to increase as the duration of treatment increases (160, 183, 206, 214 - 216). Other studies, however, have failed to find such deteriorations (208, 217 - 219). Nevertheless, it has been suggested that if the data are age-adjusted, then there is a tendency for glucose levels to rise over the long term (217). It also appears that glucose tolerance deteriorates initially and women are prone to unpredictable effects during the first few cycles of treatment (220). However, an adaptive mechanism returns glucose tolerance to normal in individuals treated with contraceptive steroids over a long period (221 - 223). Although there is still some uncertainty regarding the influence of treatment duration on carbohydrate homeostasis, the evidence overwhelmingly suggests that treatment duration does have an effect.

(iv) ADMINISTRATION ROUTE

The route employed to administer contraceptive steroids

undoubtedly affects the metabolic clearence rate and this may alter the steroids influence on carbohydrate metabolism. The effects of different administration routes are discussed in Chapter 5, with particular reference to clinical studies. There is also evidence in animal studies that the route of administration affects carbohydrate homeostasis. For example, norethynodrel does not alter glucose tolerance when given subcutaneously (179), but impairs tolerance following oral administration (216). In the clinical situation, the oral route of administration is preferred for convenience, but there is increasing use of depot and intravaginal application of contraceptive steroids.

(v) INDIVIDUAL DIFFERENCES

Several lines of evidence indicate that a continuum of sensitivity to synthetic contraceptive steroids exists among users, and that the magnitude of deterioration may be affected by age, weight, hormonal complications, parity and pancreatic β cell function (3 & 5). The deterioration of glucose tolerance in oral contraceptive users is greater in women who carry one or more recognised diabetic risk factors such as obesity, a family history of diabetes and large babies (5, 144, 217 & 224). Other studies have not confirmed this association (225). Indeed, it has been suggested that neither an abnormal obstetrical history nor a family history of diabetes are good indicators of diabetic risk because there is no accurate correlation between these criteria and the diagnosis of diabetes following synthetic steroid therapy (146). It is possible that women who develop abnormal tolerance whilst taking oral contraceptives may be undiagnosed subclinical diabetics. A rather surprising finding was disclosed by the work of Wynn (226) and Phillips (217) who showed that women with the poorest pre-test tolerance manifested the least deterioration following treatment with these drugs. If synthetic

contraceptives are indeed diabetogenic, this finding is contrary to expectation. In older pre-menopausal women (30 - 50 years) it is generally agreed that there is an increased incidence of abnormalities following oral contraceptive therapy (5 & 202). It is uncertain, however, if this is due to an increased expressivity of diabetic genes, and/or due to the non-specific effects of ageing (227).

Individual differences, both genetic and environmental, are an unavoidable integral part of clinical studies but they can be strictly controlled and even eliminated in animal studies. Furthermore, identical preparations administered under similar conditions to different species produce similar effects on carbohydrate metabolism in nearly all cases studied. Thus animal experimentation can serve as a useful indicator of possible effects that may be produced by contraceptive preparations in women. In addition, the use of animals allows the investigator to perform experiments that would be prohibited in the clinical environment.

(vi) TYPES OF PREPARATIONS AND REGIMES

There is still considerable controversy over which components in synthetic contraceptive preparations affect carbohydrate homeostasis. It has even been questioned whether there is an association between the type of steroid utilised and the glycometabolic alterations (211 & 217). It is generally held that the estrogen component of combined preparations produces the greater effect on carbohydrate metabolism (228 - 230), but the evidence for this is by no means conclusive. Although estrogens alone can often mimic the diabetogenic effects of combined contraceptives, one cannot discount the possibility that progestogens may modify this action by undefined mechanisms, or through their own estrogenicity. Indeed, there are those who have suggested that it is the progestogen component that exerts the greater influence on carbohydrate homeostasis (1, 144, 208, 231 &

232). The situation is further complicated by reports that suggest that the progestogen components do not alter the estrogen-induced alterations of carbohydrate metabolism (217 & 223).

Since there are still extensive areas to be clarified, the following sections discuss this topic in detail. For reasons of clarity, the discussion is divided into animal and clinical sections. Literature pertaining to synthetic steroids is subdivided to consider their individual effects and the effects of the combined preparations on carbohydrate metabolism.

ANIMAL - EXOGENOUS SYNTHETIC ESTROGENS

There are relatively few studies concerning the effects of synthetic contraceptive steroids on carbohydrate homeostasis in animals. Partially pancreatectomised gonadectomised rats treated with diethylstilbestrol are reported to show an amelioration of diabetes (166). This can probably be attributed to the increased islet growth (234) and increased hepatic glycogen deposition (235) reported in these animals. In intact rats, however, diethylstilbestrol has a strong diabetogenic influence (236 & 237). Thus it appears that care must be taken when comparing data from animals with and without intact functional ovaries.

Ethynyl estradiol administration does not significantly alter glucose tolerance in monkeys (165) or rats (237) but insulin sensitivity may be reduced (165). In contrast mestranol impairs glucose tolerance (216) and elevates the insulin response to secretagogues in monkeys (165). It appears that there is a species difference with respect to the alterations of insulin metabolism produced by this synthetic estrogen. Whereas, the hypoglycaemic action of insulin is reduced in monkeys (165), neither hepatic nor muscle glucose uptake was affected in rats (216) treated with mestranol.

ANIMAL - EXOGENOUS SYNTHETIC PROGESTOGENS

It is often stated that synthetic progestogens administered alone do not significantly alter glucose tolerance in animals. This has been demonstrated in many animal species including monkeys (165 & 179), rats (238) and dogs (239 & 240). These particular studies have shown that progestogen treatment is associated with an elevation of the plasma insulin titre. These findings suggest that insulin sensitivity is impaired in these animals and indeed this has been confirmed in norethynodrel treated rats (216).

ANIMAL - EXOGENOUS COMBINED PREPARATIONS

It is evident that combined preparations containing mestranol are associated with a higher incidence of impaired glucose tolerance than those containing ethynyl estradiol (165 & 221). This is consistent with the alterations of glucose homeostasis produced by these synthetic estrogens on their own. A preparation containing mestranol and norethynodrel has a diabetogenic influence in rats and insulin sensitivity is reduced (216). Similar alterations have been observed in other animal species receiving this combined preparation (165 & 221). In rats treated with mestranol and norethnodrel , the plasma glucose levels observed were higher than those produced by either of the two components when given alone (216). Thus an interaction between the two components is indicated which exacerbates the diabetogenic effects.

Ethynyl estradiol in combination with either norgestrel or norethisterone acetate did not alter fasting glucose levels in rats (233). Indeed, impairments of glucose tolerance are infrequently reported in animals treated with combined preparations containing ethynyl estradiol (241).

CLINICAL - EXOGENOUS SYNTHETIC ESTROGENS

The effects of synthetic estrogens on carbohydrate metabolism in normal healthy women are dependent on the type of steroid employed. Diethylstilbestrol is diabetogenic since glucose tolerance is significantly impaired in women receiving this estrogen (3 & 206). This particular compound, however, is no longer used in contraceptive preparations due to an association with endometrial carcinoma. The synthetic estrogens predominantly included in present day combined preparations are mestranol and ethynyl estradiol. Although some investigators have failed to demonstrate any alterations of carbohydrate tolerance with either of these estrogens (224 & 242) it is the consensus that certainly mestranol produces a deterioration of glucose tolerance in women (3, 207, 221 & 243). However, circulating insulin titres may be unaltered by mestranol (196 & 224).

Ethynyl estradiol does not appear to produce hyperglycaemia as readily as mestranol. Women treated with ethynyl estradiol frequently exhibit no alterations of glucose tolerance (205, 221, 224, 242 & 243). Fasting plasma insulin is also often unaltered following ethynyl estradiol therapy (196 & 244). The peripheral tissue sensitivity to the hypoglycaemic action of insulin may be reduced (205). There have been reports of impaired glucose tolerance in women taking ethynyl estradiol (3 & 245), hence some doubt remains concerning the effects of this estrogen on carbohydrate homeostasis.

CLINCAL - EXOGENOUS SYNTHETIC PROGESTOGENS

The effects of synthetic progestogens on carbohydrate homeostasis in normal healthy women are often contradictory and further investigations are necessary, particularly in view of the increased use of these steroids in mini - pills and intrauterine devices (IUDs). Norethisterone therapy has been extensively studied in women and it is evident that this progestogen does not significantly alter glucose

tolerance following either oral or intravenous glucose tolerance tests (244, 246 - 249). Fasting glucose and insulin levels have been variously reported as unaltered, elevated and reduced (246, 248 & 249). The plasma insulin response to glucose, however, is frequently elevated in norethisterone treated women (248 & 249). Thus it can be inferred that the hypoglycaemic action of insulin is reduced in these women. Neither norethisterone oenanthate (250 & 251) nor norethisterone acetate (243 & 252) have been reported to alter glucose tolerance significantly in women.

Norgestrel administration over a period of three years was reported to improve glucose tolerance (204), but insulin sensitivity was not altered in these patients. Norethnodrel therapy over a twelve month interval did not produce any abnormalities of carbohydrate metabolism (202 & 220) and a similar picture was observed following megestrol acetate administration (245).

The effects of ethynodiol diacetate on carbohydrate metabolism are uncertain. Impairments of glucose tolerance have been reported (202, 229, 253 & 256), but there is an equal number of reports that tolerance is unaltered during ethynodiol diacetate treatment (202, 220, 257 & 258). Although there are considerable variations in fasting glucose and insulin titres (202, 229, 256 - 258), it appears that insulin sensitivity is not significantly altered by ethynodiol diacetate (229).

Oral GTTs in women receiving chlormadione acetate suggest that tolerance is not altered significantly (245 & 259). However, intravenous tests conducted in women using this steroid for more than twelve months have shown both an improvement (260) and a deterioration (246) of glucose tolerance. There were no consistent alterations in the plasma insulin response to glucose in these women.

Depot administration of medroxyprogesterone acetate has been

reported to have no adverse effects on glucose tolerance after various periods of treatment (249, 258, 261 - 263). It appears, however, that plasma insulin levels are elevated in women using this progestogen (261). Oral administration medroxyprogesterone acetate does not influence glucose or insulin metabolism (264).

It is evident from the literature that most synthetic progestogens do not significantly alter glucose tolerance in normal healthy women of child-bearing age. Nevertheless, the plasma insulin response to glucose is often elevated and the hypoglycaemic action of insulin may be reduced in women receiving synthetic progestogens.

CLINICAL - EXOGENOUS COMBINED PREPARATIONS

Enovid, a preparation containing mestranol and norethnodrel , was one of the first synthetic combined contraceptive steroids prescribed and thus has been extensively studied. It is evident that this oral contraceptive is frequently associated with a deterioration of glucose tolerance in the presence of hyperinsulinaemia (165, 207, 213, 214, 228, 256, 258 & 265). These data suggest that insulin sensitivity is reduced and this has been confirmed by insulin hypoglycaemia tests in patients using this combination (265). The deterioration of glucose homeostasis is consistent with decreased hepatic glycogen levels (168). Treatment with mestranol and norethnodrel is associated with an elevation of fasting glucose and insulin in many patients (193, 256 & 258). There have been reports that glucose tolerance is unaltered during this treatment, but these reports are generally in studies using the intravenous GTT (209, 212, 218, 219 & 266).

Similar results are available from studies using a mestranol and norethisterone preparation. Again impairment of glucose tolerance is the "norm" (203, 267 - 269) and these patients often show elevated plasma titres of insulin (203, 226, 259 & 269).

Likewise, mestranol in combination with ethynodiol diacetate is

associated with a significant deterioration of glucose tolerance (270 & 271), indeed these studies suggest that up to 50% of users show a significant deterioration. Hyperinsulinaemia is noted in most women using this preparation (271 - 273). Fasting glucose levels, however, may be unaltered (225 & 255) or elevated in some women (272).

Women using the combined preparation containing mestranol and lynoestrenol show a deterioration of glucose tolerance during therapy (226 & 274) and the insulin reponse to glucose is generally elevated in these women (226 & 274).

There is a lower incidence of impaired carbohydrate homeostasis in women receiving oral preparations containing ethynyl estradiol. For example, preparations containing ethynyl estradiol and norgestrel do not appear to alter glucose tolerance significantly in most women (270, 275 & 276). However, an improvement of glucose tolerance has been noted in a majority of users in one study (277). Although fasting insulin appears to be unaffected by ethynyl estradiol and norgestrel therapy (278), the insulin response to glucose is elevated (277 & 278). However, the hypoglycaemic action of insulin does not appear to be reduced significantly (278).

Glucose tolerance is not significantly affected in most women receiving combined preparations which contain ethynyl estradiol and norethisterone acetate (221 & 279). There have been reports of a slight deterioration of tolerance in some users (211) but it was less than the deterioration observed in women taking mestranol and norethisterone acetate preparations. Likewise, carbohydrate homeostasis was not significantly altered in women taking either ethynyl estradiol and ethynodiol diacetate (196 & 244) or ethynyl estradiol and megestrol acetate (279).

CLINICAL - SEQUENTIAL PREPARATIONS

A number of sequential preparations have been employed in the past,

but they are not presently favoured by the medical profession and thus are now infrequently prescribed. The alterations in carbohydrate homeostasis are again dependent on the steroidal components in the various preparations.

Sequential contraceptives have been reported to produce a significant deterioration of glucose tolerance (207, 226 & 269). However, improvements of tolerance were seen in women using these agents in intravenous GTTs (280). Some sequential preparations improved glucose tolerance (260) or produced no apparent effects (205, 269 & 281). In each of these investigations sequential contraceptive preparations were associated with elevated plasma insulin titres.

DIABETIC WOMEN - SYNTHETIC SEX STEROIDS

It is believed that synthetic sex steroids have an undesirable influence on glucose metabolism in women suffering from diabetes. This applies to potential (229, 282 - 284), asymptomatic (2 & 5), latent insulin-dependent and latent non-insulin dependent (5, 285 & 286) and maturity onset (287) diabetic women. It appears that diabetics with an adequate insulin reserve may show an improvement of glucose tolerance as a result of the hyperinsulinaemia produced by synthetic steroid administration (288 & 289). Whereas, those diabetic women with a compromised insulin reserve may show a deterioration of glucose tolerance due to their inadaptability (287, 290 & 291). It seems likely, therefore, that women with a predisposition to diabetes may be more susceptible to the diabetogenic effects of certain synthetic contraceptive steroids than non-diabetic women (165 & 217).

Synthetic estrogens alone appear to improve carbohydrate metabolism in diabetic women (288). This is similar to the protective influence exerted by natural estrogens in diabetic animals. Furthermore, synthetic progestogens have not been shown to adversely

affect carbohydrate metabolism in diabetic women (229 - ethynodiol diacetate, 261 - medroxyprogesterone acetate). However, significant elevations of plasma insulin have been reported during depot-medroxyprogesterone acetate therapy in non-insulin dependent diabetics (261 & 288).

Oral combined contraceptive preparations containing mestranol have varying effects on carbohydrate homeostasis in diabetic women. Many of these women manifest a deterioration of glucose tolerance when taking mestranol containing preparations (2, 218, 219, 274, 287 & 292). These findings are consistent with the impairments of glucose tolerance reported in many non-diabetic women receiving mestranol containing preparations. The basal insulin titre was unaltered in non-insulin dependent diabetics during mestranol treatment (274 & 287) but there may be a delay in the insulin response to glucose (293) and a reduction in insulin sensitivity (287) in these women.

Ethynyl estradiol containing oral combined preparations also produce a deterioration of glucose tolerance in diabetic women (292 & 294) but to a lesser extent than the mestranol containing preparations. A group of diabetic women taking ethynyl estradiol and norgestrel over a five year period exhibited an increased insulin response to glucose (278). These data indicate that the diabetic syndrome may be ameliorated by long-term administration of certain synthetic preparations.

Sequential regimes appear to produce an initial deterioration of glucose tolerance in diabetic women (273) but as the treatment period increases, tolerance is generally improved (283 & 284). Sequential preparations also produce hypoinsulinaemia in non-insulin dependent diabetic women (273, 283 & 284)

POST - MENOPAUSAL WOMEN - SYNTHETIC SEX STEROIDS

Post-menopausal women treated with synthetic estrogens often

manifest a deterioration of carbohydrate homeostasis. Diethlystilbestrol produced an impairment of glucose tolerance following both oral and intravenous GTTs (206). Likewise, a high percentage of users developed abnormalities when taking mestranol (221 & 224), however, these deteriorations were usually transitory (5 & 221). In contrast, ethynyl estradiol does not have such an adverse effect on carbohydrate metabolism in post-menopausal women. This is consistent with the effects of this particular estrogen in normal women, diabetic women and animals. A group of women receiving ethynyl estradiol showed no significant alterations of glucose tolerance (221). Other studies, however, suggested this estrogen produces a slight impairment of glucose tolerance in post-menopausal women (224).

There are few reports in the literature concerning the effects of synthetic progestogens on carbohydrate homeostasis in post-menopausal women. Nevertheless, it is evident from these few studies that the administration of synthetic progestrogens generally does not significantly affect glucose tolerance (221 - norethisterone acetate, 275 - depot-medroxyprogesterone acetate). Short-term administration of ethynodiol diacetate may be associated with a slight deterioration of glucose tolerance and an increase in plasma insulin (287).

The most extensively studied oral combined preparation in post-menopausal women is one containing mestranol and norethnodrel (Enovid) (160, 194, 206, 221 & 281). These reports corroborate the adverse effects of mestranol containing preparations in healthy pre-menopausal and diabetic women. It has been suggested that the impairment of glucose tolerance is only seen in women following oral GTTs, and that tolerance is usually unchanged when intravenous GTTs are performed (206). This finding was not confirmed by Yen and Vela (281). It also appears that the deterioration of carbohydrate homeostasis is temporary and it returns to normal during long-term

treatment with enovid. Plasma insulin levels are not significantly affected in post- menopausal women using contraceptive steroids (194). Mestranol in combination with ethynodiol diacetate also impairs glucose tolerance in post- menopausal women (270) but sequential preparations do not significantly influence carbohydrate metabolism in those women (295).

CONCLUSIONS

The foregoing review of the literature shows that synthetic contraceptive steroids influence carbohydrate metabolism in women and many animal species. One of the characteristic features is the hyperinsulinaemia produced by these drugs. Glucose tolerance, however, may be improved in some individuals but impaired in others depending upon the steroids involved, dose, duration and the physiological condition of the recipient. It appears that although glucose tolerance is initially impaired during contraceptive administration, it is frequently followed by a return to normal with continued use of these agents. This adaption is dependent on the ability of the β cells to respond to an increased insulin demand. Thus the possible consequence of β cell exhaustion and the precipitation of diabetes must be closely examined. It may be argued that truely non-diabetic women do not develop abnormal carbohydrate tolerance whilst taking synthetic steroids even though their glucose curves are frequently higher than they were before using these agents. Indeed, it appears that oral contraceptives cause a small shift in the glucose curves of women in the third decade to that characteristic in women twenty years older (296). It has also been suggested that oral contraceptive users have higher plasma glucose levels following a meal than non-users (296). The health consequences of the small elevations of plasma glucose extended over many years of steroid contraceptive use are uncertain, but in view of reports that certain regimes induce inappropriate hyperinsulinaemia it

is important to clarify the situation.

Synthetic contraceptive steroids rarely impair glucose metabolism to the extent of chemical diabetes if there are no indications of abnormalities prior to therapy. However, these drugs can induce an acquired form of subclinical diabetes in some non-diabetic women that is reversed when treatment is discontinued (225 & 278). While the magnitude of changes induced by synthetic contraceptive steroids is generally small, there remain many preparations in common use which create a diabetogenic stress, particularly in obese and diabetic women (221). Whether prolonged administration will jeopardise carbohydrate metabolism regardless of susceptibility is not known. Indeed, studies in women using these agents for more than five years are in total disagreement (3, 202 & 269). This emphasises that many questions concerning contraceptive steroids and carbohydrate metabolism remain to be answered.

RAISON D'ETRE FOR THE PRESENT STUDY

On the basis of the literature review presented in this chapter, this thesis investigates the influence of the steroidal components, administration route and duration of treatment on carbohydrate homeostasis in female mice. This investigation includes an examination of plasma glucose, plasma insulin and tissue glycogen concentrations; the distribution of sex steroids *in vivo*; and peripheral tissue sensitivity to the hypoglycaemic action of insulin *in vitro*.
It is hoped that this thesis will contribute towards a better understanding of the association between carbohydrate metabolism and sex steroids.

Chapter 2

MATERIALS AND METHODS

CHAPTER 2

MATERIALS AND METHODS

This chapter describes the materials and methods used in the present study. All chemicals were of the highest purity commercially available and double-distilled water was used throughout.

ANIMALS

Adult female mice of the Theillers Original (T/O) strain were obtained from Bantin & Kingman, Hull, and maintained in the University of Aston animal house for at least two weeks prior to use. Only mice of 10 - 15 weeks, weighing 30 $\pm$ 2g were included at the start of each experiment. The animals were maintained in a constant controlled environment of 20 - 22° C and 50% humidity, with a 10 : 14 hours light : dark schedule. Unless otherwise stated, free access was allowed to food (Heygates Breeding Diet, Heygates & Sons Ltd., Northampton) and water.

VAGINAL SMEARS

The reproductive periodicity of the adult female mammal is clearly illustrated by changes in vaginal cytology (297). During the estrous cycle changes occurring in the vaginal epithelium of young adult female mice can be identified by the vaginal smear technique described by Zarrow (298). Only those animals cycling regularly every four days were used. In all studies, the control animals were tested during the diestrus stage of the estrous cycle which corresponds to the time of minimal circulating sex steroid levels (118 & 299). The stages of the estrous cycle were defined as follows -

PROESTRUS - many nucleated and a few cornified epithelial cells, approximately 1 day (rapid growth of vaginal epithelium).

ESTRUS - remnants of nuclear and many cornified epithelial cells, 1 - 2 days (maximum vaginal growth).

METESTRUS - fragmented cornified epithelial cells and many leucocytes,

1 day (desquamation).

DIESTRUS - a few leucocytes and epithelial cells,

approximately 1 day (quiescence).

BILATERAL OVARIECTOMY

All surgical procedures were carried out with the maximum amount of asepsis possible. Instruments and sutures were sterilised in a 2% solution of Hibitane (chlorhexidine digluconate, ICI Ltd., Macclesfield) before contact with animal tissues.

Bilateral ovariectomy of young adult female mice was performed as described by D'Amour (300). The animal was anaesthetised with Nembutal (sodium pentobarbitone, 60mg/kg body weight, intraperitoneal (ip), Abbott Labs. Ltd., Kent) and further control was achieved by inhalation of ether. The operation sites were shaved and cleaned with Hibitane. Each ovary was removed by the following procedure - A longitudinal-mediolateral incision of 3mm was made in the skin of the abdomen, followed by a similar incision in the abdominal wall. The ovary, embedded in parametrial fat, was carefully exposed and a ligature (size 0000, polyamide 6/6 suture, Armour Pharmaceutical Co. Ltd., Eastbourne) was applied around the fallopian tube and associated fat and blood vessels. The ovary was excised and the remaining tissue carefully returned into the abdomen. The abdominal wall was closed with one suture, and the skin was closed with one or two sutures using a curved triangular needle (size 18, Phillip Harris Med. Ltd., Birmingham). The wound was treated with penicillin G powder (Sigma Chem. Ltd., Kingston-upon-Thames). An ambient temperature of 30°C was maintained throughout the operation and during the recovery period. The animals were returned to clean cages to reduce the risk of post - operative infection.

BLOOD SAMPLING

A varity of methods are available for obtaining samples of blood

from small laboratory rodents. The suitability of each method depends on the particular requirements of the experiment, for example the volume required, sample site, use of anaesthetics, amount of stress caused and whether the procedure is terminal or part of a sequential regime. A large volume of blood is easily obtained, without trauma, by decapitation and this technique was used for all terminal samples. Among the methods available for sequential sampling, cardiac puncture (301) and jugular venipuncture (302) yield large volumes of blood rapidly, but produce considerable stress in the absence of anaesthesia. The technique chosen for sequential sampling in the present study was a modification of the tail-tip amputation procedure of Grice (303). The method is efficient and rapid, causing minimal stress to the animal (304) and requiring no anaesthetics.

The mouse was gently introduced into a restrainer (a hollow glass cyclinder, 80 x 33 x 2 mm) with a cork at each end containing apertures to allow free access to the tail and for aeration. Animals were confined in the restrainer for less than two minutes while blood was collected. The extreme tip of the tail was cut with a sharp scalpel blade and blood was collected by a continuous gentle milking of the tail. The flow of blood was stemmed by gentle pressure at the cut tip. Subsequent samples were obtained up to one hour later during any single test by re-opening the wound with a further milking action. Blood was collected in small polythene tubes (250ul capacity, Beckman-Riic Ltd., Fife) which were pre-washed with heparin, 500U/ml (BDH Chem. Ltd., Poole) and dried in air. Samples were centrifuged in a Beckman 152 Microfuge (Beckman-Riic Ltd., Fife) at 10,000g for 25 seconds. Plasma was removed and stored at -20° C until analysed.

RADIOISOTOPES

Special precautions in accordance with the code of practice of the University of Aston were observed when working with the radioisotopes.

Contaminated glassware was immersed for at least a week in a 5% solution of detergent (Lipsol Liquid Concentrate, Lip Ltd., Yorks.), then immersed in water for a week prior to the normal washing procedure described below.

CLEANING OF GLASSWARE

Glassware was rinsed thoroughly in tap water and immersed overnight in a detergent solution. After rinsing several times in tap water, distilled water and finally double-distilled water, all glassware was dried overnight in an oven at 45°C.

STATISTICS

Statistical analyses were performed using an Olivetti Programma 101 (British Olivetti Ltd., London). The mean ($\bar{x}$), standard deviation (SD) and the standard error of the mean (SEM) were calculated. Data were prepared using the Students "t"- Test for unpaired samples and differences were considered significant for probability values of $p < 0.05$ (305).

EXPERIMENTAL DESIGN

Although precise details of the experimental design are given in the materials and methods section of each chapter, the following details apply generally.

Control animals were injected with the vehicle, arachis oil, 0.05ml/kg/day (Hopkins & Williams, Essex). A daily check was maintained of the estrous cycle and all experiments were performed during the diestrus stage.

Steroids (Sigma Chem. Ltd., Kingston-upon-Thames) were administered in arachis oil at the following doses : Estradiol - 17β and ethynyl estradiol, 5µg/kg/day; Progesterone, Norethnodrel and norethisterone acetate, 1mg/kg/day. Steroids were also administered in combinations at the same dosage. The combinations used were as follows: Estradiol and progesterone; Ethynyl estradiol and norethnodrel ; and

Ethynyl estradiol and norethisterone acetate.

Three routes of administration were used for all the preparations, subcutaneous injection (given in the supra -scapular region), intramuscular injection (given in the femoral muscle), and oral administration.

Experiments were performed after 1, 4 and 6 cycles of treatment, 4 days being the mean estrous cycle length.

At least one month was allowed to elapse between bilateral ovariectomy and experimentation.

GLUCOSE TOLERANCE TEST

The GTT has been widely used as a sensitive tool to assess alterations of carbohydrate homeostasis (306 - 309). When corresponding plasma insulin levels are also measured, a useful indication of the efficiency of insulin in the glucose homeostatic system in the body is provided (310 & 311). In the present study the ipGTT was used to assess glucose homeostasis in mice. The tests were performed without anaesthesia. This method offers the advantage of simplicity and provides highly reproducible results, as shown by the low SEM of the control groups (see figure 17). Tests were performed at 9.00am on animals previously deprived of food for 24 hours. All tests were performed at this time to prevent interference from diurnal variations (312 & 313). 100µl blood were collected from the tip of the tail immediately before and exactly 30 and 60 minutes after an ip injection of 80mg glucose (equivalent to 2.7g/kg body weight in a 40% w/v solution). Plasma was separated by centrifugation and 20µl aliquots were dispensed for radioimmunoassay, and 15µl aliquots for glucose assay. Samples were stored at -20°C until analysed. Food and water were witheld during the test. Precautions were taken to minimise stress and maintain identical environments in all studies. The results were evaluated using individual glucose and insulin levels at the

various time intervals, and also the total areas under the tolerance 'curves', as described in Chapter 3. These parameters provide a simple and accurate assessment of glucose tolerance (130).

PLASMA GLUCOSE DETERMINATION

Numerous methods are available for the quantitative estimation of glucose in blood, based on either the reductive properties of glucose (314 & 315) or the specific oxidation of glucose by either glucose oxidase, hexokinase or glucose-6-phosphate dehydrogenase (316 & 317).

In this study glucose was measured using an automated glucose oxidase method on a Beckman Glucose Analyser (Beckman-Riic Ltd., Fife). The analyser uses the following principles. A sample of plasma is added to a solution containing glucose oxidase. This enzyme ensures the specific determination of glucose, that is the so-called 'true' glucose measure (316). Dissolved oxygen consumed by the reaction is directly proportional to the amount of glucose present. Oxygen combines with glucose according to the reaction :-

$$\text{Glucose} + \text{Oxygen} \xrightarrow[\substack{\text{Glucose}\\\text{oxidase}\\H_2O}]{} \text{Glucoronic Acid} + H_2O_2$$

Oxygen disappearance is measured by an oxygen electrode which is calibrated to translate the equivalent glucose concentration onto a digital display. If unopposed, oxygen would dissociate from the hydrogen peroxide formed in the initial reaction. This is prevented by the presence of ethanol, catalase, iodide and molybate which enable the following reactions to take place -

$$H_2O_2 + \text{Ethanol} \xrightarrow[\text{Catalase}]{} CH_3CHO + H_2O$$

$$H_2O + 2H^+ + 2I \xrightarrow[\text{Molybate}]{} I_2 + H_2O$$

A particular advantage of the Beckman Glucose Analyser is the

small volume of plasma required, namely 10µl, which is particularly useful in experiments on small animals where sequential withdrawal of blood samples could seriously distort fluid and electrolyte balance. A high degree of accuracy and reproducibility is achieved by the analyser, as evidenced by the low cofficient of variation between replicate samples (318). Furthermore, no deproteinisation of plasma is required (318). Plasma is preferred to whole blood because non-specific interference is reduced: saccharoids producing falsely high values are mainly confined to erthrocytes; glycolysis is less active in plasma; and in addition, differences in haemocrit are avoided by the use of plasma (319).

RADIOIMMUNOASSAY OF INSULIN

For many years investigators have sought an assay for insulin which could accurately measure the minute concentrations of this hormone in the circulation. Early attempts were based on the biological assay of insulin by assessment of hypoglycaemia *in vivo* (320 & 321) or glucose uptake and glycogen deposition in diaphragm, muscle and fat *in vitro* (322 - 324). These methods have been replaced in recent years by immunological techniques, especially radioimmunoassay (RIA) which provides a unique combination of specificity, sensitivity, precision, practicability and reproducibilty for the microdetermination of insulin in unfractionated mixtures (325 - 328).

RIA is based on the principle of isotopic dilution (329) which is dependent on competition between the labelled and unlabelled antigen (Ag) for binding sites on specific antibodies (Ab) (330). Increasing amounts of unlabelled Ag in the sample produce a proportional decrease in the binding of labelled Ag to the Ab (331). Thus the level of radioactivity associated with the Ab - Ag complex is related to the concentration of unlabelled Ag in the original sample. The latter is obtained by comparing the bound radioactivity with that

produced by standard solutions containing known amounts of unlabelled Ag. The preparation of Ab and labelled hormone and the selection of assay design parameters have been extensively discussed elsewhere (327, 332, 333 & 354). The present consideration is restricted to the techniques adopted in this study, namely a procedure based on the double-Ab RIA method of Hales & Randle (334). This method employs a second Ab to seperate the Ab-bound and free insulin in the reaction mixture. The method was adopted because of its relative simplicity, rapidity and sensitivity (119). A further advantage is that commercially available antisera and labelled insulin can be obtained. The pre-precipitation procedure described by Hales & Randle (334) was selected since it produces rapid results and involves easy separation of the bound and free labelled Ag by centrifugation. The modifications recommended by the Radiochemical Centre (Amersham), as developed in the Department of Biological Sciences at the University of Aston in Birmingham were used in the present study. In the present method the first Ab (an anti-insulin Ab) was raised to porcine insulin in guinea-pigs and the second Ab (induced in rabbits against the γ - globulin of the guinea-pig) was used to precipitate the primary Ab - Ag complex.

(i) REAGENTS

ANTISERUM - The double-Ab (anti-insulin, anti-guinea-pig-γ- globulin serum) was obtained as a freeze-dried preparation from Wellcome Reagents Ltd., Beckenham. The preparation was stored at 4°C and reconstituted in assay buffer on the day of the assay and used at the dilution recommended by the supplier, usually about 1:16,000. At this dilution approximately 40% of a selected dose of labelled insulin at the lower end of the physiological range (0.25ng) is bound.

LABELLED INSULIN - Insulin was iodinated with ^{125}insulin by the Chloramine-T method (335) and purified by gel-filtration

(The Radiochemical Centre, Amersham). The preparation was stored at 4° C.

STANDARD INSULIN - Mouse insulin was obtained as a freeze-dried preparation (The Novo Research Institute, Bagsvaerd, Denmark). The preparation was extracted from mouse pancreas and recrystallised twice. The biological potency was determined using the mouse convulsion test as 22.4± 3.2IU/mg (327). Mouse insulin consists of two species of insulin in approximately equal amounts (336 & 337) which differ from each other at residues B_9 and B_{29}. Even repeatedly recrystallised preparations are not homogeneous (338 & 339) and may contain small amounts of precursor compounds (340 & 341) and other immunologically active contaminants which represent a source of error (see 342). The standard and endogenous insulin appear to be immunologically identical when analysed under routine assay conditions used in this study (327, 343 & 344). Subtle conformational changes may occur during manipulation and storage of the standard so the following protocol was devised to minimise these alterations. The freeze-dried mouse insulin containing 1mg human albumin was diluted in double-distilled deionised water to give a stock solution (100µg/ml) which was divided into 120µl aliquots. From each aliquot, a working solution (1µg/ml) was prepared in assay buffer and divided into 200µl aliquots. The solutions were stored at -20°C in tightly capped tubes. A series of standard dilutions (0.5 - 10ng/ml) was prepared from the working solution for each assay. Thus freezing and thawing of the standard was minimised. There is no evidence that deterioration of the standard occurred over the two years it was used (327). This regime was based on the general recommendations of Bangham & Cotes (345).

ASSAY BUFFER - 0.4mM/l phosphate buffer, pH 7.4, containing 10.2g BSA (fraction v, Miles Labs. Ltd., Stoke Poges), 12.4g $NaH_2PO_4.2H_2O$ and 0.5g thiomersalate per litre made up in deionised double-distilled

water was used. The buffer was stored at 4°C.

PLASMA - RIA were conducted on 20µl plasma using polystyrene tubes (LP 2, Luckhams Ltd., Burgess Hill) fitted with plastic caps.

(ii) PROCEDURE

The RIA and incubations were performed at 4°C and all additions were made using an Eppendorf - Marberg micropipette equipped with disposable plastic tips (Anachem Ltd., Luton). Reactants were mixed without frothing using a variable speed rotomixer (Hook & Tucker Ltd., London). The protocol used for the assay of insulin is shown in figure 1. Each assay was conducted on the unknown samples and triplicate sets of standard insulin solutions (0.5 - 10ng/ml),zero, blank and total counts. Briefly the procedure was performed as follows. The initial reactants (standard or unknown plus antisera) were mixed and incubated at 4°C for 24 hours. Labelled insulin was added, the reactants mixed and incubated at 4°C for 6 hours. Following the second incubation, the reactants were diluted in buffer, mixed and then the free and Ab-bound insulin separated by centrifigation at 1500g (Mistral Coolspin Centrifuge, MSE Scientific Instruments, Surrey) for 30 minutes. The supernatant, containing the free labelled insulin, was decanted and the final drops of fluid at the lip of the tubes were aspirated with a pasteur pipette connected to a vacuum pump. The radioactivity in the precipitated Ab - Ag complex was counted simultaneously on two channels for one minute using a well-type crystal scintillation counter (ICN Tracer Lab Gamma Set 500, ICN Pharmaceuticals, London) connected to a teletype machine (Model 33ASR, Teletype Corporation, Illinois, USA). A standard curve, using the logarithm of the concentration against the bound radioactive count, was prepared and the insulin in the unknown samples was interpolated from this graph (figure 2). The criteria for a successful assay were :-

(a) Good agreement between replicates of standards, zero, blank and

Figure 1 PROTOCOL FOR INSULIN RADIOIMMUNOASSAY

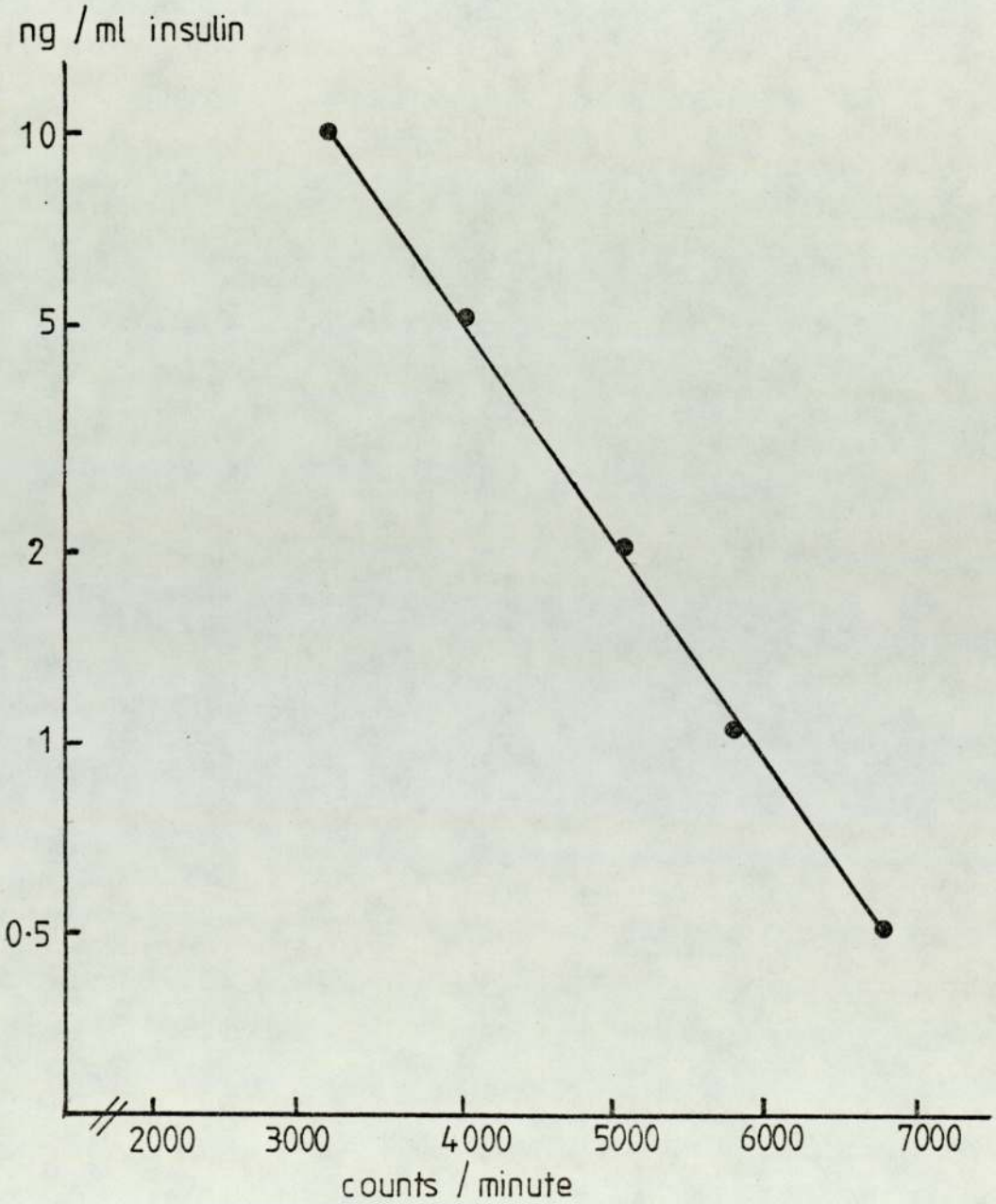
	Buffer	Mouse insulin standard	Unknown plasma sample	Binding agent	Labelled insulin (I^{125})	Buffer
Total count	-	-	-	-	20µl	-
Blank	40µl	-	-	-	20µl	200µl
Zero count	20µl	-	-	20µl	20µl	200µl
STANDARDS 0.5ng/ml	-	20µl	-	20µl	20µl	200µl
1.0ng/ml	-	20µl	-	20µl	20µl	200µl
2.0ng/ml	-	20µl	-	20µl	20µl	200µl
5.0ng/ml	-	20µl	-	20µl	20µl	200µl
10ng/ml	-	20µl	-	20µl	20µl	200µl
UNKNOWN SAMPLES 1	-	-	20µl	20µl	20µl	200µl
2	-	-	20µl	20µl	20µl	200µl
etc.						

After addition of binding reagent, mix & incubate for 6 hrs. at 4°C.

After addition of label, mix & incubate for 24hrs. at 4°C.

After 2nd addition of buffer, mix, centrifuge, decant (except total counts) & count.

Figure 2 Standard curve - insulin



total counts. Replicates differing by more than 200 counts/minute were discarded.

(b) A zero count that corresponds to a theoretical percentage of the total count. At the dilution used, 40% of the total label will be bound. The zero count evaluates the amount of radioactivity of the insulin-Ab complex in the absence of unlabelled insulin.

(c) A blank count less than 5% of the total count. Non-specific binding can be assessed by assaying blank tubes which contain only the buffer and label. An acceptable limit for the percentage of label bound is 5% or less of the total count added. Good agreement between blank replicates indicates a constant error. The blank count also evaluates the washing procedure of the antibody precipitate.

The sensitivity of the assay as defined by Midgly (346) was 0.18ng/ml and the intra-assay coefficient of variation was 3.58%.

TISSUE GLYCOGEN DETERMINATION

Numerous methods have been employed for the determination of glycogen in animal tissues. The most popular techniques involve digestion of tissues with caustic alkali followed by a colorimetric reaction using either copper (347 & 348), anthrone reagent (349 & 350) or phenol-sulphuric acid (351 & 352). The procedure used in the present study was a modified version of the phenol-sulphuric acid method of Lo et al. (353). This method is rapid, relatively simple and has the advantages of both stable reagents and a stable colour reaction.

Animals were killed by cervical disslocation. 35-50mg pieces of liver, uterus, abdominal, cardiac and femoral muscle were rapidly excised, frozen in liquid nitrogen and stored at -20°C until assayed. On the day of the assay, each tissue sample was weighed and placed in a glass tube containing 0.5ml 30% potassium hydroxide saturated with sodium sulphate. Tissue digestion was performed by boiling the tubes

for 30 minutes to produce a homogeneous solution. 0.5ml 95% ethanol was added and the tubes transferred to an ice-bath for 30 minutes to precipitate the glycogen from the digestate. Separation of the precipitate was achieved by centrifugation at 1500g (Mistral Coolspin Centrifuge, MSE Scientific Instruments, Surrey) for 30 minutes. The supernatant was carefully aspirated and the glycogen precipitate dissolved in 3ml double-distilled water. Aliquots, 0.1 - 0.9 ml (in 0.1ml graduations), were pipetted into strong glass tubes and made up to 1ml with double-distilled water. The aliquots selected varied, according to the type of tissue assayed and the steroid treatment given, in accordance with the concentration of glycogen anticipated. 1 ml 5% phenol solution immediately followed by 5 ml 98% conc. sulphuric acid were added, and the contents thoroughly mixed. The colour was allowed to develop at 27° C for 20 minutes. Blank solutions were prepared in a similar manner, using 1 ml double-distilled water in place of the glycogen solution. Aliquots (0.1 ml) of standard glycogen solution (rabbit liver glycogen, Hopkins & Williams, Romford), from 50-800µg/ml were treated in the same manner as the unknown glycogen solutions. Absorbance of the unknown and standard solutions was measured at 490nm against the blank solutions on a Beckman DB Spectrophotometer (Beckman-Riie Ltd., Fife). Triplicate samples of each standard were assayed, and a linear figure was prepared by plotting the glycogen concentration against absorbance (see figure 3). Tissue glycogen content was calculated from the following formula -

$$\text{mg glycogen/g wet weight} = \frac{A \times V \times 10^{-1}}{K \times v \times W}$$

where A = absorbance at 490nm

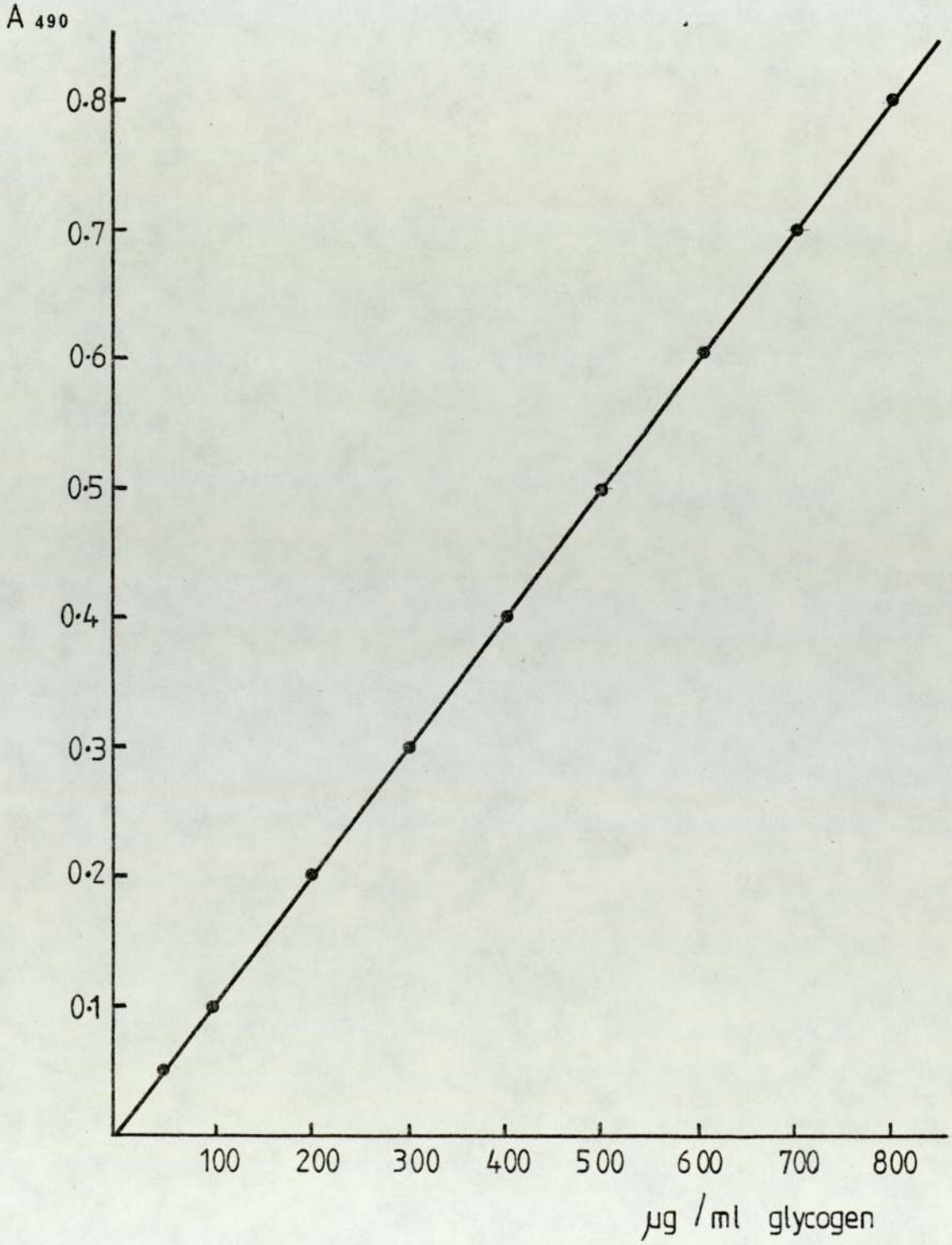
V = volume of glycogen solution (3 ml)

K = slope of the standard curve (0.001)

v = aliquot of glycogen solution assayed (0.1 - 0.9 ml)

W = tissue weight (mg)

Figure 3 Standard curve - glycogen.



LIQUID SCINTILLATION COUNTING - DISTRIBUTION & PERIPHERAL STUDIES

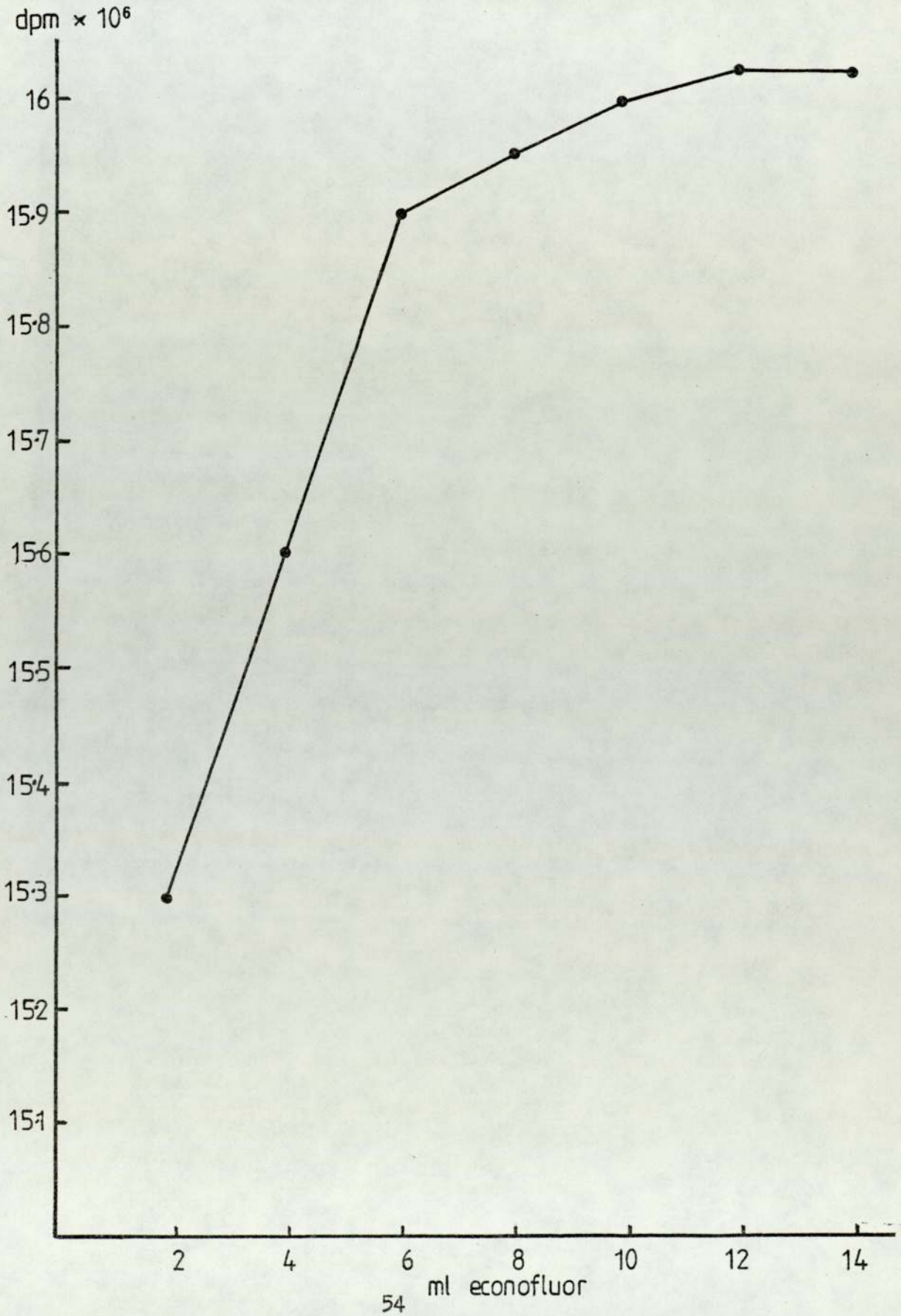
This section describes the application of liquid scintillation counting (LSC) to the in vivo distribution of steroids and to peripheral tissue sensitivity in vitro in the adult female mouse. The effects of administration route, ovariectomy and steroid treatment were investigated.

Many techniques are available for the measurement of radioactivity. The most popular techniques include, the ionisation of gases and solids, autoradiography and scintillation counting. In recent years, LSC has become the preferred method, especially for weak β-emiters such as ^{14}C, which was used in the present study, because of the sensitivity and versatility of the technique (355 - 357). The success of LSC for counting ^{14}C labelled compounds in animal tissues has been reported on numerous occasions (358 - 361). The problems of self-absorption of emissions in solid samples are absent or considerably reduced in LSC and this is critical where weak β emitters are concerned (362). Futhermore, LSC has a detection efficiency approaching 100% (356 & 357) even with biological samples (358, 359 & 361). LSC also offers the advantage that counting is mechanised to deal with a large number of samples (357). The principles of LSC are fully described elsewhere (see 363 - 368).

(i) SCINTILLANT - Preliminary experiments were conducted to select the amount of scintillant that provided the maximum counting efficiency and specific activity (see figure 4). For amounts of scintillant greater than 10ml, the advantageous effects were negligable. Thus for purposes of economy, 10ml scintillant were used for all experiments.

(ii) SAMPLE PREPARATION - Sample preparation was designed to give a high, uniform and stable counting efficiency. To fulfil these conditions tissue solubilisation was utilised in this study. Tissue solubilisation is achieved by hydrolysis with either sodium hydroxide,

Figure 4 Specific activity - effect of amount of scintillant.

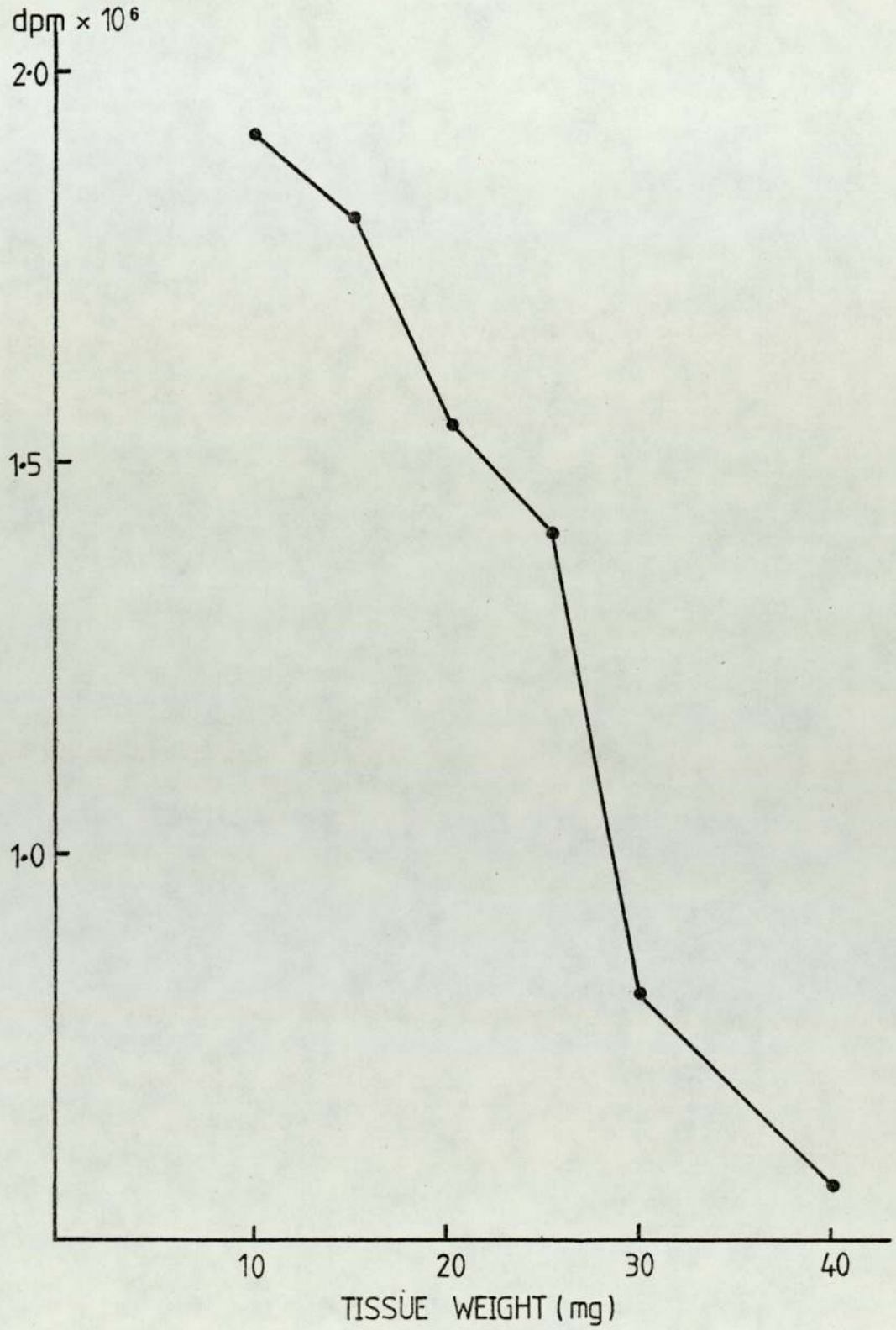


potassium hydroxide, formamide or quaternary ammonium salts to yield soluble products of a lower molecular weight. Samples solubilised by these methods are completely miscible with the toluene and xylene based scintillators used in the present study, forming homogenous counting systems. Some tissue solubilisers may affect the stability of fluors in the scintillation system (369 & 370), but hyamine hydroxide and protosol do not do this. Hyamine hydroxide is frequently associated with high levels of quenching (368), thus protosol was preferred. Protosol (New England Nuclear, Boston, USA) accepts aqueous samples and solubilises most types of whole animal tissue with low quenching and high efficiency at ambient or refigerated temperatures (NEN, technical bulletin, 1977).

Wet tissue samples were used for solubilisation because they digest easily (371). Water facilitates digestion, solubilisation and reduces chemiluminesence thus improving the overall counting efficiency (NEN, technical bulletin, 1977). Tissue weights greater than 30mg significantly reduced the specific activity of the LSC system (see figure 5). Furthermore, different types of tissue produced different coloured LSC systems and hence different quench characteristics. For example, muscle produced yellow and liver red solutions. For a constant weight, however, this should be consistent for each type of tissue. Mean tissue weights of 15mg in the distribution study and 25mg in the peripheral sensitivity study were used to minimise colour quenching and ensure a high counting efficiency.

(iii) RADIOLABELLED STEROIDS - ^{14}C labelled steroids were utilised in the present study to increase detection efficiency and minimise errors. The ^{14}C was incorporated in the A ring of the steroids thus increasing the certainty that the radioactivity measured in a tissue sample is still associated with the steroid molecule. A further advantage is that samples can be stored for up to four weeks without any significant

Figure 5 Specific activity - effect of tissue weight.



losses of radioactivity (see figure 6) (357). The dose of radiolabel administered in the distribution study was selected to provide sufficient counts in the tissue samples within a limited period to enable satisfactory analysis of the results. Excessive amounts of isotope are not only economically unsound, but may also result in abnormal metabolism of the hormone.

(iv) QUENCHING - In LSC quench correction and efficiency determination are synonymous. Thus quenching has to be accurately measured. The methods used to determine quenching and counting efficiency were the the external and the channels-ratio standardisation techniques.

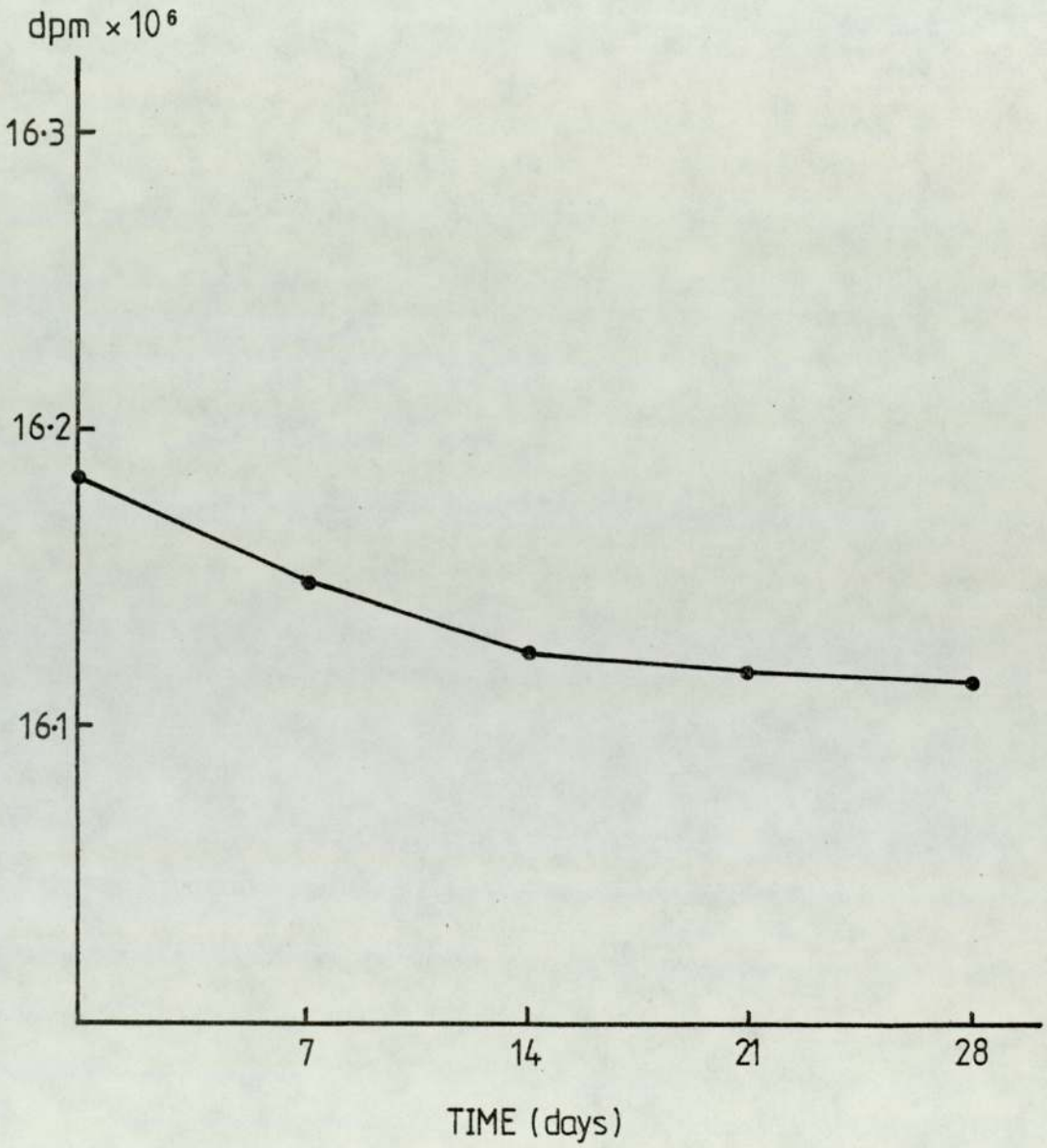
External standardisation is based on a comparison between the sample counted alone and counted whilst irradiated by an external standard to yield the external standard ratio (ESR). This technique is adequately discussed elsewhere (368, 372 - 374). ESR was used in the peripheral tissue sensitivity experiments where highly radioactive samples were produced.

Channels-ratio standardisation is based on a downward shift of the pulse-height spectrum of a radionuclide in the presence of a quenching agent. The extent of this shift is related to the counting efficiency of the LSC system. The channels-ratio (CR) technique is described elsewhere (374 - 376). This method is particularly suitable for counting large numbers of samples and low-counting samples. CR was utilised in the distribution studies.

(v) VIALS - The counting vials influence the optimum assay conditions, accuracy, reproducibility and contribute to the background count (368). Low potassium-40 glass vials were used due to the low cost, practicability, infrequency of phosphoresence and the facility of re-use after cleaning (368 & 377).

(vi) CHEMILUMINESENCE - Chemiluminesence can produce a spurious high count rate. The problem of chemiluminesence in LSC systems is

Figure 6 Specific activity - effect of storage time.



described by (378 & 379). Chemiluminesence is temperature-dependent and at low temperatures may be suppressed (379). Hence, low temperatures were employed in the present study to reduce chemiluminesence. It can also be eliminated by acidification of the alkaline tissue digests to pH 6 - 7 prior to addition of the scintillator (368 & 380). Acetic acid was used in the present study.

(vii) PHOSPHORESENCE - Many proteinaceous LSC systems produce spuriously high counts due to the development of phosphoresence (368). To prevent the emission of light as phosphoresence during photoactivation, samples were stored in the dark and acidified prior to counting (371). Acetic acid was used.

(viii) HETEROGENEOUS SYSTEMS - Samples containing aqueous and polar compounds may precipitate or crystallise out of solution producing a heterogeneous LSC system which is counted with a lowered efficiency (368). Thus care was taken to discard obvious heterogeneous samples.

(ix) BACKGROUND ACTIVITY - Background activity can arise in numerous ways (366, 381 & 382). It is important to minimise the background count so that low sample counts are not obscured and in addition, it must be accurately assessed to allow effective standardisation.

IN VIVO DISTRIBUTION OF LABELLED STEROIDS

The assay design was a modification of the method of Oksanen & Tuohima (360). Commercially available scintillation solution and solubiliser were used to attain high levels of efficiency and reproducibility. Econofluor (New England Nuclear, Boston, USA), lot 167EC7, a scintillant containing several aromatic hydrocarbons and all the necessary solutes was used. The scintillant was stored in the dark at room temperature for safety and stability.

The tissue solubiliser , protosol (New England Nuclear, Boston, USA), was a 0.5M quaternary ammonium hydroxide solution. It was stored in the dark at room temperature to prevent degradation and

crystallisation.

The labelled steroids (4 - ^{14}C) progesterone, batch 45, and (4 - ^{14}C) estradiol-17 β, batch 38, were obtained from the Radiochemical Centre (Amersham). They were stored at 4°C until required.

(i) QUENCH CURVE - Quenching was assessed by the CR technique. A quench curve was prepared by adding increasing amounts of chloroform, a quenching agent, (70 - 700 µl) to a LSC system containing 0.5ml protosol, 10ml econofluor and 0.01µCi ^{14}C-alanine and recounting the vials after each addition. This produces a series of increasingly quenched standards. A graphical plot (see figure 7) of the CR against the counting efficiency was produced. The CR of the unknown samples was read from this quench curve to give the detection efficiency and hence the specific activity in disintegrations per minute/mg wet weight (dpm/mg).

(ii) PROCEDURE - The mice received the labelled steroids either by the subcutaneous, intramuscular or oral route of administration at a dose of 5µCi/0.05ml solvent. Animals were decapitated two hours later. Preliminary experiments indicated a high uptake at this time (figure 8). The two hour period allows sufficient time for the steroid to be distributed throughout the body yet minimises the time available for degradation. Blood was centrifuged immediately and 100µl plasma was transferred to tightly capped glass scintillation vials containing 0.5ml protosol. The tissue samples were rapidly excised, trimmed, blotted to remove surface blood, weighed and placed in tightly capped glass scintillation vials containing 0.5ml protosol. A mean tissue weight of 15mg was utilised. The <u>in vivo</u> distribution of labelled steroids was investigated in liver (anterior margin of medium lobe), skeletal muscle (femoral quadriceps), cardiac muscle (ventricular apex), uterus (mid-section of one horn), pancreas (splenic region), adipose

Figure 7 Quench curve - distribution studies

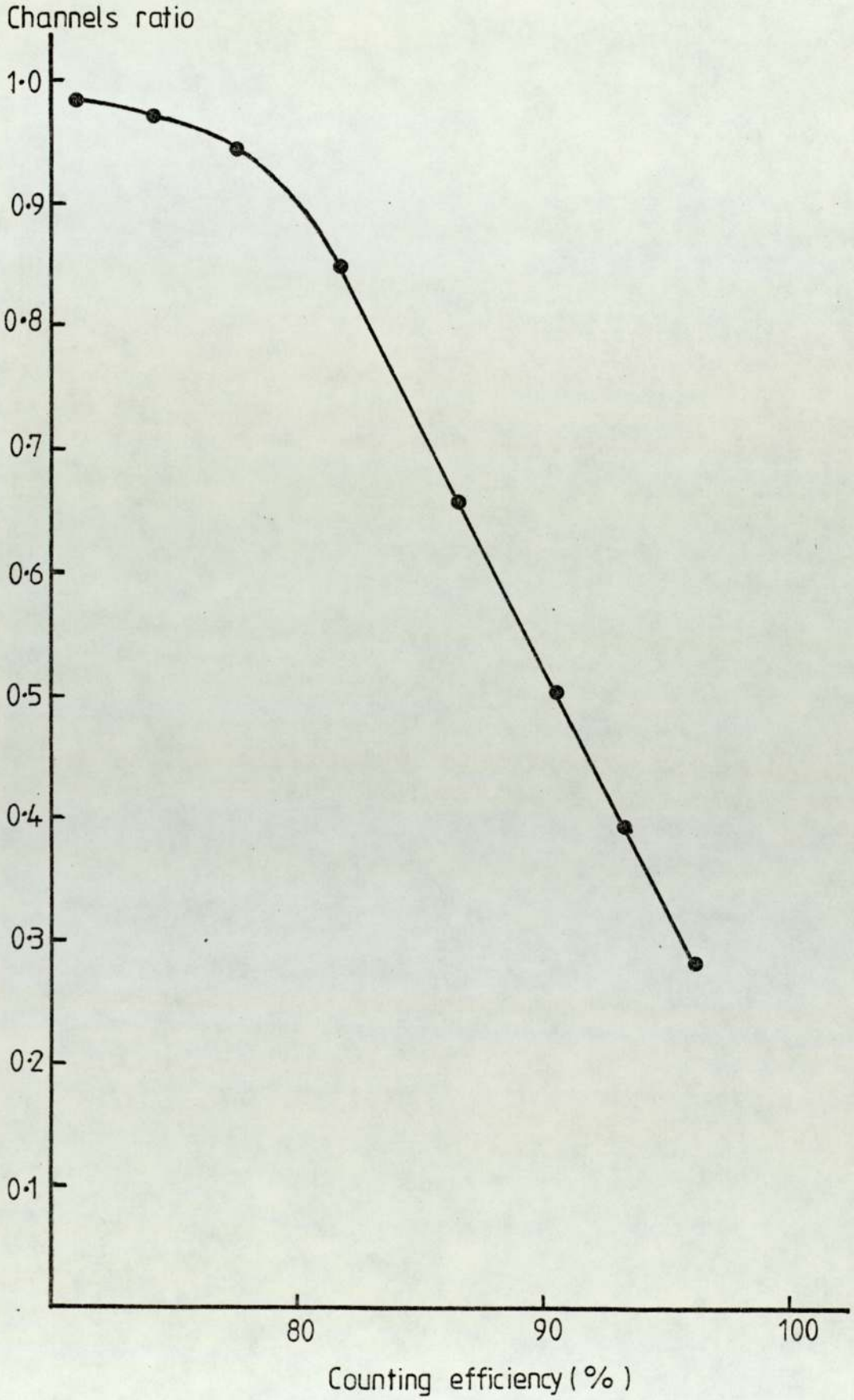
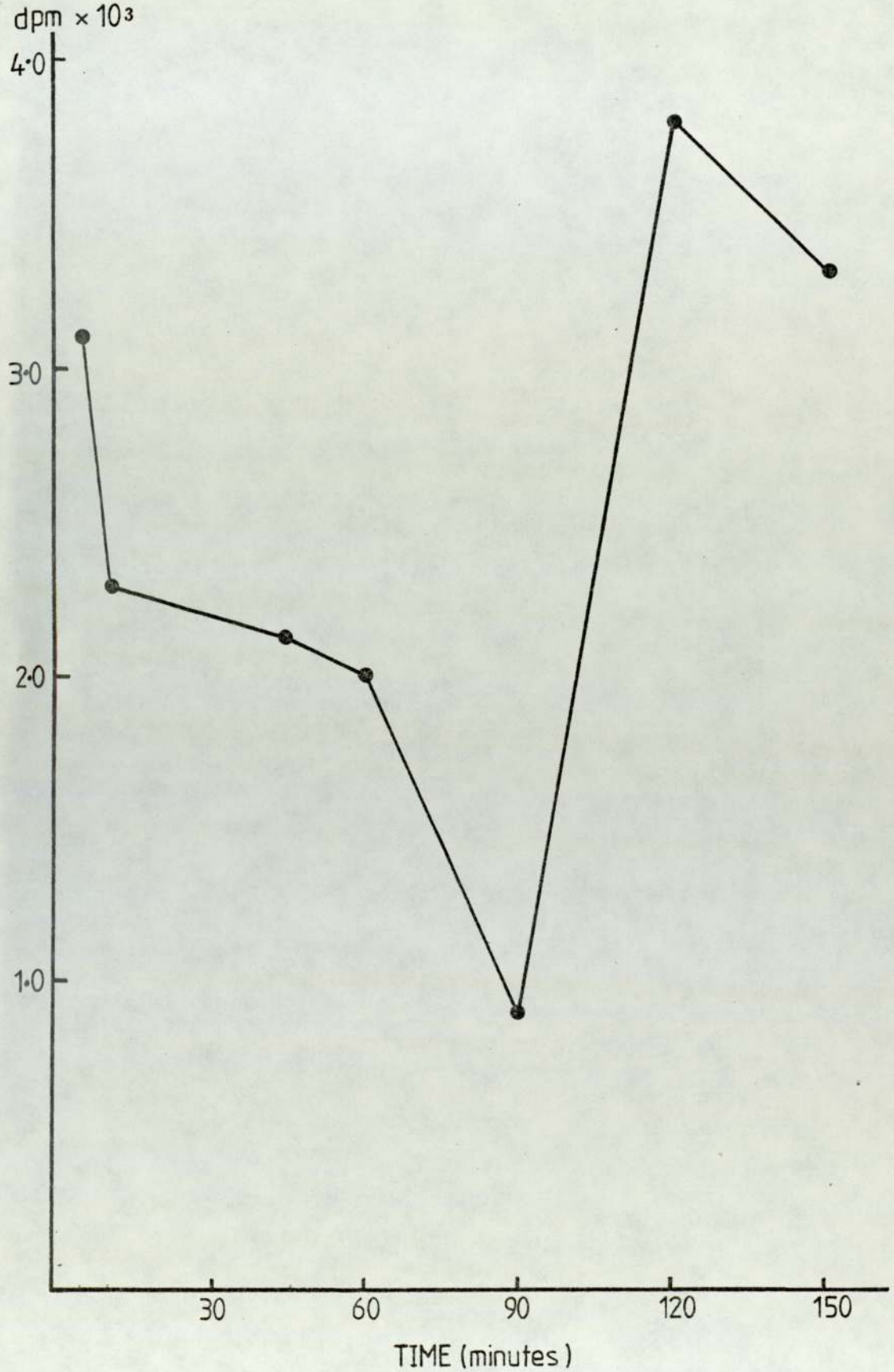


Figure 8 Liver radioactivity levels following subcutaneous administration of 5μCi ^{14}C progesterone.



tissue (parametrial lateral margin) and pituitary (total).

All samples were digested at 48°C in a shaking water-bath until solubilisation was complete, between 2-24hrs.. Constant retightening of the caps prevented evaporation. The alkaline digests were neutralised with a small amount of glacial acetic acid and after cooling, 10ml econofluor was added. The contents of the vials were thoroughly mixed and stored at 4°C for 24hrs. in the dark. Radioactivity was counted on a Beckman LSE Scintillation Counter (Beckman- Riic Ltd., Fife). preset for either a maximum of ten minutes or an error of 1%. Gain settings were selected to produce a maximum ratio of sample to background count. The specific activity of each sample in dpm/mg wet tissue weight was determined by correcting for tissue weight, background and quenching.

PERIPHERAL TISSUE SENSITIVITY STUDIES

Tissue incubations were originally used for the bioassay of insulin (320 & 383) but they were soon extended to include metabolic studies. Peripheral tissue sensitivity to insulin has been examined in many tissues (103, 324, 384 - 387) but muscle and adipose tissue have been studied in particular depth.

Various muscle preparations have been utilised, especially rat diaphragm and hemidiaphragm (386). The use of these preparations has been severely criticised on many occasions. Intact diaphragm does not respond well to exogenous estrogen (390 & 391) and is not a representative skeletal muscle because of the continual inherent contractions (390). The rat hemidiaphragm demands extreme technical care to achieve reproducibility and adequate sensitivity. Pooled samples from several animals are required and a large uptake of glucose occurs in the absence of insulin (386). Furthermore, hemidiaphragm and intact diaphragm preparations differ considerably in metabolic studies (392). Heart muscle incubations are also considered

unrepresentative (103). Intact soleus muscle preparations have also used. This muscle is considered to be a representative skeletal muscle (389) which can be prepared intact without breaking or cutting the muscle fibres. It yields quantitative measurements of insulin binding which correspond with the biological effects of insulin in the preparation (389 & 393). The soleus muscle responds well to insulin and is thin enough to allow rapid diffusion of metabolites and gases (389, 390, 393 - 395). In addition, this intact preparation exhibits very little degrading activity towards insulin and maintains optimal sensitivity to the hormone (393). The advantages afforded by the the intact soleus muscle, together with the relative simplicity of isolation and preparation, make this muscle preparation the natural choice for the present study.

The sensitivity of adipose tissue to minute concentrations of insulin have been confirmed on several occasions (396) and this preparation was originally exploited for insulin bioassay (386, 397 & 398). More recently, it has been recognised that insulin acts directly on the adipose tissue both in vivo (399) and in vitro (400). In vitro investigations of insulin sensitivity and resistance have relied heavily on the isolated adipocyte as a cell model (100). The separation and isolation of adipocytes is a time consuming and complicated technique (see 401), futhermore, the results are often unreproducible. Collagenase digestion is frequently used, but recent evidence indicates that cell membranes are damaged during the digestion procedure (402). Glucose uptake is consequently altered and the preparation might be considered to be invalidated. Rodbell (401), however, maintains that adipocytes prepared in this way keep the intrinsic metabolic characteristics of the tissue. Glucose metabolism is also affected by cell size (403 & 404). Thus, a further disadvantage of this preparation is that cell size must be standardised

for each sample. Adipose tissue incubations have also been performed using isolated fat pads (405 & 406) and pieces of adipose tissue (386, 407 & 408). These preparations are relativly simple and rapid to perform. Glucose enters the cell rapidly in such incubations, provided that small pieces of tissue are used (402). Accurate and reproducible results can be obtained with these preparations (405 & 408). The sensitivity of the adipose tissue within a given depot varies (398 & 409). To achieve reproducibility, samples must be taken from the same part of the fat depot. Adipose tissue incubations performed in the present study utilised parametrial fat tissue pieces from the lateral margin of this depot. Tissue pieces were selected in preference to isolated adipocytes due to the relative simplicity and reproducibility that the technique affords.

(i) MATERIALS - To ensure maximum detection efficiency, commercially available pre-mixed scintillant was used (Econofluor, New England Nuclear, Boston, USA). Labelled glucose, glucose D-(^{14}C (U)), was obtained from the Radiochemical Centre (Amersham) and stored at 4°C.

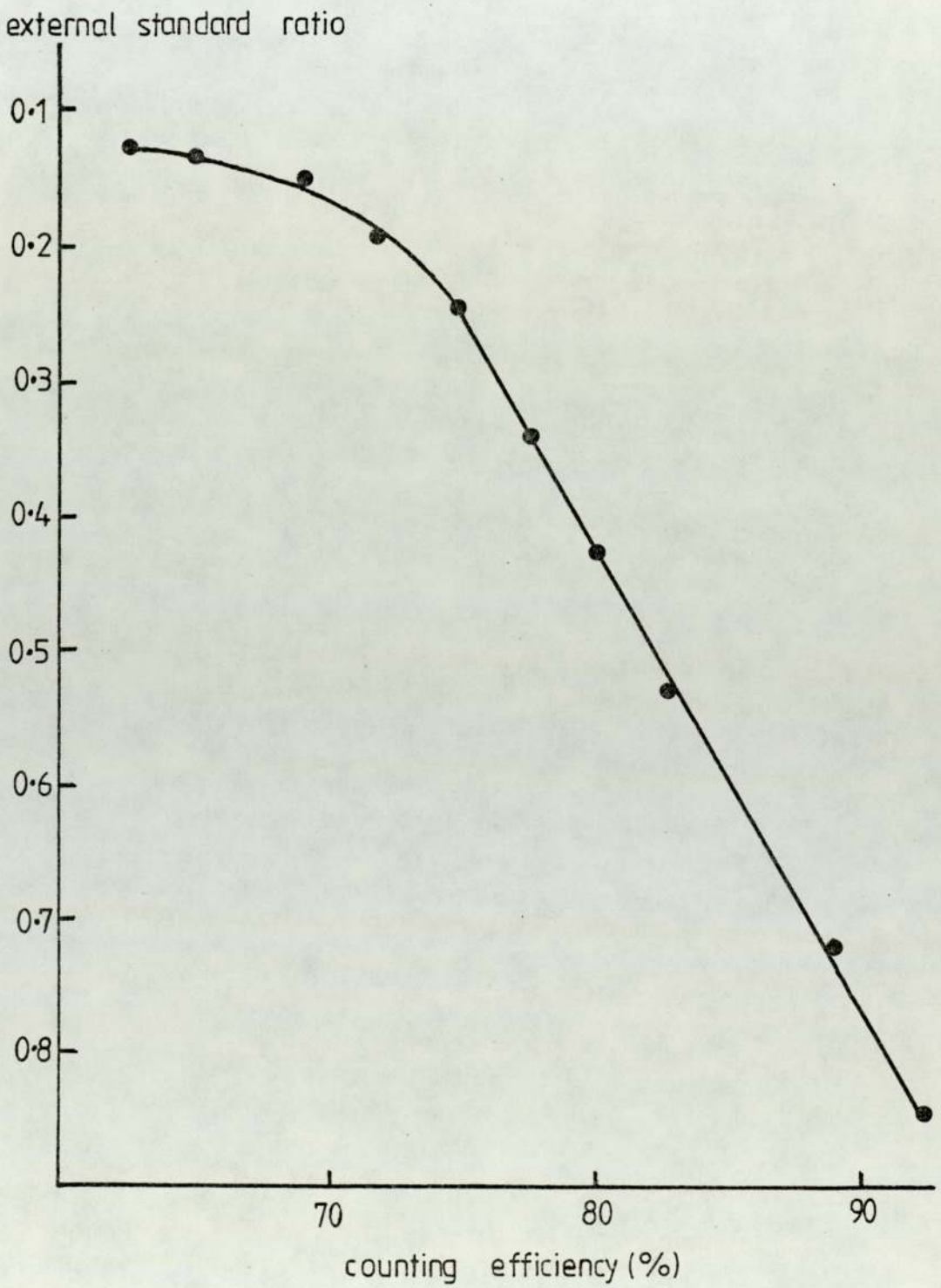
A modified Krebs-Ringer bicarbonate buffer was used containing :- g/l NaCl-6.92, KCl-0.35, $CaCl_2$-0.14, KH_2PO_4-0.16, $MgSO_4 7H_2O$-0.29, $NaHCO_3$-2.1, Bovine serum albumin-1.0, Glucose-1.0. Bovine serum albumin was used as a carrier protein to pervent adsorption of insulin to glassware. The buffer contained half the normal concentration of calcium to prevent the formation of insoluble calcium phosphates on standing. The buffer was sterilised by vacuum distillation, stored at 4°C and adjusted to pH 7.4 prior to use. Insulin (Crystalline Bovine Insulin, Sigma Chem. Co., Poole, Dorset) was dissolved in acidified buffer. Solutions were prepared by serial dilution, stored at -20°C until required and maintained in ice-bath when in use. Each solution was frozen and thawed only once.

(ii) QUENCH CURVE - Quenching was assessed by the ESR technique. A

quench curve was prepared by adding increasing amounts of chloroform, a quenching agent, (70 - 700µl) to a LSC system containing 0.5ml protosol, 10ml econofluor and 0.01µCi ^{14}C glucose. A graphical plot of the ESR against efficiency was prepared from a series of quenched standards (see figure 9). The ESR of the test samples was used to determine the counting efficiency from the quench curve plot, and hence the specific activity in dpm/mg wet weight.

(iii) PROCEDURE - Animals were killed by cervical dislocation. The two soleus muscles and a portion of adipose tissue, from the parametrial lateral margin, were rapidly excised and placed in warm (37°C) buffer oxygenated with O_2 and CO_2 (95 : 5). The muscles were cleared of excess tissue, blotted, weighed and placed in separate incubation vials. The adipose tissue was blotted, divided into three 25mg pieces which were placed in separate incubation vials. These procedures were performed rapidly to minimise chilling and tissue damage. The glass incubation vials contained 2ml oxygenated buffer at 37°C. They were capped, oxygenated and gently shaken throughout the incubation period. A preliminary incubation (37°C for 10 minutes) was performed prior to the addition of labelled glucose and insulin. The second incubation was performed at 37°C for 60 minutes with continuous gentle oxygenation and shaking. Tissues were removed from the incubation vials, rinsed quickly in warm buffer, blotted and placed in scintillation vials containing 0.5ml protosol. Samples were digested at 48°C in a shaking water-bath until solubilisation was complete, between 2 - 24hrs.. Constant retightening of the vial caps prevents evaporation. The alkaline digests were neutralised with glacial acetic acid and after cooling 10ml econofluor was added. Following thorough mixing, the vials were stored at 4°C for 24hrs. in the dark. Radioactivity was counted on a Packard Scintillation Counter preset for a maximum count of 5 minutes or an error of 1%. The specific activity of each sample in dpm/mg wet

Figure 9 Quench curve - sensitivity studies



was determined by correcting for tissue weight, background and quenching.

Chapter 3

EFFECTS OF SEX STEROIDS ON GLUCOSE TOLERANCE AND PLASMA INSULIN

CHAPTER 3

GLUCOSE TOLERANCE TESTS

INTRODUCTION

There is considerable evidence to suggest an association between sex steroids and intermediary metabolism (4). Changes of both glucose and insulin homeostasis during the estrous cycle, the menstrual cycle and following ovariectomy (119, 126 & 139) suggest that sex hormones participate in the regulation of carbohydrate metabolism. Literature has also accumulated over the past twenty years to suggest an association between the synthetic sex steroids used for contraceptive purposes and carbohydrate metabolism (1, 2, 5, 201 & 230). However, the precise nature of the effects of natural and synthetic sex steroids on carbohydrate metabolism still remains to be clearly determined. This is reflected by the many discrepancies that have been reported in the medical and scientific literature and also in the many anomalous reports that have appeared in the popular press. It has been very difficult to concisely analyse the literature as frequently studies do not give full details about the subjects, the contraceptive regimes or the investigative procedures used. All of these factors are likely to have an important influence on the results.

To provide a more detailed analysis of the effect of contraceptive steroids on the regulation of glucose homeostasis, the present study examines the influence of the type of preparation used, the route of administration and the duration of treatment under a strictly controlled genetic and external environment on the ipGTT and plasma insulin titres in mice. The basal plasma insulin and glucose levels following steroid administration are described. In the following sections the effect of arachis oil, estrogen treatment, progestogen treatment and combined treatment on ipGTT and plasma insulin levels are discussed.

METHODS

Intact adult female mice were divided into nine groups and treated as described below -

Groups 1 Controls - arachis oil

2 Estradiol-17β (E_2) - 5μg/kg (body wt.) /d

3 Ethynyl estradiol (EE) - 5μg/kg (body wt.) /d

4 Progesterone (P) - 1mg/kg (body wt.) /d

5 Norethnodrel (N) - 1mg/kg (body wt.) /d

6 Norethisterone acetate (NA) - 1mg/kg (body wt.) /d

7 E_2 & P - 5μg & 1mg/kg (body wt.) /d

8 EE & N - 5μg & 1mg/kg (body wt.) /d

9 EE & NA - 5μg & 1mg/kg (body wt.) /d

Within each group the subcutaneous (Sc), intramuscular (Im) and oral routes of administration were examined. After one, four and six cycles of treatment, basal levels of plasma glucose and insulin were measured after a twenty-four hour fast. Then ipGTTs with simultaneous measurement of plasma insulin concentrations were performed using a glucose challenge of 2.7g/kg (body wt.). The control animals were tested during the diestrus stage of the estrous cycle. Areas under the glucose and insulin 'curves' were determined using the following formula -

Area = (2B + A + C) x 15

where A, B and C are the readings taken at 0, 30 and 60 mins. respectively.

RESULTS

(i) Basal plasma insulin and glucose levels

The effect of steroid treatment on the basal levels of plasma insulin and glucose after a twenty-four hour fast are shown in figures 10 - 15. The results clearly show that both components were influenced by the type of steroid used, the route of administration and the duration of treatment.

Estrogen treatment produced higher basal insulin levels than progestogen treatment, with one exception, following Im P administration (see figure 11). The results also clearly demonstrated that the natural steroids, E_2 and P, produced greater rises of plasma insulin than their synthetic counterparts. P, however, was slower than E_2 in eliciting this response (figure 12). Whereas E_2 and P singularly markedly elevated insulin, together in a combined preparation they did not produce this response. A similar effect was also noted with synthetic steroids. Whilst EE elevated basal plasma insulin, in combination with either N or NA, insulin levels were frequently not significantly different to control values. Thus the combined preparations exerted the smallest stimulatory influence on basal plasma insulin. These data are indicative of antagonism between the two steroids in the combined preparations, particularly with regard to the natural sex steroids. In the E_2, EE and P groups, basal insulin titres were all significantly higher than the controls at four and six cycles. The levels were more variable in the synthetic progestogen and combined steroid groups. On occasions insulin levels were significantly lowered in these groups (figure 10). However, there was a tendency for basal insulin concentrations to increase as the duration of treatment increased in all steroid treated animals (see figure 10).

The basal plasma glucose levels are illustrated in figures 13 - 15. These data show that at no time during the study were basal glucose

levels significantly higher than the diestrus control levels. Estrogen treatment was associated with lower plasma glucose titres than progestogen treatment (figure 13). Indeed, progestogen administration often produced glucose titres that were not significantly different to those seen in the control mice. In addition, the results show that the natural steroids, E_2 and P, lowered basal glucose to a greater extent than their synthetic counterparts, EE, N and NA (figure 14). Over the long term, only Sc EE administration lowered plasma glucose titres significantly (figure 13). E_2 and P, given singularly or together, lowered basal glucose. However, after combined E_2 and P treatment glucose levels were similar , and not as one might expect lower, than those seen in mice receiving the single components. These data suggest some antagonism was present in this combined group. The reverse effect was noted during synthetic combined treatment. The hypoglycaemic tendency of EE was substantiated by the presence of either N or NA ie. plasma glucose levels were lower in the combined synthetic mice than in either the EE, N or NA treated mice (figure 15). These results suggest synergism between the steroids, with respect to reducing basal glucose occurred in the combined EE and N, and EE and NA treated mice. Alterations of basal glucose concentrations with treatment duration were observed. Consistent with the time-dependent elevation of insulin, basal glucose titres decreased as the duration of treatment increased (figure 13). Following NA treatment, however, the reverse was seen. That is, basal glucose titres increased as treatment duration increased (figure 15).

The results of the ipGTTs performed in diestrus control mice are shown in figures 16 and 17. Neither the administration route nor treatment duration had a significant influence on the outcome of the GTTs in the control group. Thus it seemed appropriate to use the average of all the control data (including all three routes and all

three test periods) as an indicator of control plasma insulin and glucose titres for comparison with experimental groups. Hence this value is the control mean ± SEM shown in all subsequent figures in this chapter. The statistics, however, were calculated for each administration route at each time and compared as such with the steriod treated mice.

(ii) ip GTT - ESTROGEN TREATMENT

Typical ipGTT 'curves' with simultaneous determination of plasma insulin seen after estrogen treatment are shown in figures 18 - 21. Throughout the study, the plasma insulin response to a glucose challenge was significantly elevated in comparison with the control levels. EE was slower in eliciting this response than E_2 (figures 20 and 21). In addition, the natural steroid had a greater influence than the synthetic steroid, EE, on plasma insulin titres. Alterations of insulin with treatment duration differed between the two estrogen treatments. In the E_2 treated mice, plasma insulin increased reaching a maximum by the end of the study (figure 20). Whereas during EE treatment, the maximum response occurred at four cycles and levels fell at six cycles (figure 21). The administration route also influenced the plasma insulin titre, particularly in the EE treated mice (figure 21). The smallest stimulatory effect on the insulin response to glucose was associated with the Sc route, however, the route associated with the greatest response varied in the two groups. Im administration of EE and oral administration of E_2 produced the highest plasma insulin elevations. Both steroids also altered the plasma insulin pattern observed in the diestrus control mice (see figure 16 for comparison).

Estrogen administration significantly lowered plasma glucose concentrations in comparison with the control levels. Only at one cycle

during oral EE treatment were levels significantly higher than the diestrus controls (figure 21). Comparison of the two estrogens shows that the natural steroid, E_2, had a greater influence than the synthetic steroid on plasma glucose titres during the ipGTT. Time-dependent alterations of the plasma glucose titre were variable, especially in the E_2 treated mice (figure 20), but there was a tendency for levels to rise towards control levels by the end of the study (figure 20). The Sc route of administration, however, during E_2 and EE treatment, produced a consistent fall in plasma glucose which increased as treatment duration increased (figure 20 and 21). Thus the lowest glucose titres were seen in the Sc estrogen treated animals. In addition, the highest glucose titres were observed in the oral estrogen treated animals.

Comparison of the plasma insulin with the plasma glucose data shows that during EE administration and during Sc E_2 treatment, the glucose titre altered according to changes of insulin concentration ie. rises of the plasma insulin titre produced reductions of the plasma glucose titre (see figure 21). This association was not always obvious during E_2 treatment where insulin elevations were not always accompanied by decreases in plasma glucose levels (figure 20). Thus impairment of the hypoglycaemic action of insulin was indicated after Im and oral administration of E_2.

(iii) ipGTT - PROGESTOGEN TREATMENT

The effect of progestogen administration on the ipGT and plasma insulin 'curves' are illustrated in figures 22 -27. These data show that the plasma insulin response to a glucose challenge was affected by progestogen treatment, but significant elevations above diestrus levels were less frequent. The synthetic progestogens, N and NA, were slower

in eliciting an elevation than P (figures 25 - 27). Comparison of the effect of the three progestogens shows that the natural steroid, P, exerted a greater stimulatory influence on the insulin titre than the synthetic progestogens. Indeed, significant decreases were observed during N and NA treatment in comparison with diestrus control levels (figures 26 and 27). Furthermore, of the two synthetic steroids, NA had the lesser effect on elevating the plasma insulin response to glucose (figure 27). The time-dependent alterations of the insulin response were similar in all three groups. The plasma insulin titre was often lower than the controls at one cycle and significant elevations were most frequently seen at six cycles. Thus insulin levels increased as the duration of treatment increased (see figure 25). Likewise, the administration route-induced alterations of the insulin response to glucose were identical. The oral route of administration was associated with the highest insulin concentrations and the Sc route with the lowest levels in all three groups. In addition, progestogen treatment altered the plasma insulin pattern produced in the diestrus controls (see figure 16 for comparison).

Considerable variations of plasma glucose during the ipGTT were observed throughout progestogen administration. In comparison with the diestrus control levels, progestogen treatment produced both elevations (figure 26) and decreases (figure 27) of the plasma glucose titre. A comparison of the three progestogens shows that synthetic steroids produced both the highest (N) and the lowest (NA) plasma glucose concentrations. The synthetic progestogens produced similar time-dependent changes of the glucose titre, and a tendency for glucose levels to rise during treatment was observed. Nevertheless, plasma glucose titres following NA administration were only significantly elevated above the controls in oral administration (figure 27). The situation also occurred during oral P use (figure 25).

The other two administration routes, however, produced the opposite effect with plasma glucose levels falling as treatment duration was increased. Administration route-induced alterations of glucose during the ipGTT were similar in P and NA treated mice. The Im route was associated with the lowest, and the oral route with the highest plasma glucose titres. N administration, however, had the opposite effect (figure 26). In addition, Im administration of P and NA had a consistent stabilising influence on the plasma glucose concentration.

Comparison of the plasma insulin with the plasma glucose levels shows that during Sc and Im P treatment and oral N treatment the glucose titre altered as a result of changes in plasma insulin ie. rises of insulin produced corresponding reductions of plasma glucose (figure 25). However, at all times during NA administration; Sc and Im N administration; and oral P administration elevations of plasma insulin were not necessarily accompanied by reductions of the plasma glucose titre. Thus impairment of the hypoglycaemic action of insulin was indicated especially during synthetic progestogen treatment.

(iv) COMPARISON OF ESTROGEN AND PROGESTOGEN TREATMENT

From a comparison of the estrogen and progestogen plasma insulin and ipGTT data some trends were apparent. During steroid treatment, it was the natural steroid that produced the greatest stimulation of the plasma insulin response to a glucose challenge. P, however, was less effective in this respect than E_2. Indeed following progestogen administration, insulin levels were often significantly lower than diestrus control levels, particularly at one cycle. The effect of both estrogens and progestogens on the plasma insulin response was potentiated as treatment duration increased. That is, insulin levels

increased as treatment length increased. In addition, steroid treatment altered the insulin pattern seen in the diestrus controls during the ipGTT. Some similarities were noted with regard to the administration route-induced alterations of plasma insulin. Generally the oral route produced the highest and the Sc route of administration the lowest insulin titres.

Fewer consistent trends could be drawn from a comparison of the glucose data. Whereas during estrogen therapy the natural hormone produced the lower plasma glucose levels, during progestogen treatment NA lowered the glucose titre to the greatest extent. Significant elevations of glucose above the control levels were produced more frequently following progestogen treatment than following estrogen administration. The data also suggested that elevations of plasma glucose with increased treatment duration occurred more frequently during synthetic than during natural steroid administration. This situation was apparent with both estrogens and progestogens. Some similarities between the steroid treated animals with respect to administration route-induced alterations of plasma glucose were apparent. Generally the oral route was associated with the highest and the Sc route of administration with the lowest plasma glucose titres.

Comparison of the plasma insulin with the plasma glucose data in the estrogen and progestogen treated animals suggested that on occasions the hypoglycaemic action of insulin was reduced, particularly following Im and oral steroid administration. It was also evident that after Sc steroid treatment the opposite situation developed. That is, the hypoglycaemic action of insulin was potentiated.

(v) ipGTT - COMBINED PREPARATION TREATMENT

This section is concerned with the effect of administering a combined preparation containing both an estrogen and a progestogen on ipGTTs and plasma insulin titres. The results are shown in figures 28 - 33. Throughout the study these preparations produced few elevations of the plasma insulin response to a glucose challenge. The natural preparation containing E_2 and P was slower in eliciting these elevations than either of the two synthetic combinations. Furthermore, the synthetic combinations had a greater influence on the insulin response than the natural preparation. Following E_2 and P treatment, plasma insulin titres were frequently significantly lower than the control levels (figure 31). The greatest insulin response was produced by EE and N treatment (figure 32). In all three groups the steroid-induced elevations of plasma insulin were potentiated with time. Thus the plasma insulin titre increased as treatment duration increased. However, during EE and N, and EE and NA administration a transient fall often occurred at four cycles (figures 32 and 33). The administration route used to give the combinations also influenced the plasma insulin concentration. In all three treatment regimes the oral route was associated with the highest plasma insulin and the Sc route of administration with the lowest plasma insulin titres. The results also show that the plasma insulin pattern produced by E_2 and P treatment was similar to that seen in the diestrus mice (see figures 16 and 28 for comparison). Whereas that produced by the synthetic combinations was similar to the patterns noted in the previous sections.

Combined steroid treatment significantly decreased the plasma glucose titre in comparison with the diestrus levels. Significant elevations were seen on only two occasions, both during E_2 and P administration (figure 31). Consistent with the plasma insulin alterations, the natural preparation was slower in reducing the plasma

glucose titre than the synthetic combinations. The E_2 and P treated mice had the highest glucose concentrations of the three groups. Of the two synthetic regimes, EE and NA treatment produced the lower plasma glucose titres (figure 33). The time-dependent alterations of glucose were similar in the three groups. The fall in plasma glucose increased with the duration of treatment. However, in the Im and oral EE and NA treated mice there was a tendency for glucose levels to rise towards the end of the study (figure 33). Administration route-induced alterations of the plasma glucose response during the ipGTT occurred in all three regimes, but the results were variable. For example, the greatest and most consistent reductions of glucose were seen in the Im administration route during E_2 and P, and EE and N treatment. Following EE and NA use, however, the Sc route was associated with the lowest levels. Likewise, the highest plasma glucose concentrations were observed following oral EE and N, and EE and NA treatment. In contrast E_2 and P treatment produced the highest glucose levels when given by the Sc administration route.

Comparison of the plasma insulin with the plasma glucose data during combined treatment, in all administration routes, shows that the glucose titre altered with respect to changes in the plasma insulin titre. That is, glucose levels decreased in response to increases of plasma insulin. These results indicate that the hypoglycaemic action of insulin was not affected by combined steroid treatment.

(vi) COMPARISON OF SINGLE AND COMBINED TREATMENTS

This section compares the effects of an estrogen and a progestogen administered alone on ipGTT and plasma insulin with those produced when the two are administered together.

A comparison of the natural steroids is shown in figure 34.

This comparison shows that the elevated insulin response to a glucose challenge produced by both E_2 and P was absent in the combined preparation containing these two steroids. This may be interpreted as antagonism between the two hormones in this preparation which reduces the stimulatory influence on the plasma insulin response. There were similarities between the three regimes. For example, plasma insulin levels rose as treatment duration increased. In addition, the greatest stimulation of the insulin response was observed during oral treatment whilst the lowest insulin titres were produced in the Sc administration route. The beneficial influence of E_2 and the detrimental influence of P on plasma glucose titres were both modified during combined administration (figure 34). This modification produced intermediate glucose levels which remained lower than the diestrus levels.

In previous sections it was shown that at various times the hypoglycaemic action of insulin was unaffected by E_2 and P combined treatment, but reduced by E_2 and P when administered alone. It can be deduced that combined E_2 and P treatment improves glucose tolerance to a greater degree than either hormone administered alone. This is probably because the hormonal environment was closer to the normal one. Combined E_2 and P treatment had a reduced or absent stimulatory influence on plasma insulin levels whilst significantly lowering plasma glucose levels. Although E_2 and P to a lesser extent controlled glucose levels, this was achieved in a high circulating plasma insulin environment. Thus combined E_2 and P treatment represents a smaller diabetogenic influence than either hormone administered alone. This finding is particularly important in patients with a compromised carbohydrate metabolism or those with the potential for developing carbohydrate metabolic abnormalities.

A comparison of the EE and N data is shown in figure 35. These results show that the estrogen treated animals exhibited the highest

plasma insulin concentrations. The progestogen, N, produced the lowest plasma insulin titres. It appears that these two synthetic steroids interact in the combined preparation to produce an intermediate response. In all three treatment regimes, the lowest plasma insulin titres were observed following Sc administration. As noted previously, in the combined preparation the steroids interact to produce intermediate plasma glucose levels which are lower than diestrus levels. For reasons previously discussed, glucose tolerance was improved to the greatest extent by combined treatment which presented a smaller diabetogenic influence to the animal.

Comparison of the EE and NA results is illustrated in figure 36. The marked stimulation of the insulin response to glucose produced by EE treatment and on occasion by NA administration, was absent or reduced during EE and NA combined treatment. Indeed it appears that the stimulatory influence of EE was greatly reduced or masked by the progestogen component during combined steroid treatment. There were some similarities between the three regimes. For example, the lowest plasma insulin titres were seen after Sc administration. In addition, plasma insulin levels were similar in the EE and combined EE and NA treated mice. However, glucose levels following combined EE and NA treatment were intermediate, as previously seen in the other comparisons. In all three groups there was a tendency for plasma glucose levels to rise as treatment duration increased. Furthermore, over the long term the oral administration route was associated with the highest glucose titres. As discussed earlier, combined EE and NA treatment had the most beneficial influence on glucose tolerance and presented a smaller diabetogenic stress than either steroid administered singularly.

Figure 10

Effect of subcutaneous administratration of estrogens (5ug/kg/day) and progestogens (1mg/kg/day) for 1, 4 and 6 cycles on plasma insulin in 24 hr. fasted mice (mean± SEM for 5 determinations).

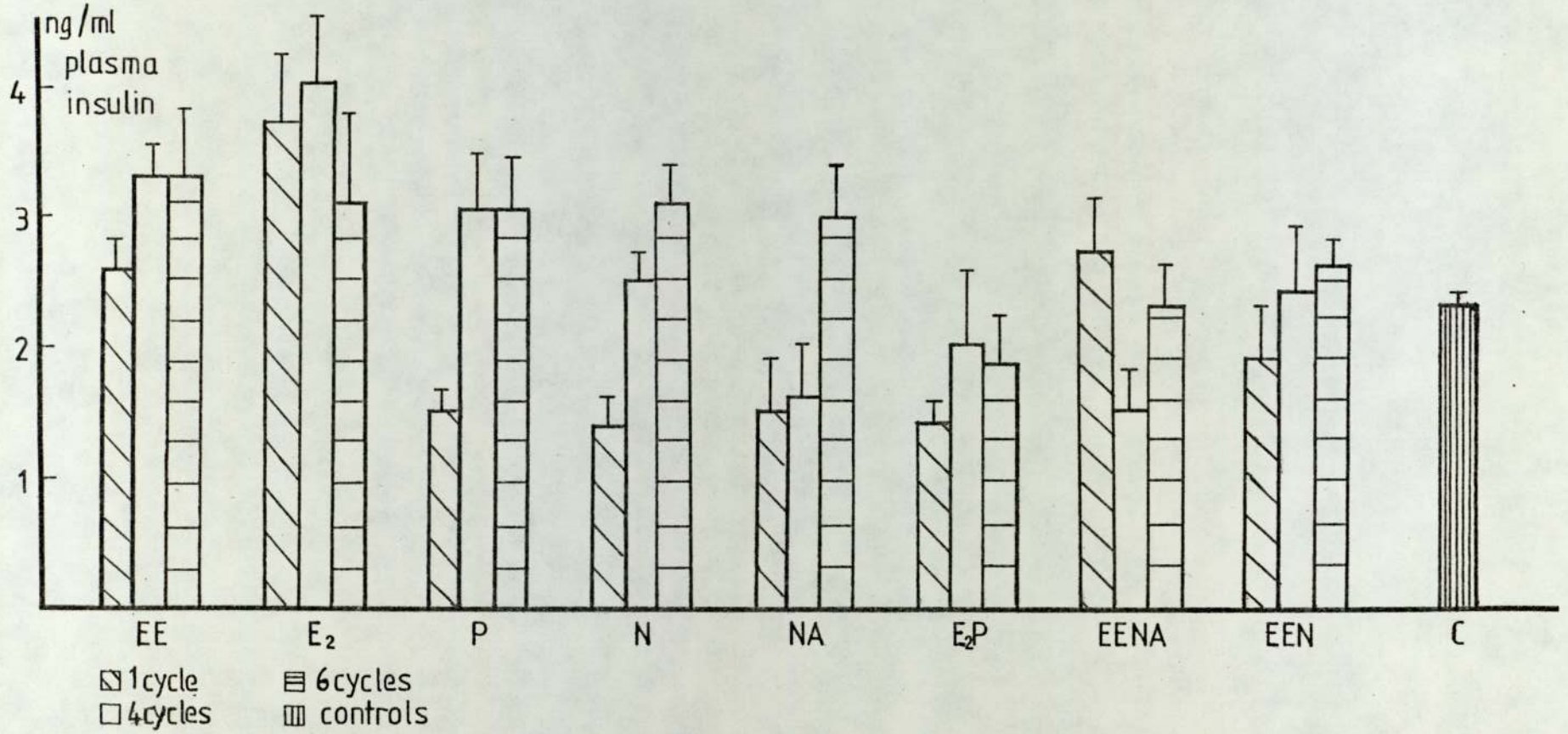


Figure 11

Effect of intramuscular adminstration of estrogens (5ug/kg/day) and progestogens (1mg/kg/day) for 1, 4 and 6 cycles on plasma insulin in 24 hr. fasted mice (mean± SEM for 5 determinations).

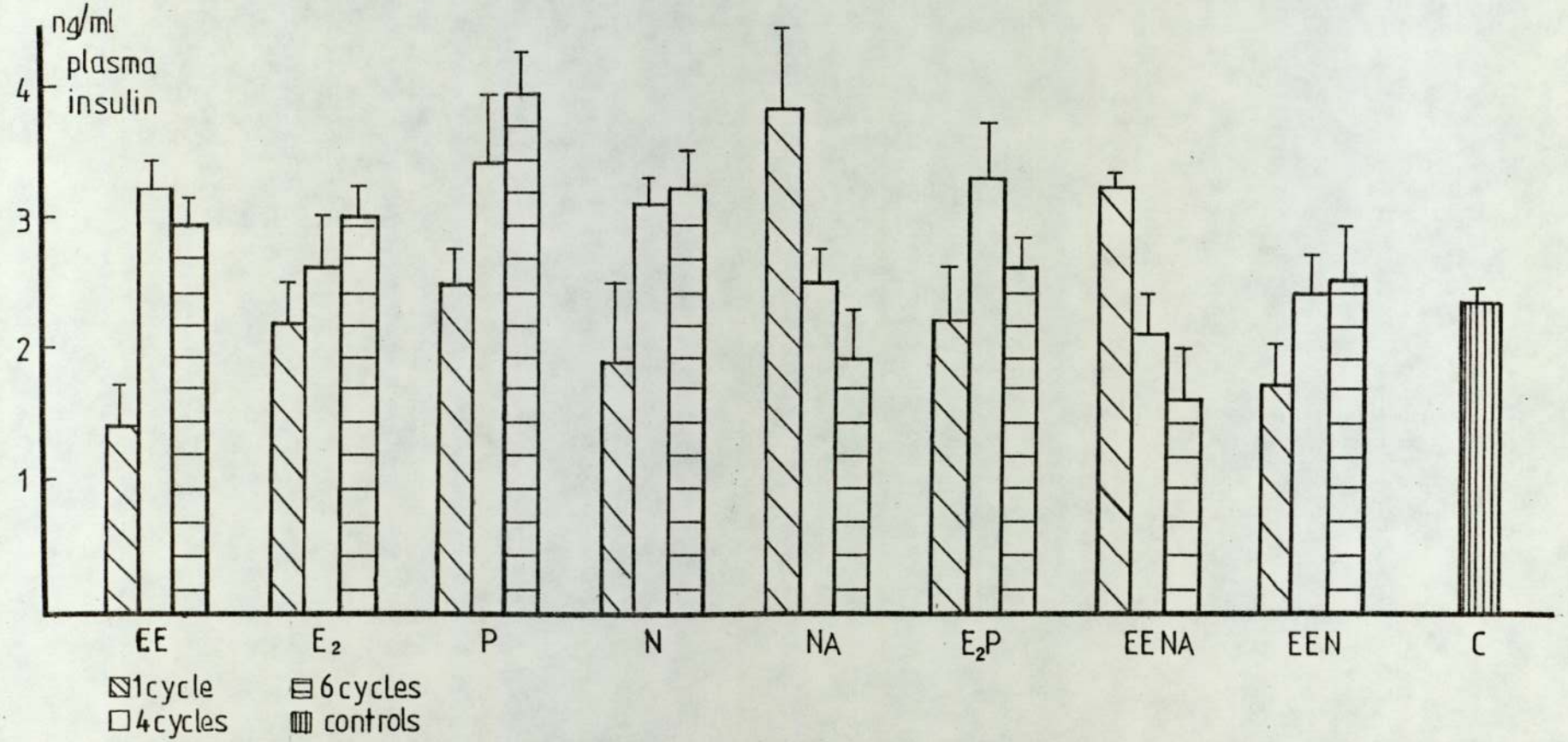


Figure 12

Effect of oral administration of estrogens (5ug/kg/day) and progestogens (1mg/kg/day) for 1, 4 and 6 cycles on plasma insulin in 24hr. fasted mice (mean± SEM for 5 determinations).

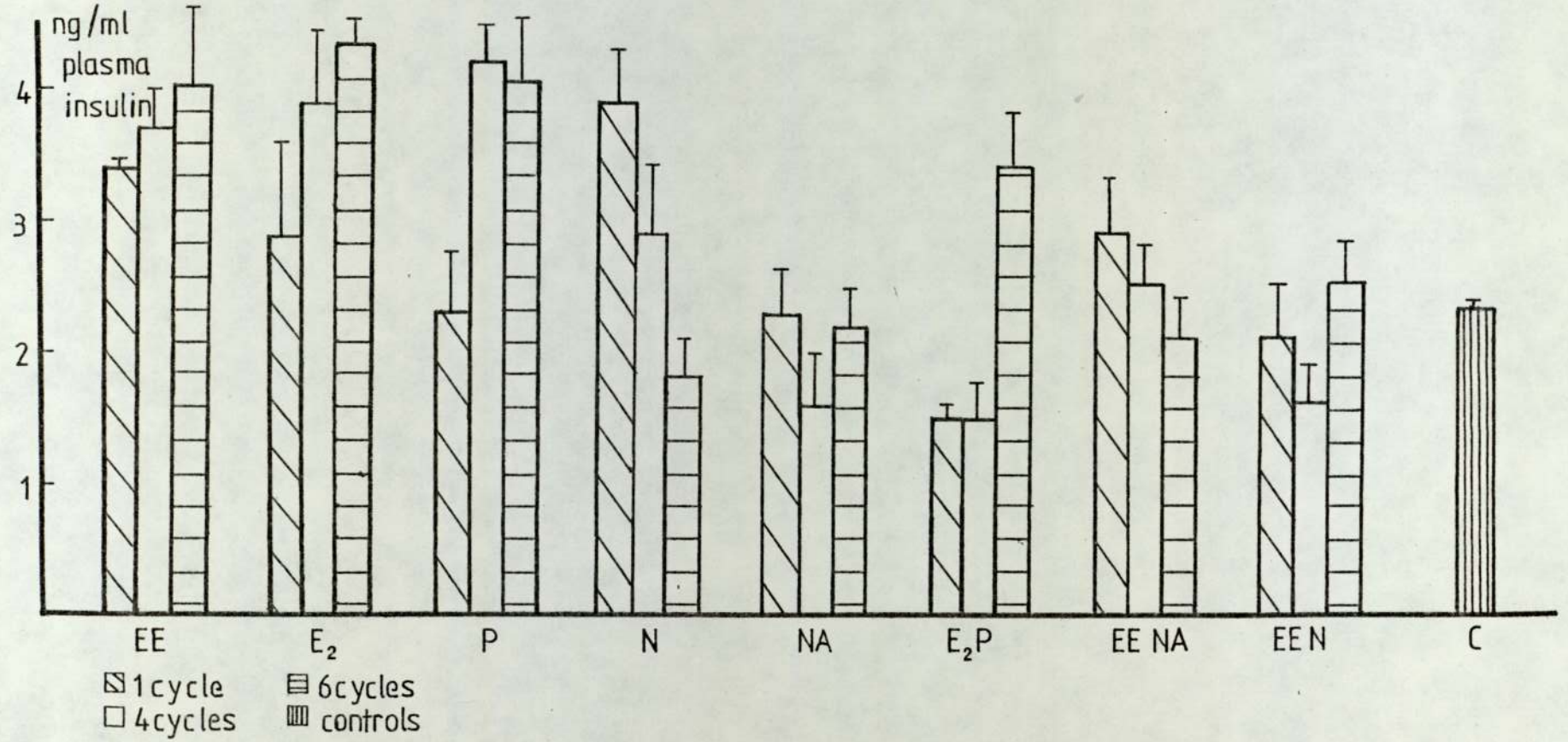


Figure 13

Effect of subcutaneous administratration of estrogens (5ug/kg/day) and progestogens (1mg/kg/day) for 1, 4 and 6 cycles on plasma glucose in 24hr. fasted mice (mean ± SEM for 5 determinations).

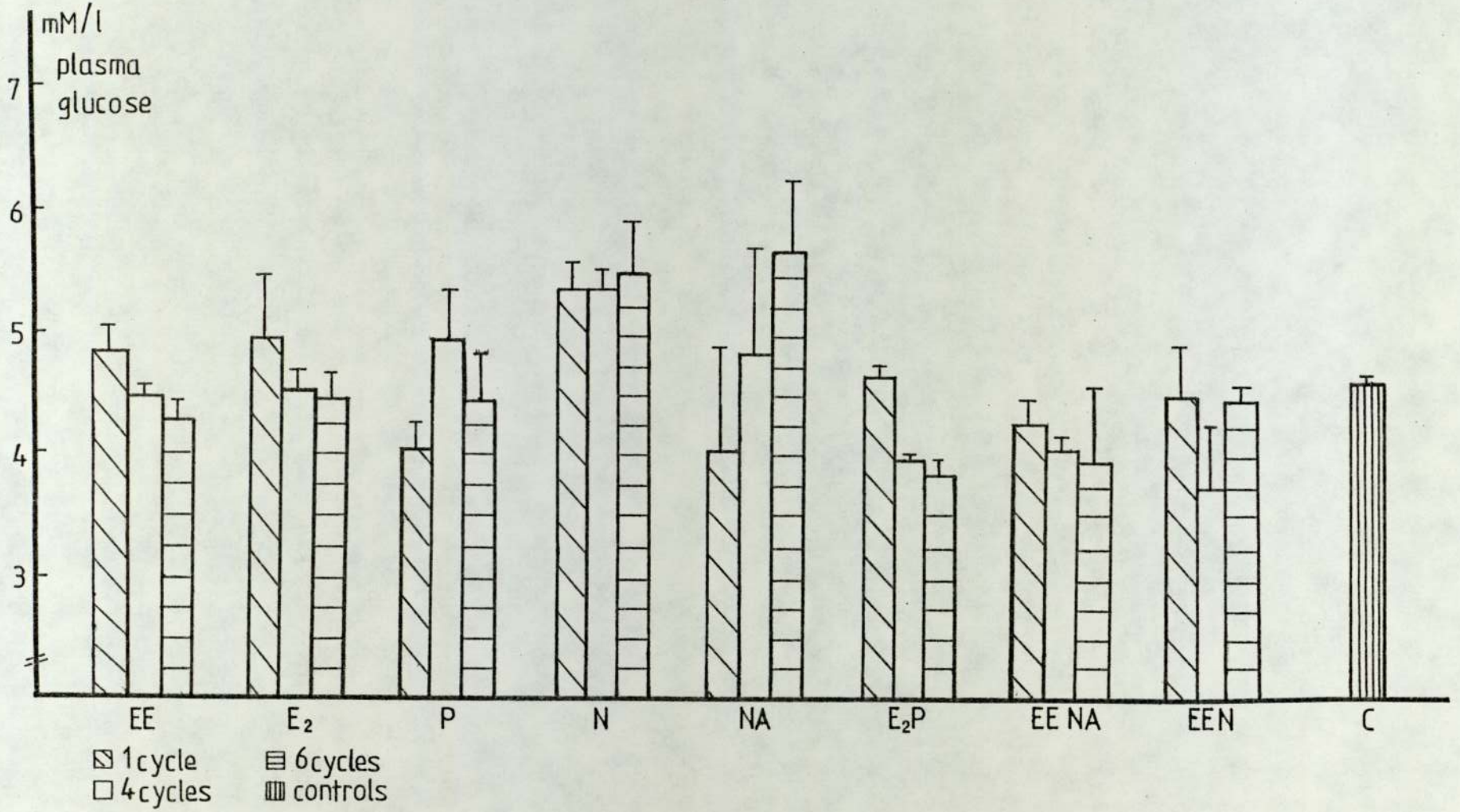


Figure 14

Effect of intramuscular administration of estrogens (5ug/kg/day) and progestogens (1mg/kg/day) for 1, 4 and 6 cycles on plasma glucose in 24 hr. fasted mice (mean ± SEM for 5 determinations).

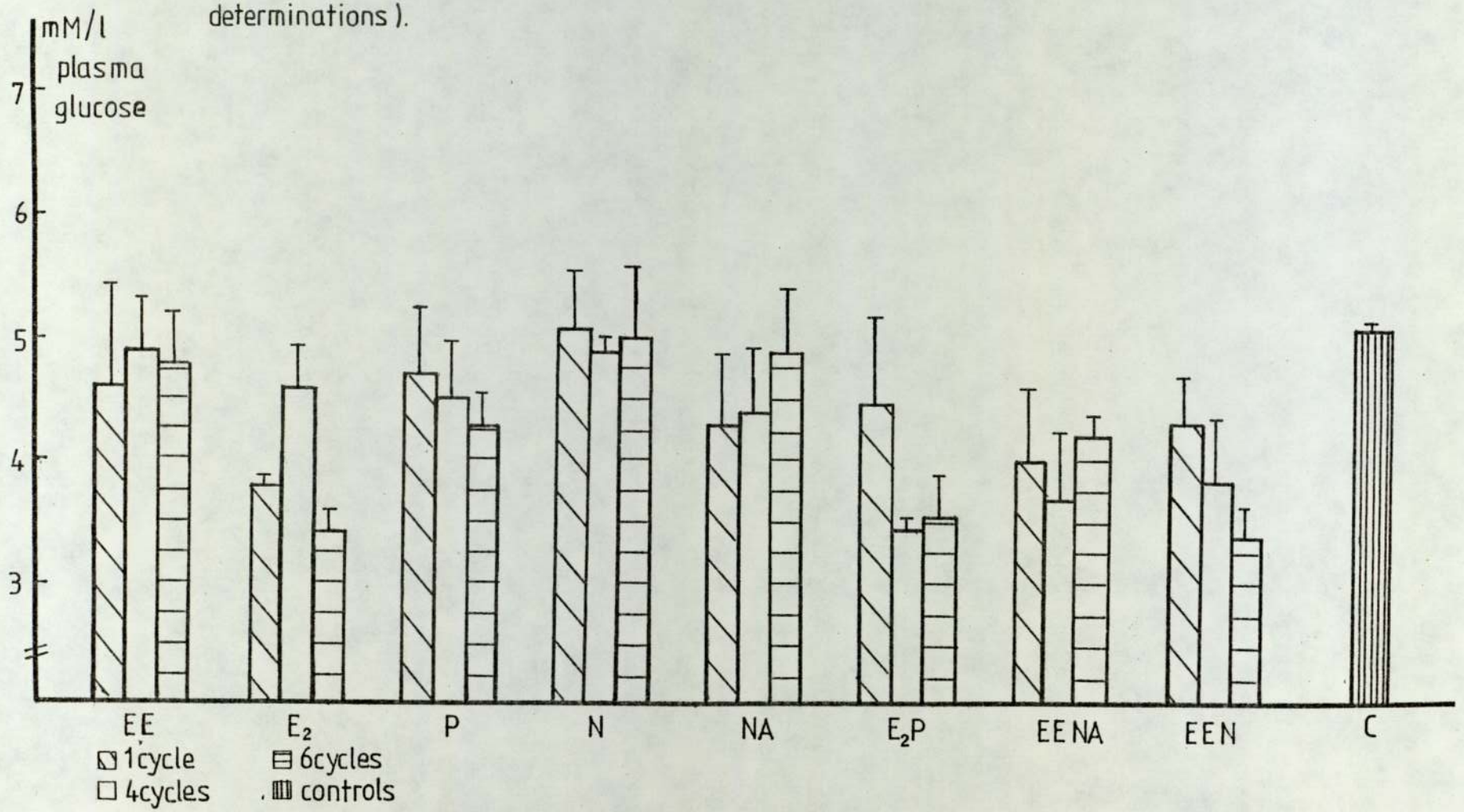


Figure 15

Effect of oral administration of estrogens(5 ug/kg/day) and progestogens (1mg/kg/day) for 1, 4 and 6 cycles on plasma glucose in 24hr. fasted mice (mean ± SEM for 5 determinations).

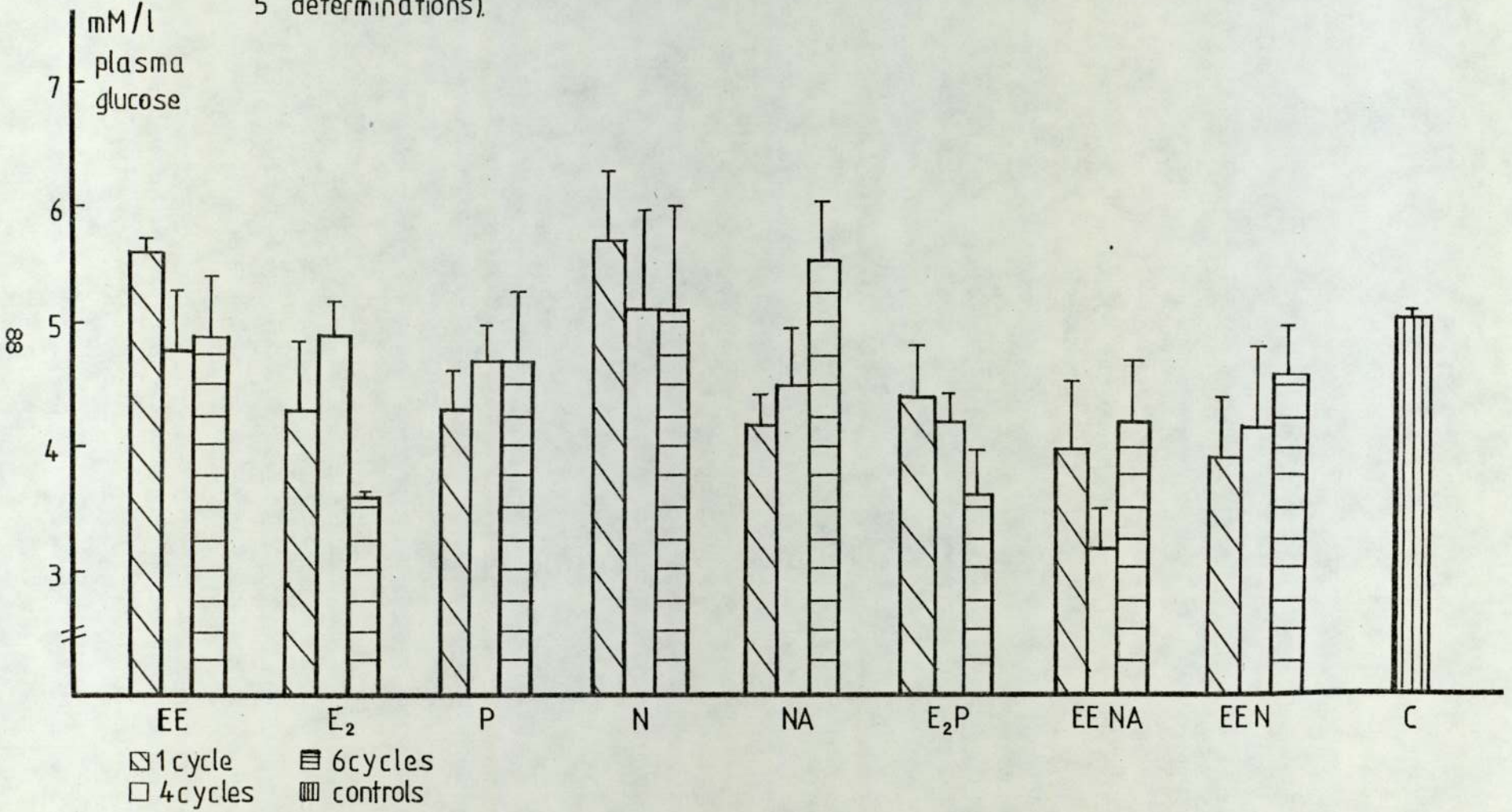
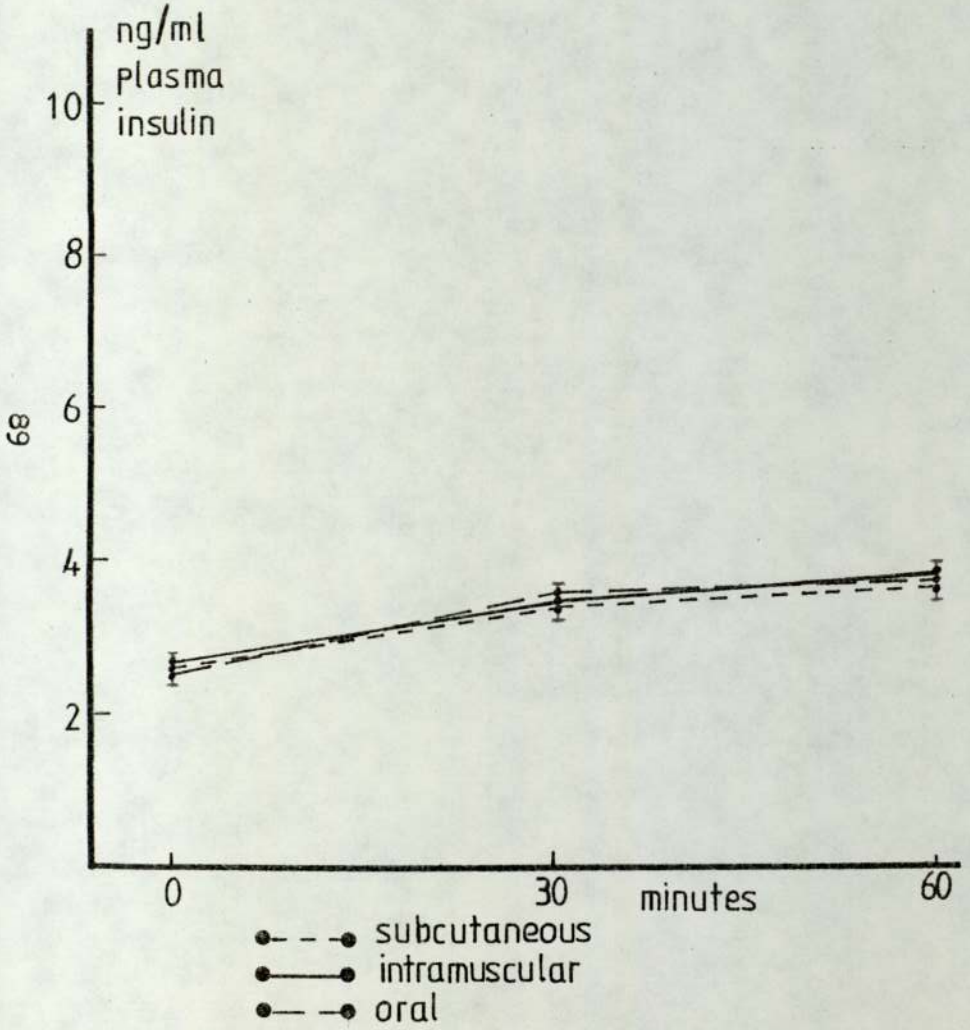
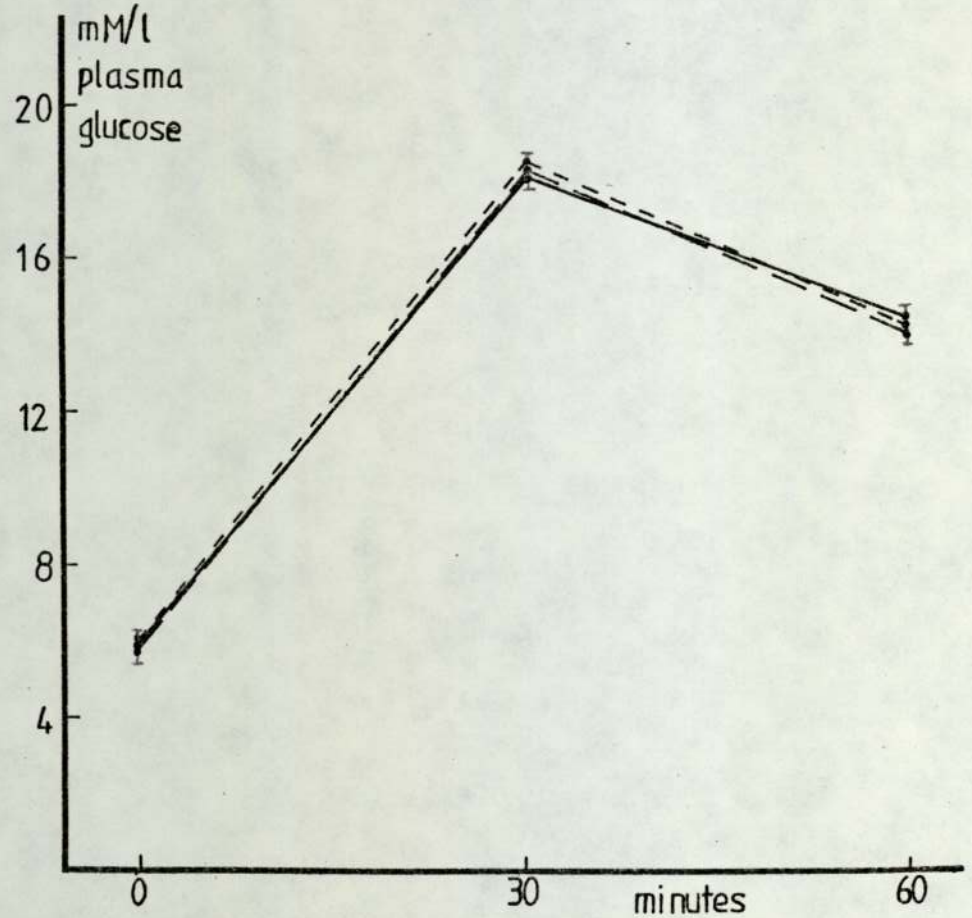


Figure 16

GTT's at 1, 4 and 6 cycles in 24 hr. fasted diestrus controls (mean±SEM for 15 determinations).





Readings were taken before (0) and after (30 and 60) ip. glucose administration.

Figure 17

Areas under the GTT curves at 1, 4 and 6 cycles in 24hr. fasted diestrus controls (mean± SEM for 5 determinations).

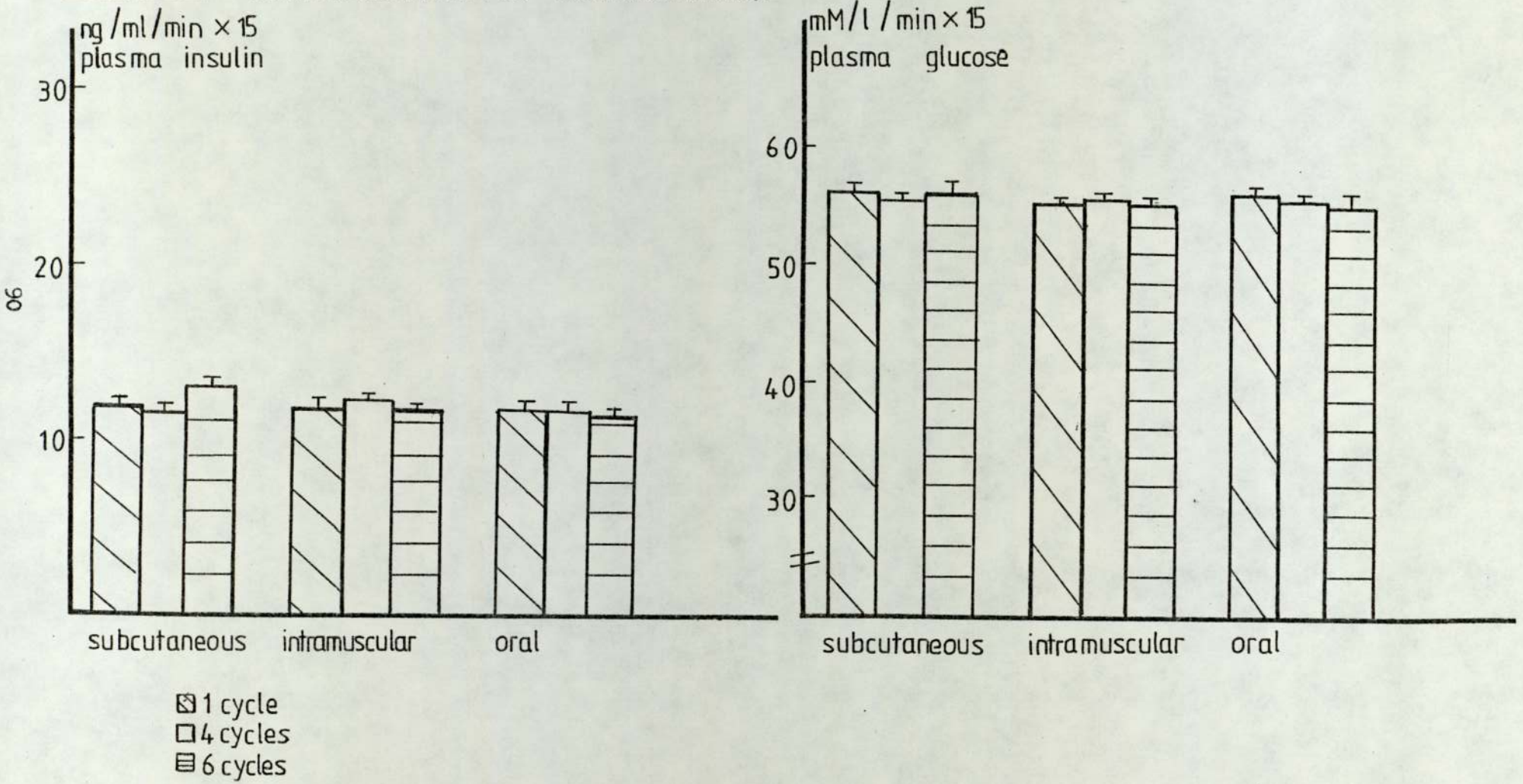
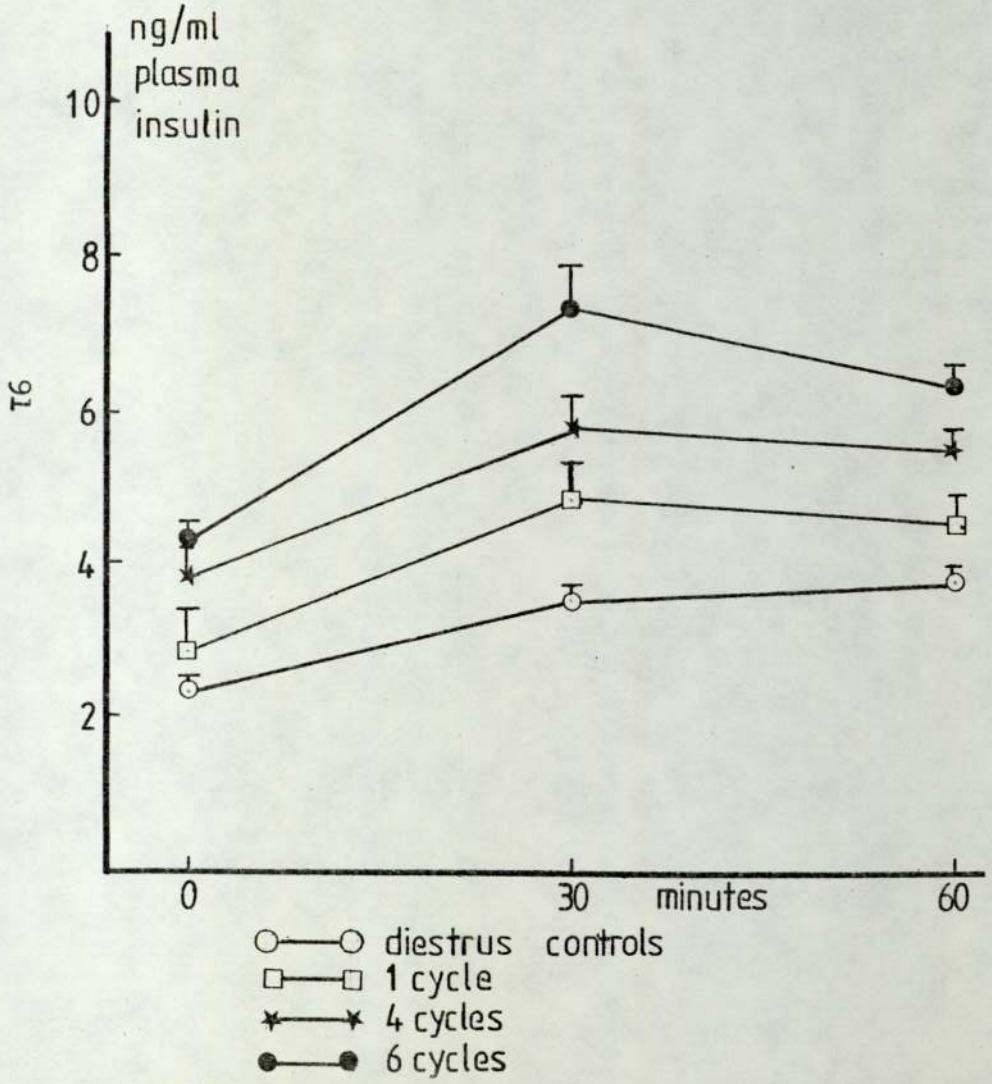
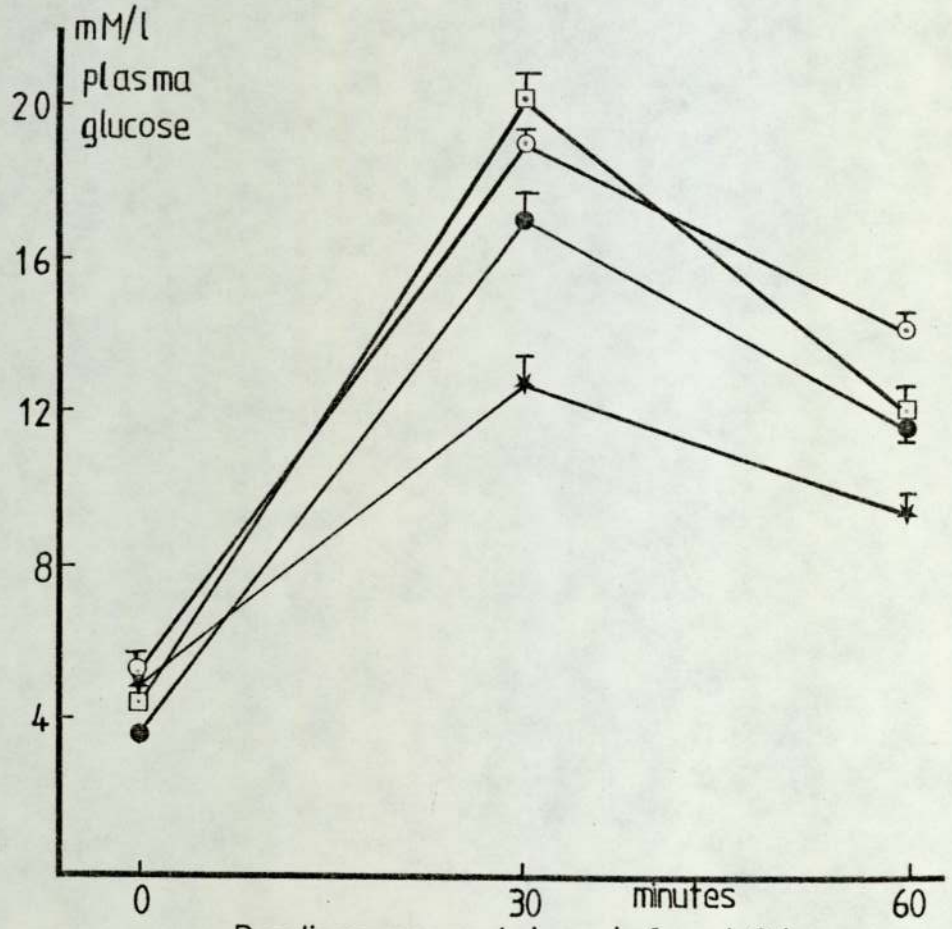


Figure 18 Effect of oral estradiol (5ug/kg/day) for 1,4 and 6 cycles on GTT in 24 hr. fasted mice (mean± SEM for 5 determinations).

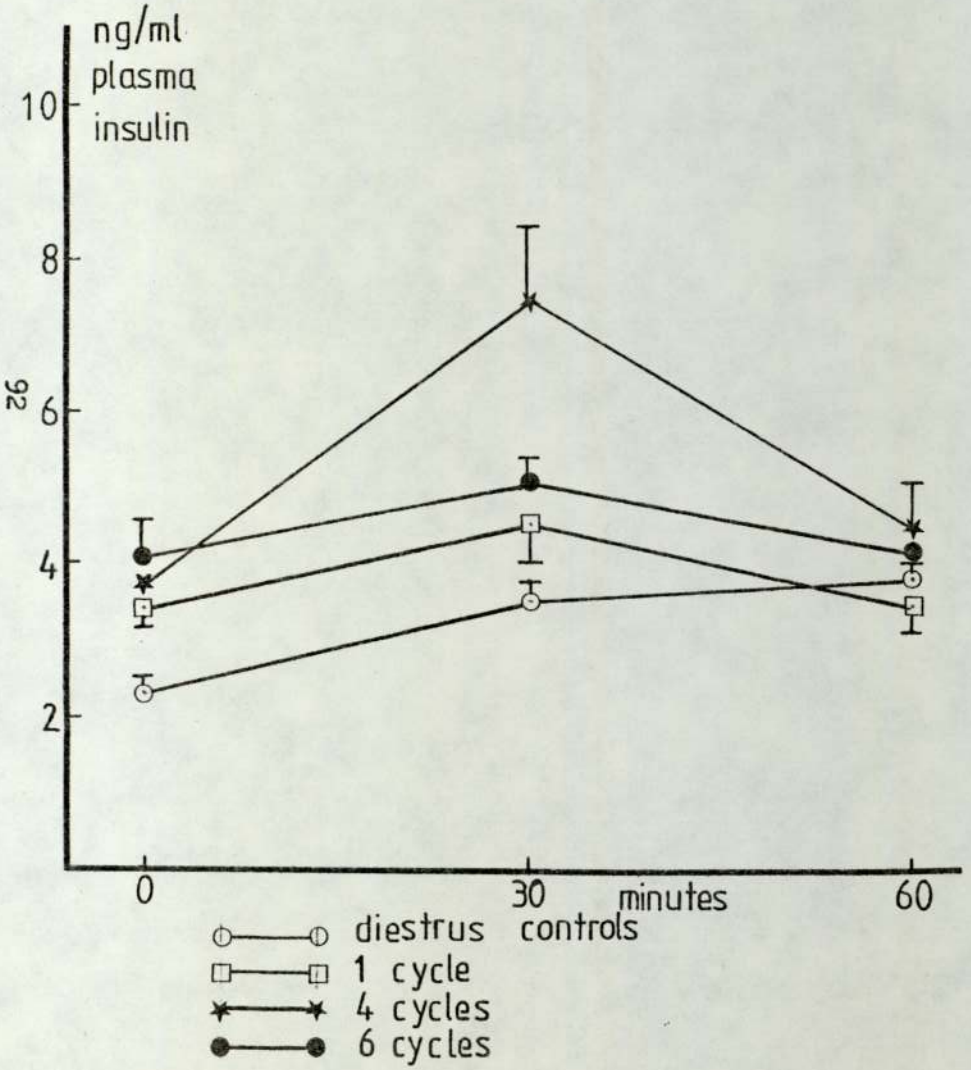


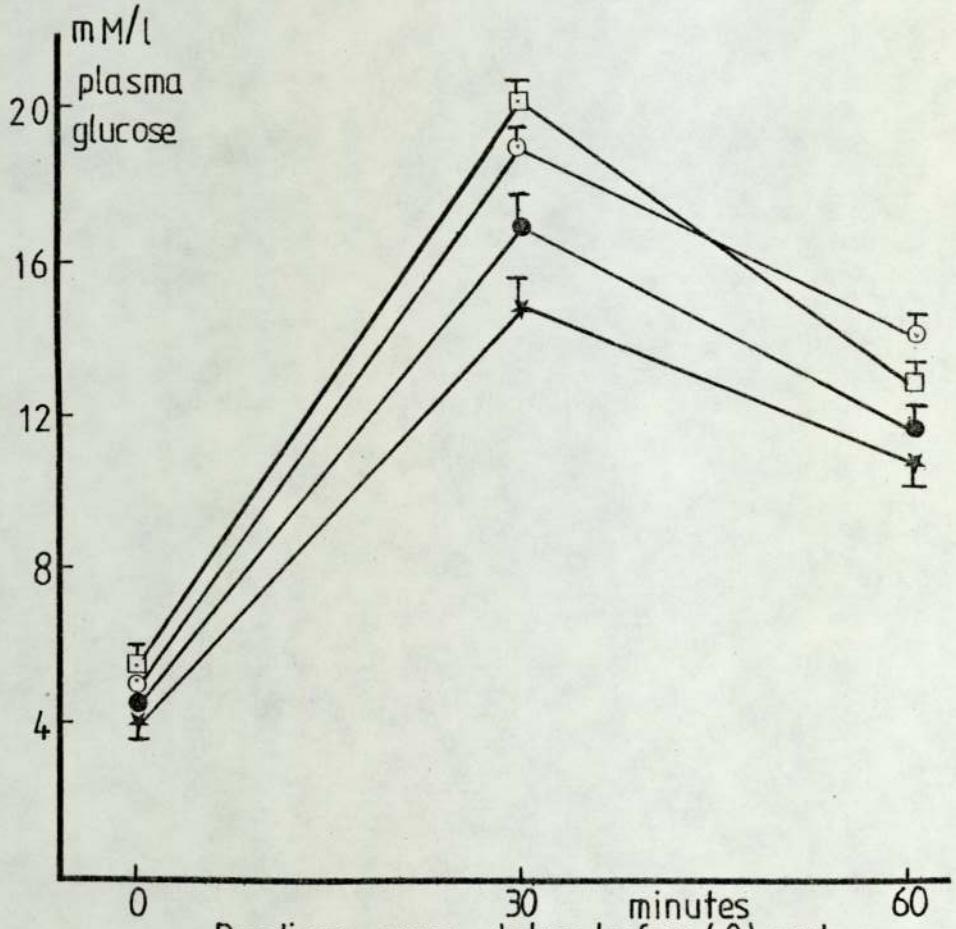


Readings were taken before (0) and after (30 and 60) ip. glucose administration.

Figure 19

Effect of oral ethynyl estradiol (5ug/kg/day) for 1, 4 and 6 cycles on GTT in 24hr. fasted mice (mean± SEM for 5 determinations).





Readings were taken before (0) and after (30 and 60 mins.) ip. glucose administration.

Figure 20

Areas under the GTT curves at 1, 4 and 6 cycles during estradiol treatment (5ug/kg/day) in 24hr. fasted mice (mean ± SEM for 5 determinations).

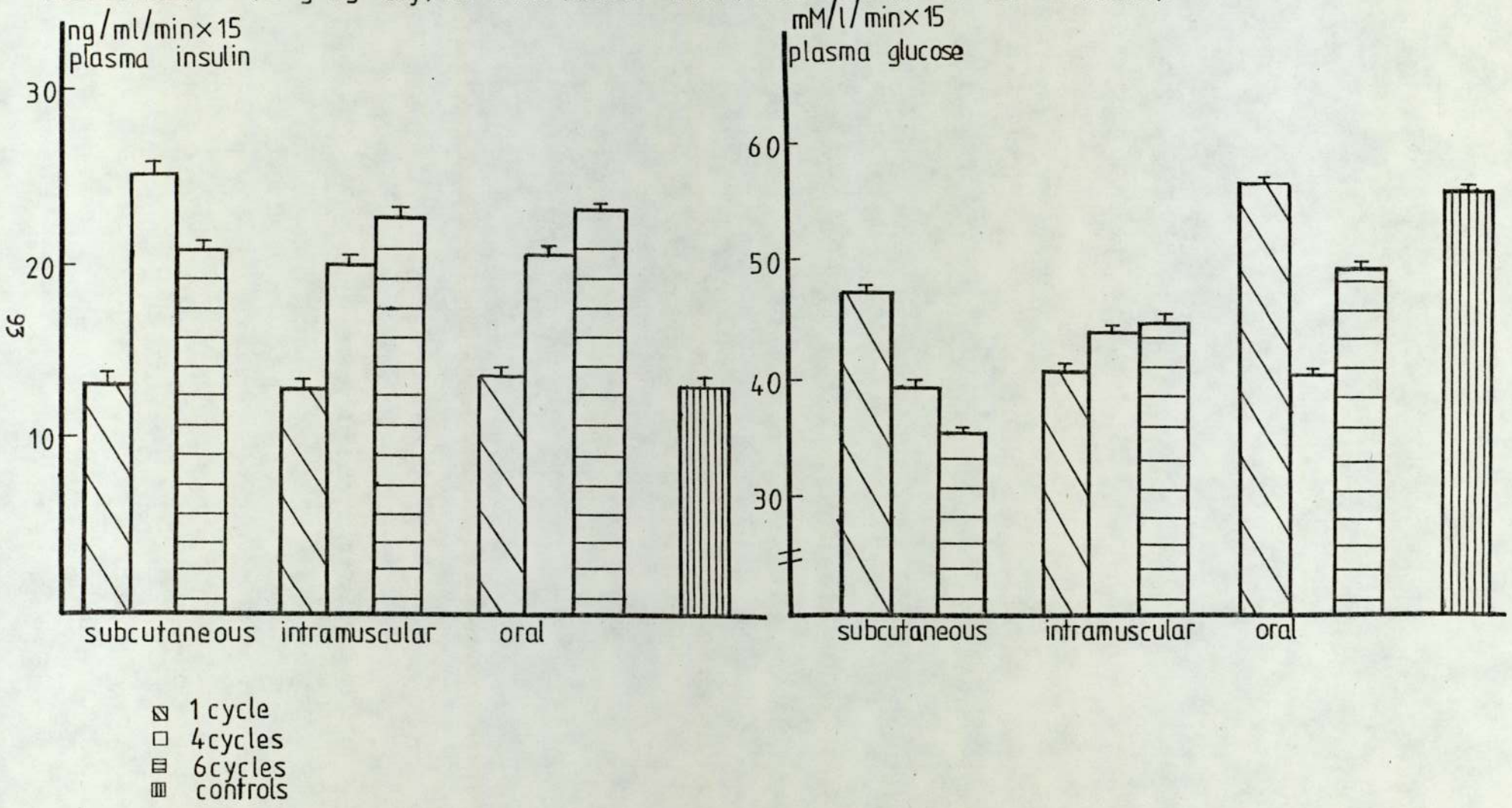


Figure 21

Areas under the GTT curves at 1, 4 and 6 cycles during ethynyl estradiol treatment (5ug/kg/day) in 24hr. fasted mice (mean± SEM for 5 determinations).

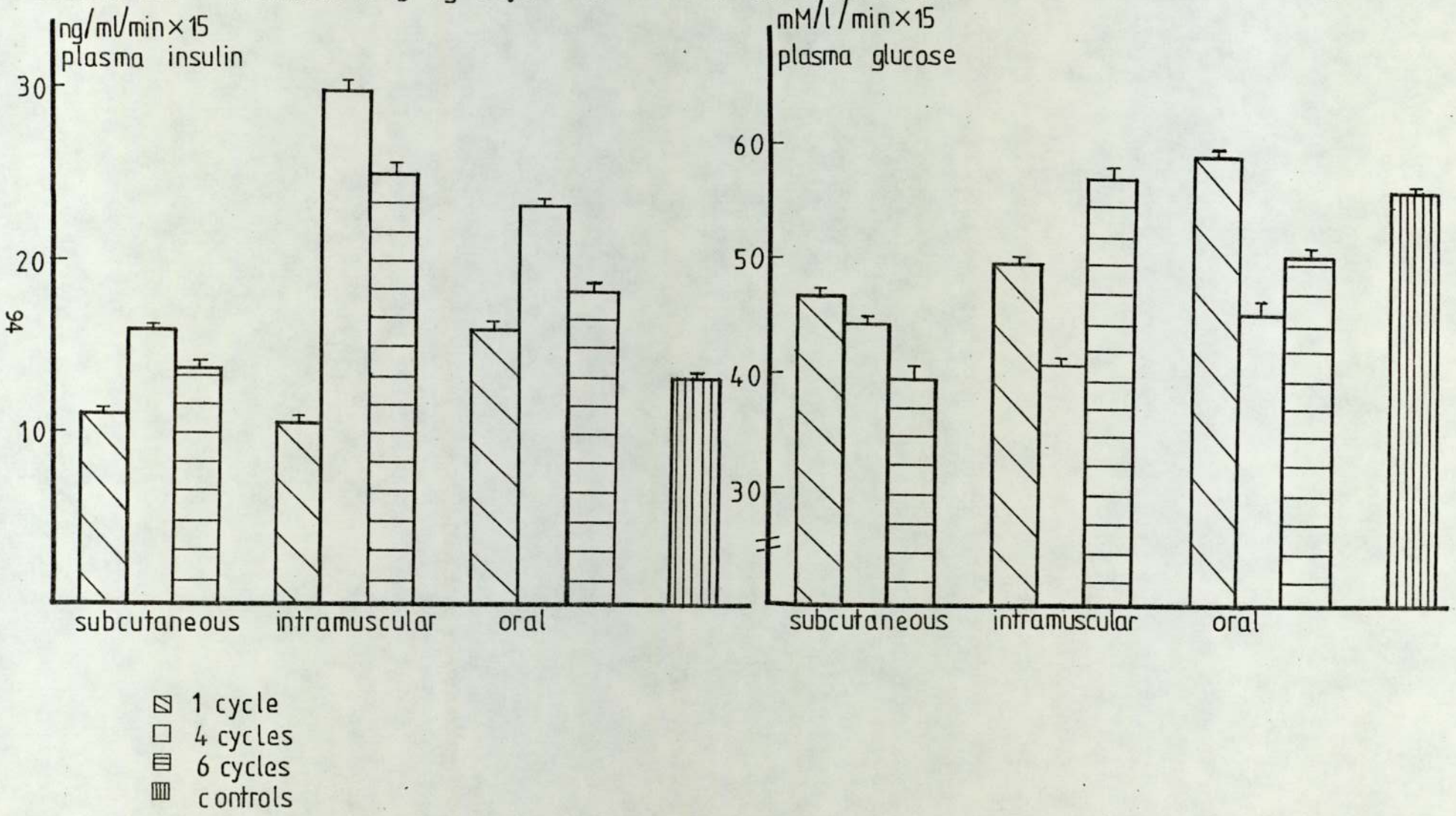
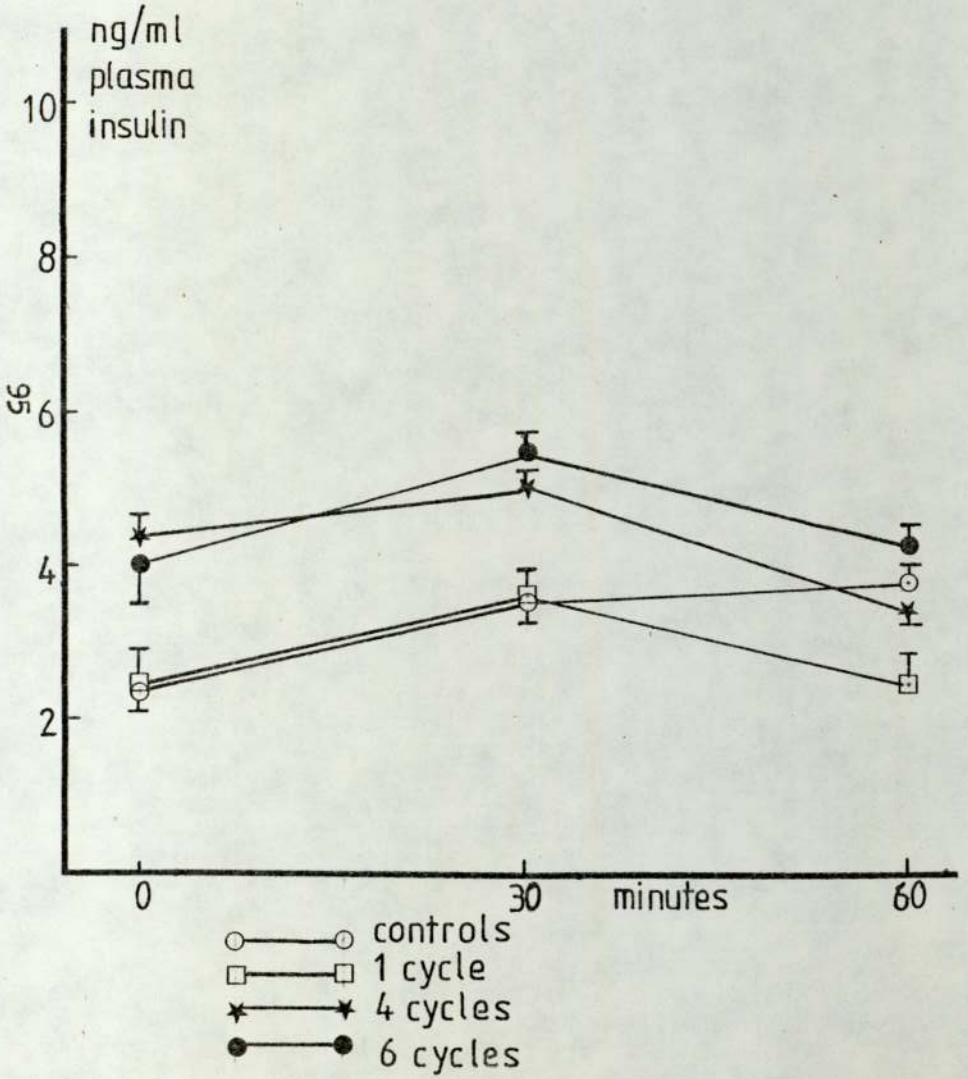
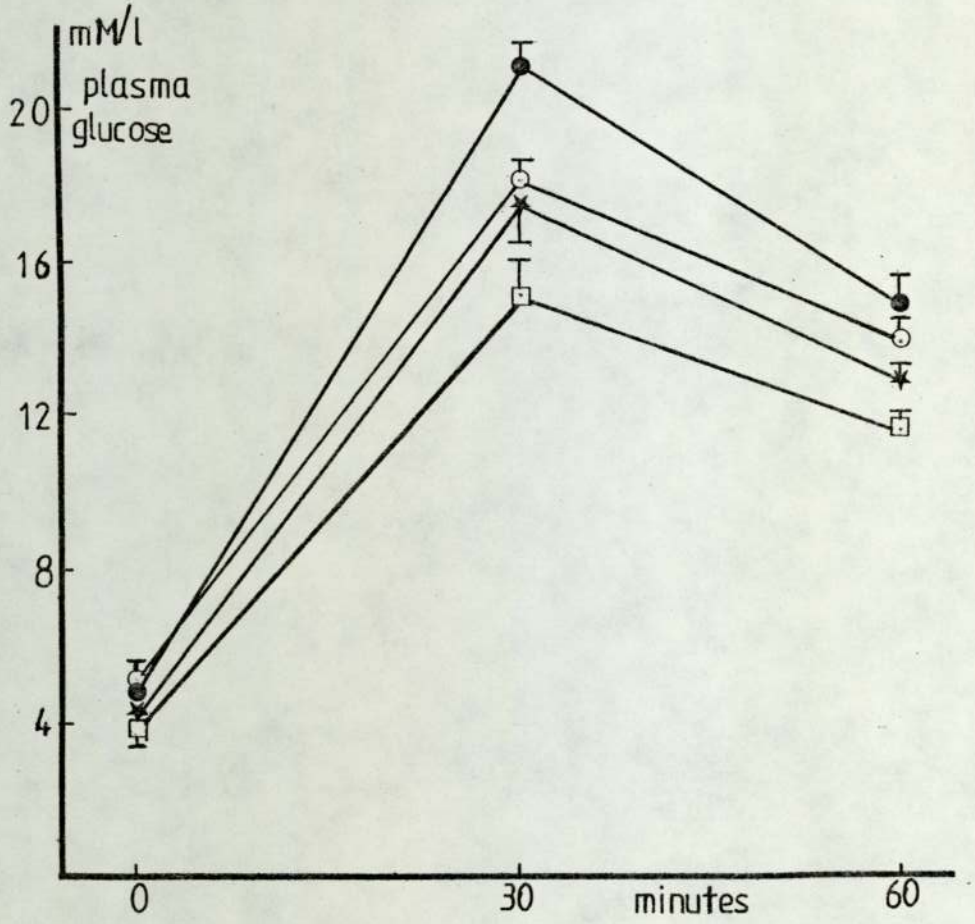


Figure 22

Effect of oral progesterone (1mg/kg/day) for 1, 4 and 6 cycles on GTT in 24 hr. fasted mice (mean ± SEM for 5 determinations).

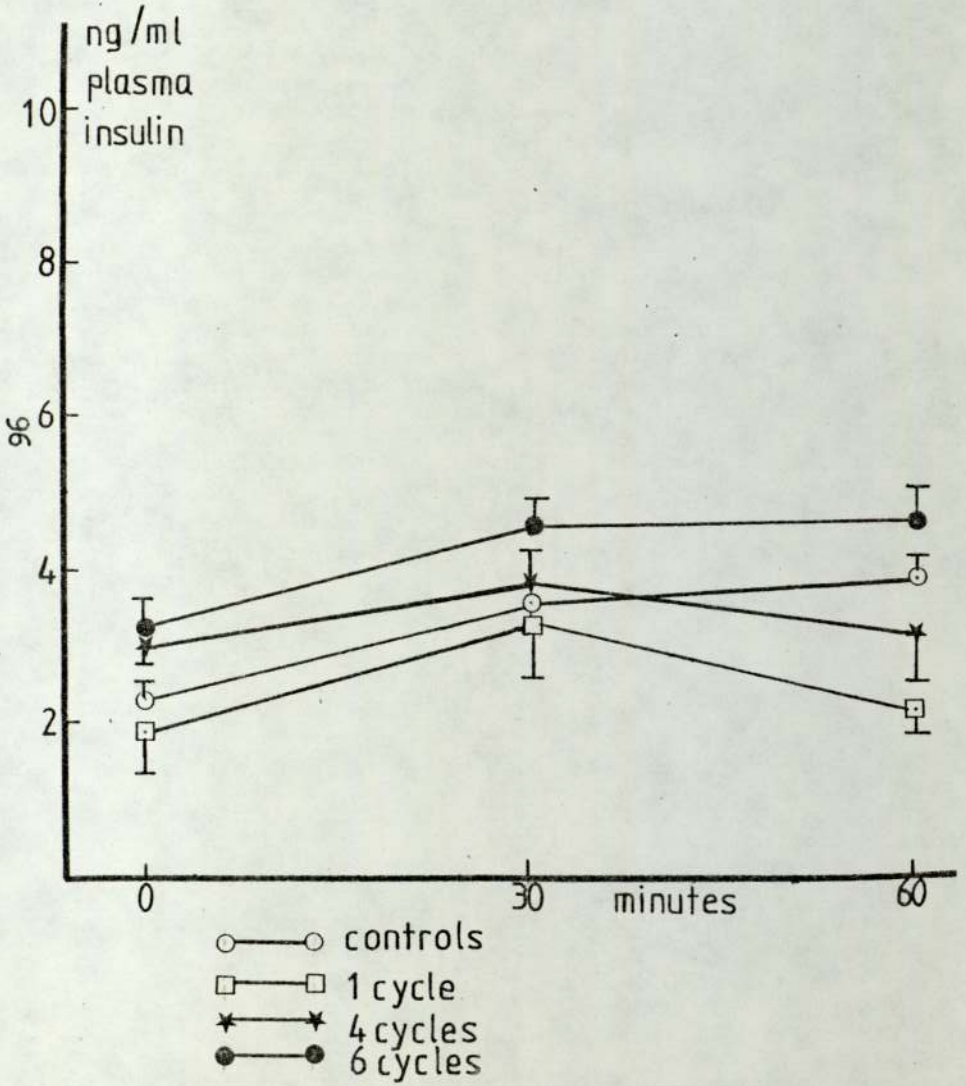


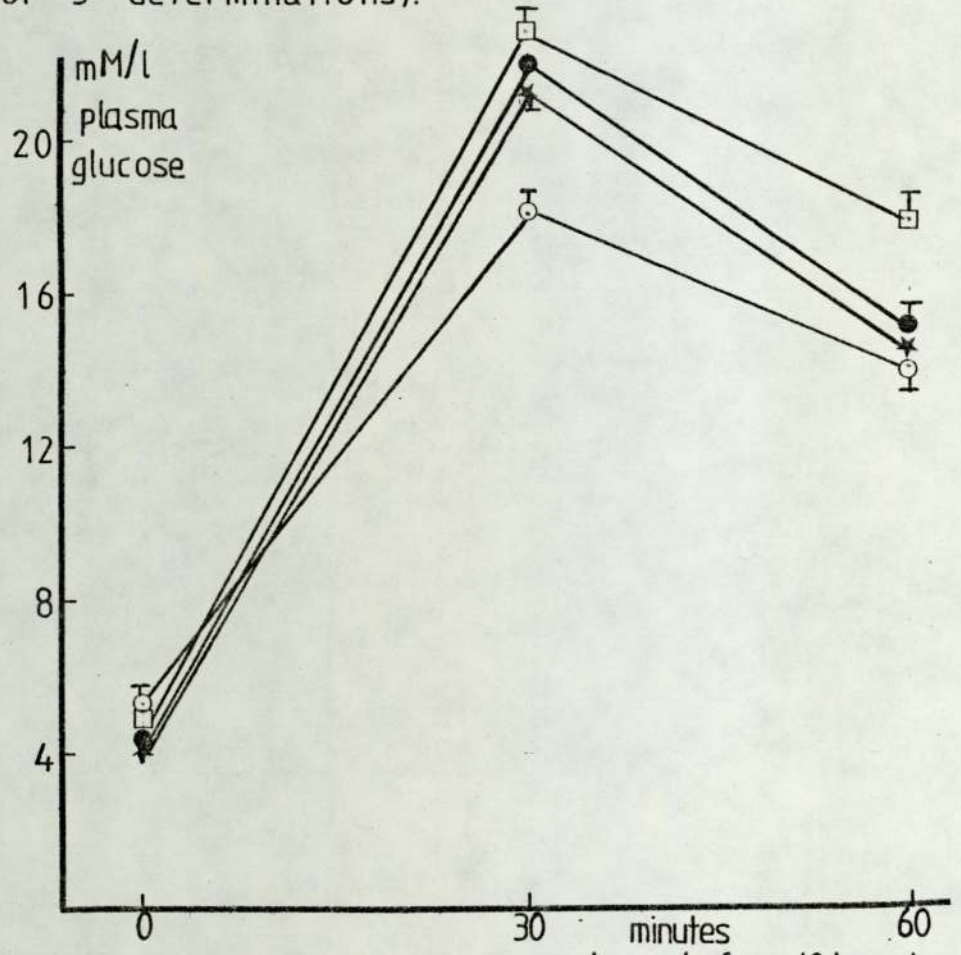


Readings were taken before (0) and 30 and 60 mins. after ip. glucose administration.

Figure 23

Effect of intramuscular norethnodrel (1mg/kg/day) for 1, 4 and 6 cycles on GTT in 24hr. fasted mice (mean ± SEM for 5 determinations).

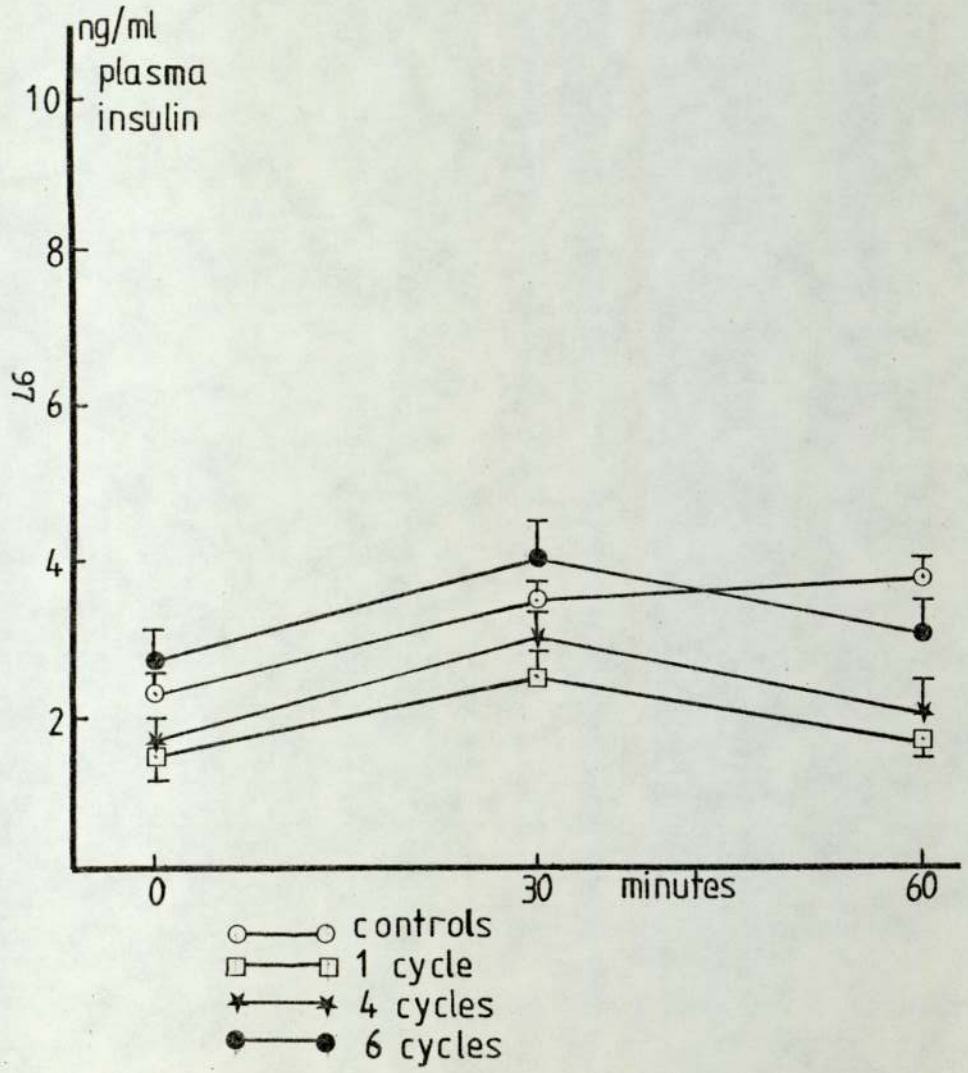


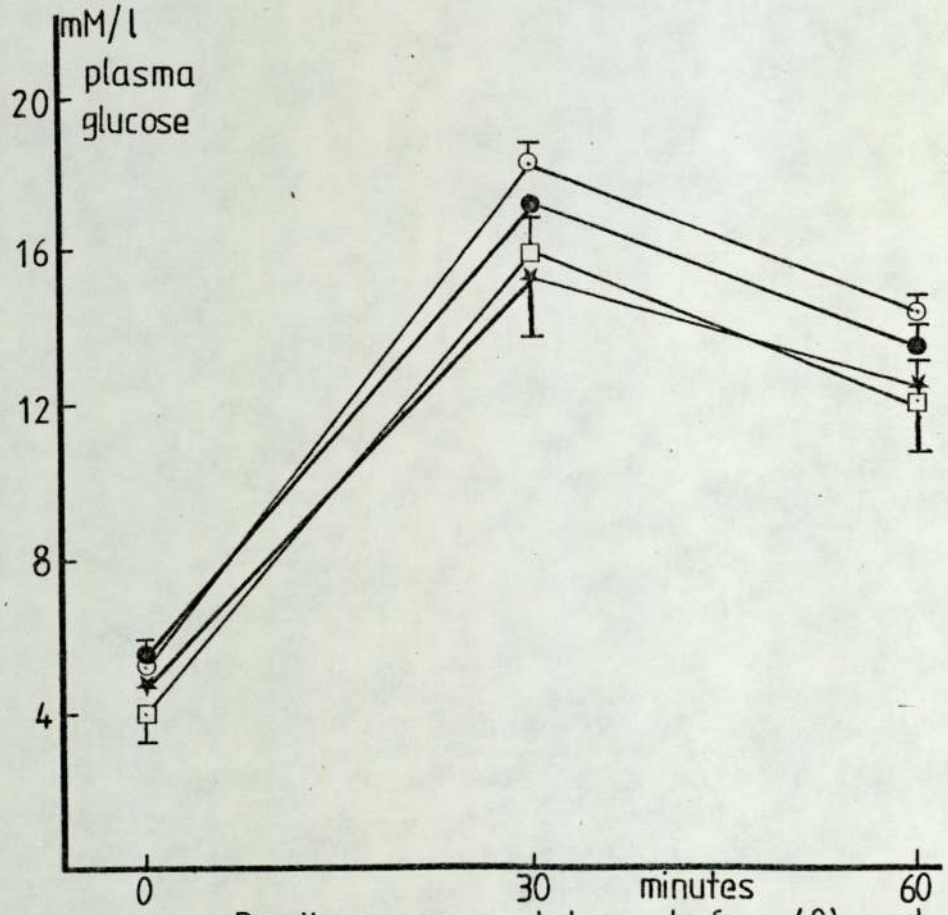


Readings were taken before (0) and 30 and 60 mins. after ip. glucose administration.

Figure 24

Effect of subcutaneous norethisterone acetate (1mg/kg/day) for 1, 4 and 6 cycles on GTT in 24hr. fasted mice (mean± SEM for 5 determinations).





Readings were taken before (0) and 30 and 60 mins. after ip. glucose administration.

Figure 25 Areas under the GTT curves at 1, 4 and 6 cycles during progesterone treatment (1mg/kg/day) in 24hr. fasted mice (mean ± SEM for 5 determinations).

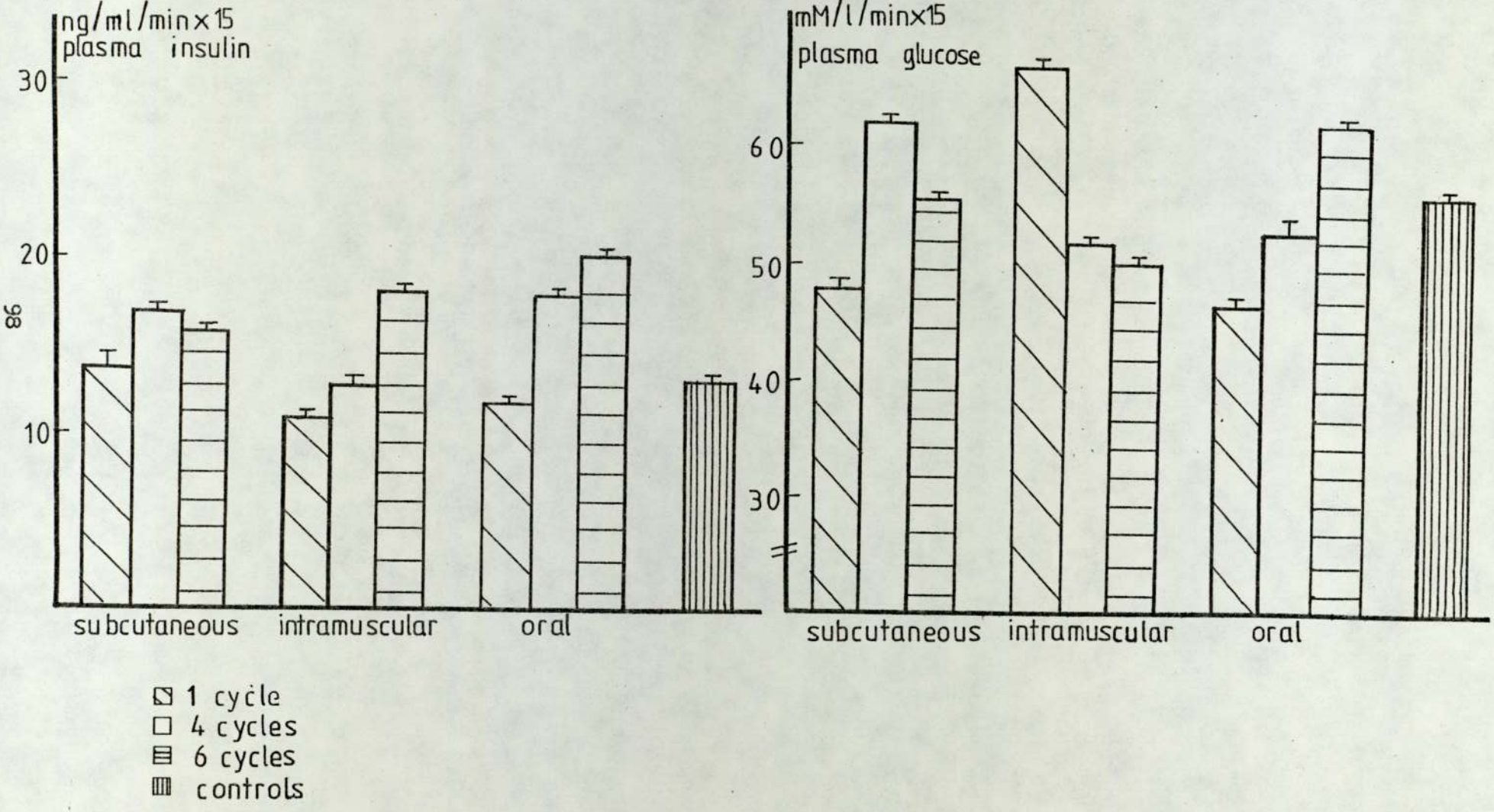


Figure 26

Areas under the GTT curves at 1,4 and 6 cycles during norethnodrel treatment (1mg/kg/day) in 24hr. fasted mice (mean± SEM for 5 determinations).

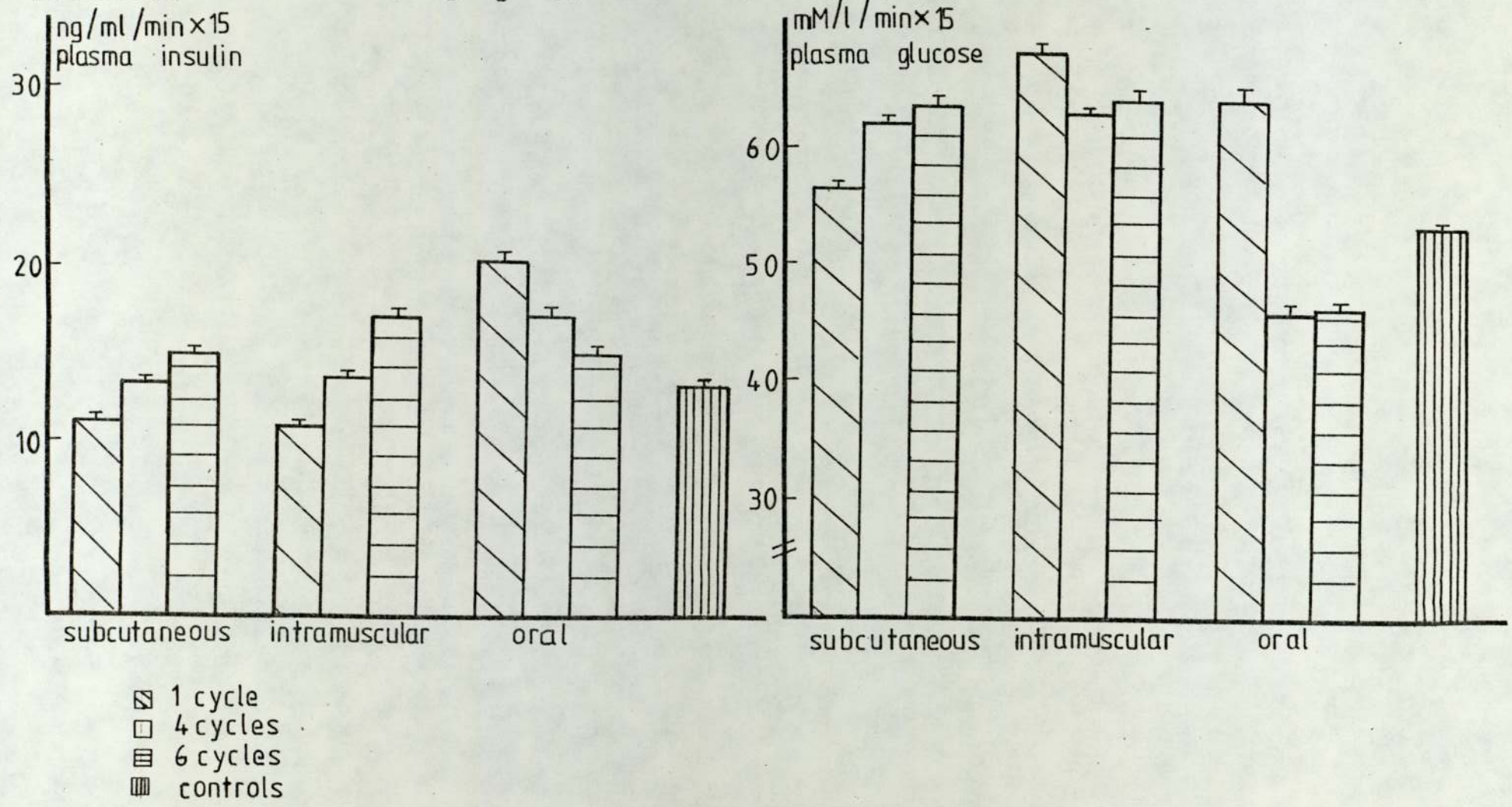


Figure 27

Areas under the GTT curves at 1, 4 and 6 cycles during norethisterone acetate treatment (1mg/kg/day) in 24hr. fasted mice (mean± SEM for 5 determinations).

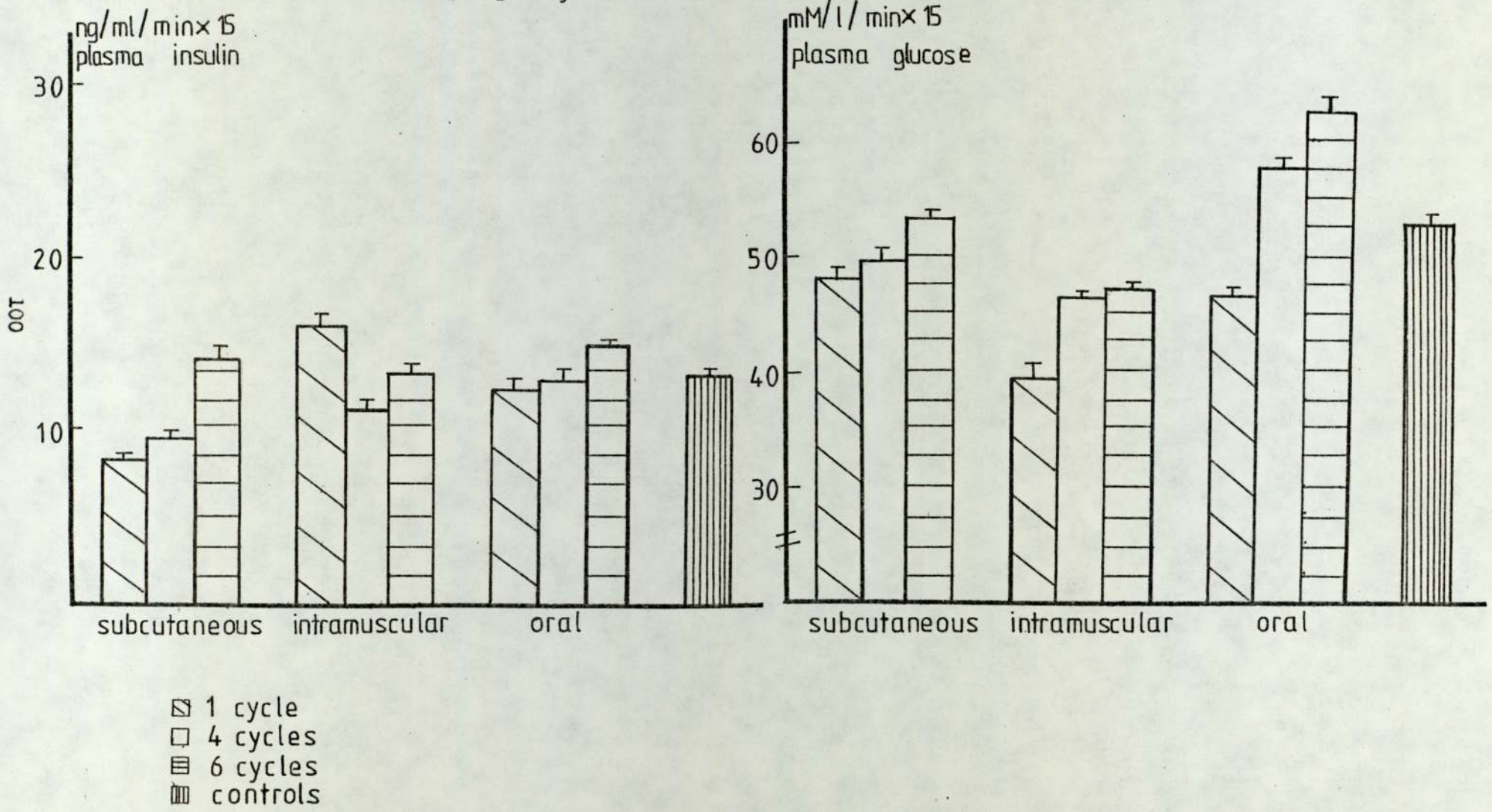
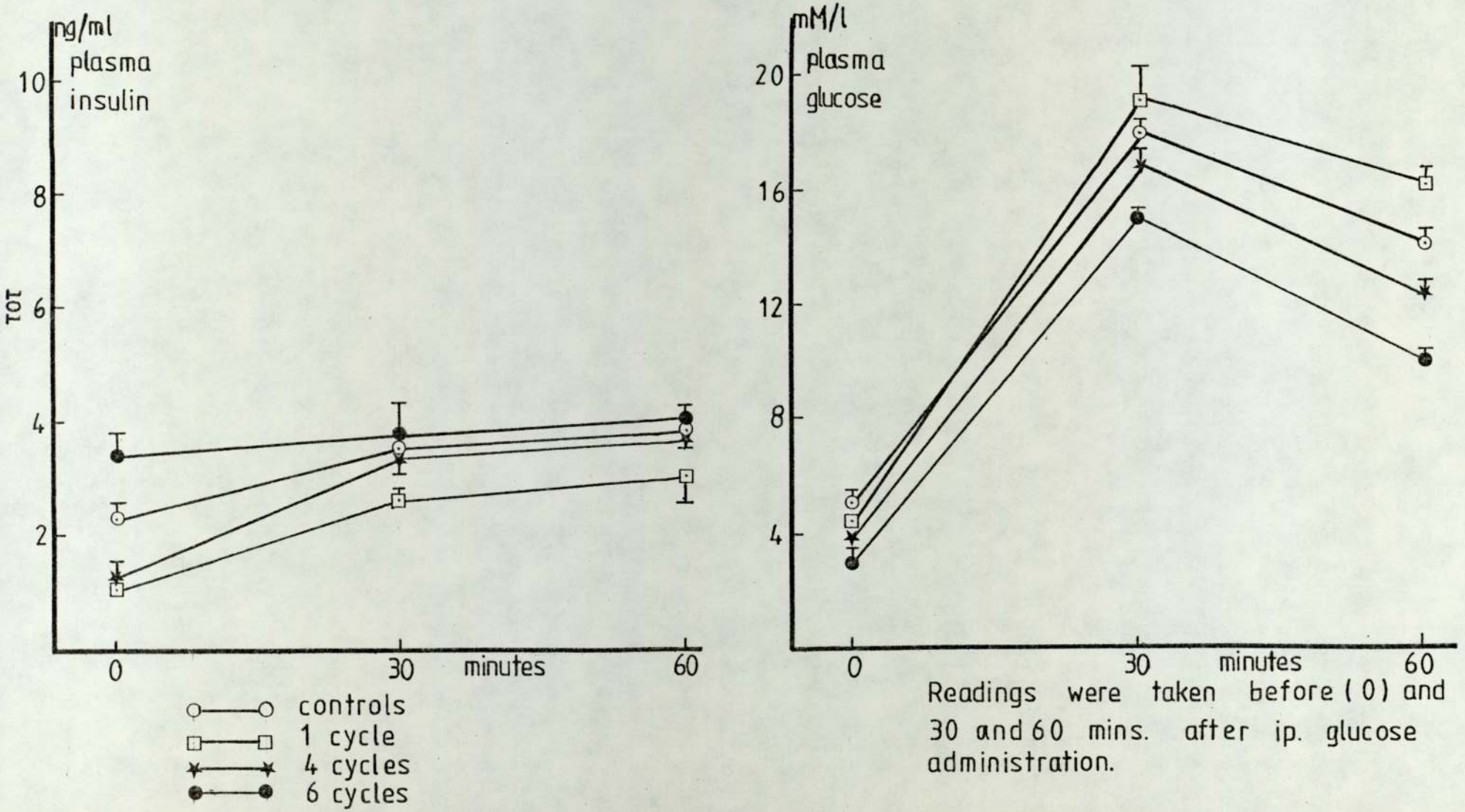


Figure 28

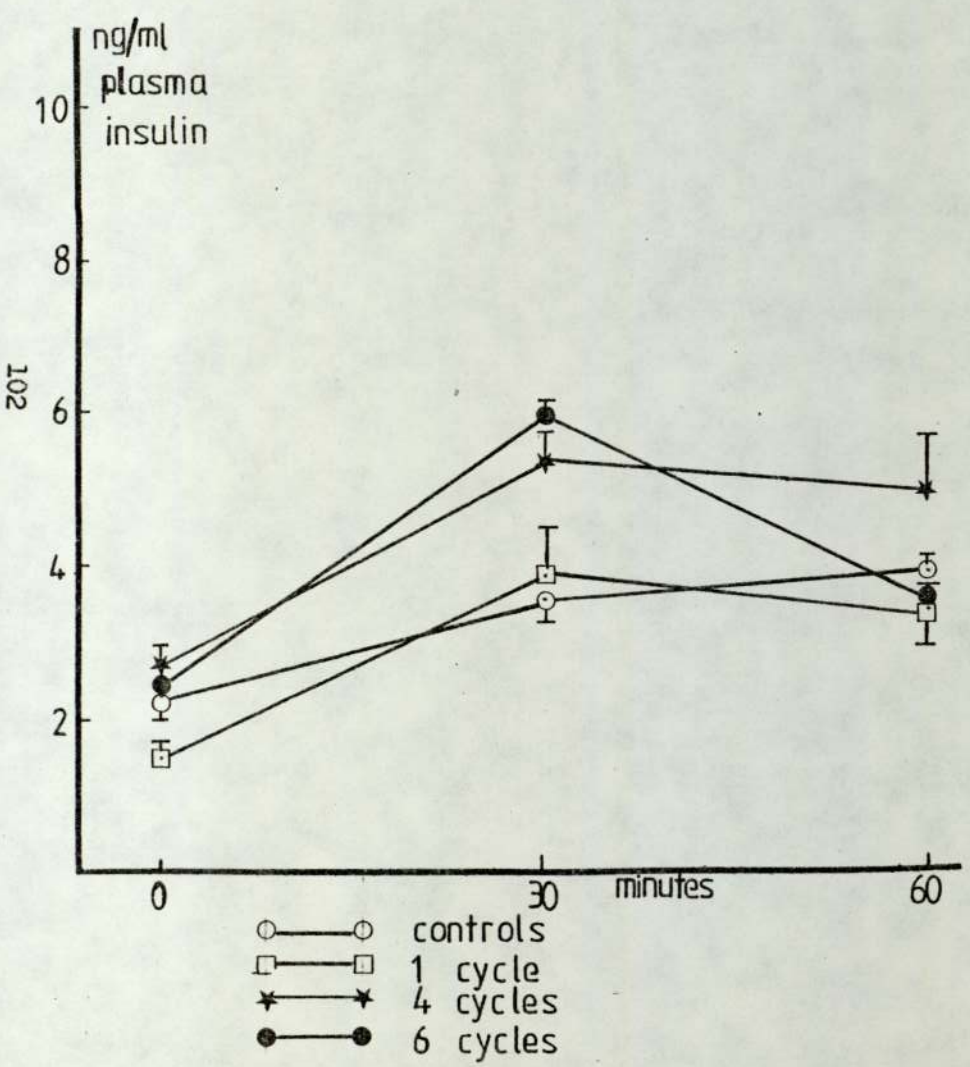
Effect of oral estradiol and progesterone (5ug + 1mg/kg/day) for 1, 4 and 6 cycles on GTT in 24hr. fasted mice (mean ± SEM for 5 determinations).

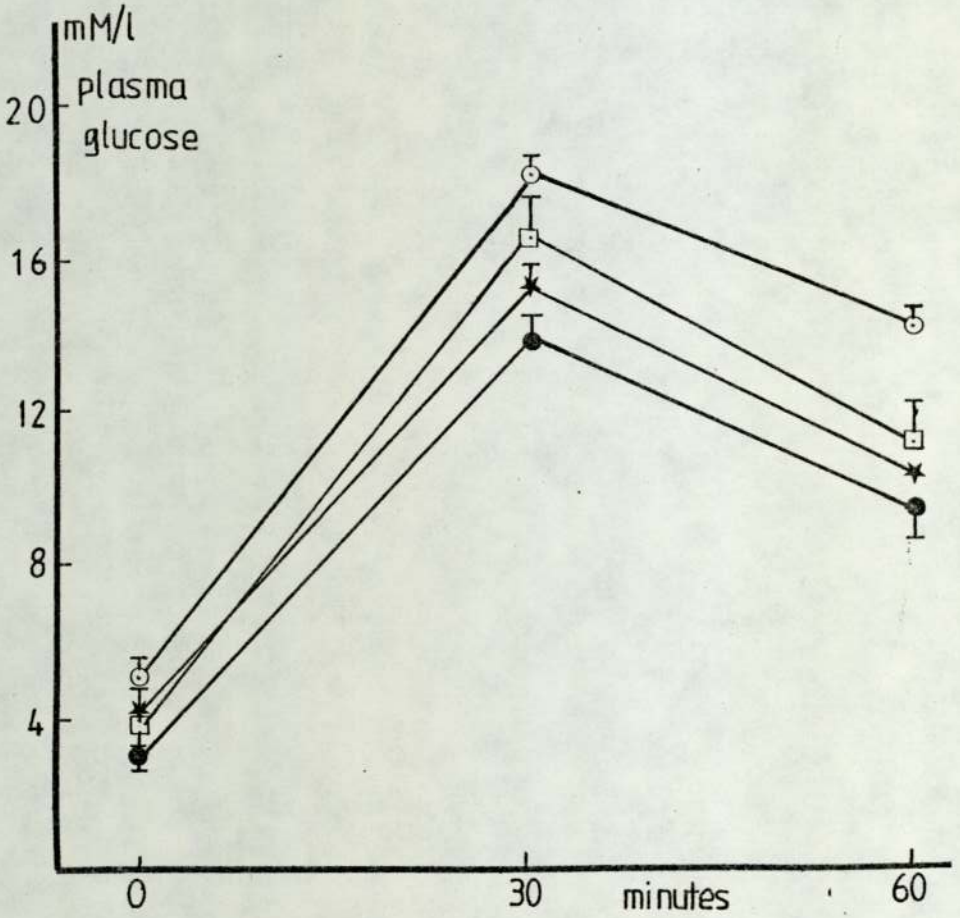


Readings were taken before (0) and 30 and 60 mins. after ip. glucose administration.

Figure 29

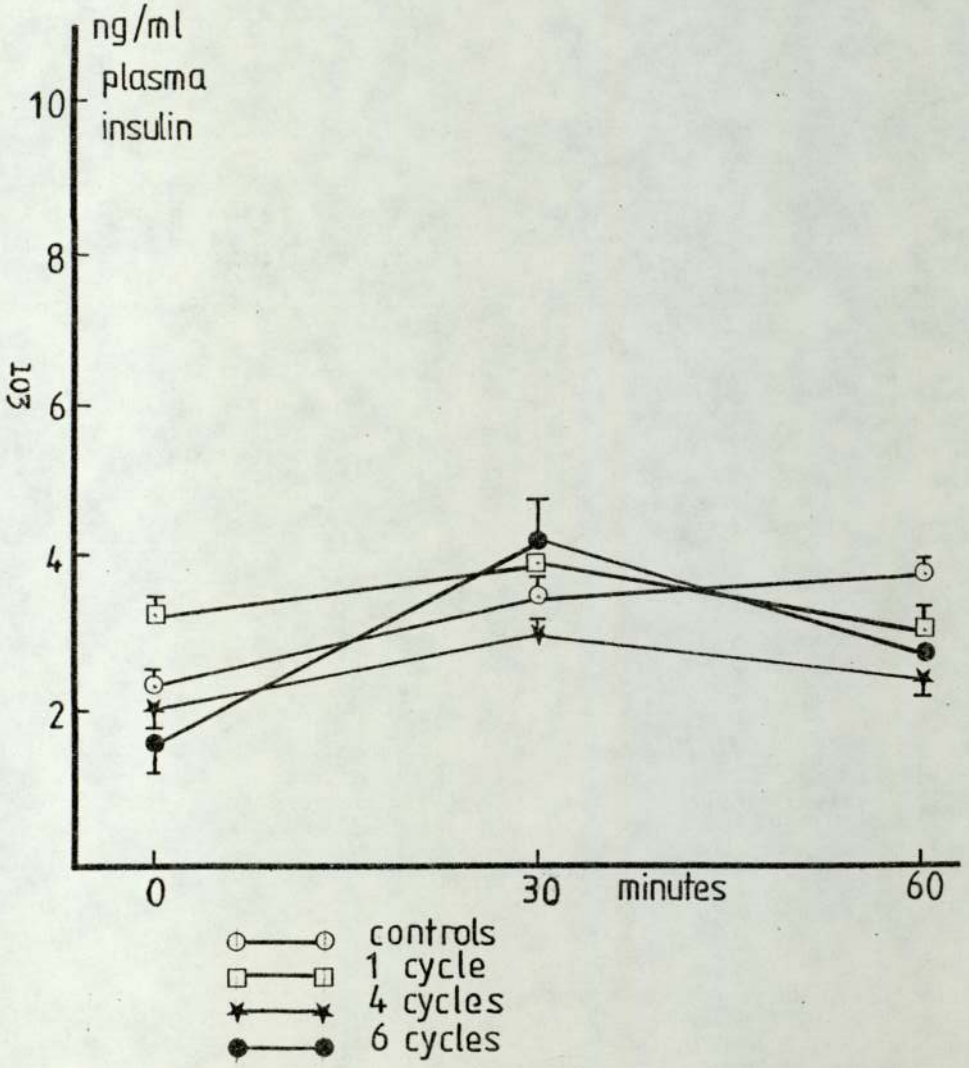
Effect of intramuscular EE and norethnodrel (5ug + 1mg/kg /day) for 1, 4 and 6 cycles on GTT in 24 hr. fasted mice (mean ± SEM for 5 determinations).

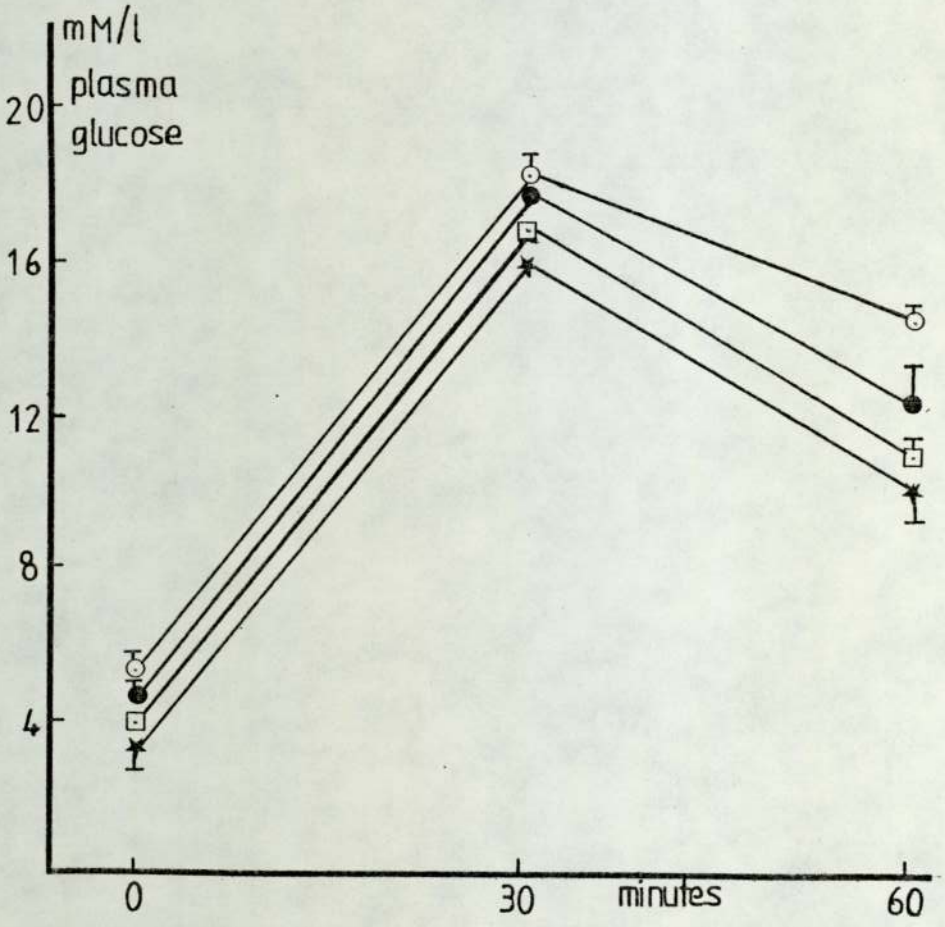




Readings were taken before (0) and 30 and 60 mins. after ip. glucose administration.

Figure 30 Effect of intramuscular EE and norethisterone acetate (5ug + 1mg/kg /day) for 1,4 and 6 cycles on GTT in 24hr. fasted mice (mean± SEM for 5 determinations).





Readings were taken before (0) and 30 and 60 mins. after ip. glucose administration.

Figure 31 Areas under the GTT curves at 1, 4 and 6 cycles during E_2 and P treatment (5ug+1mg/kg/day) in 24 hr. fasted mice (mean ± SEM for 5 determinations).

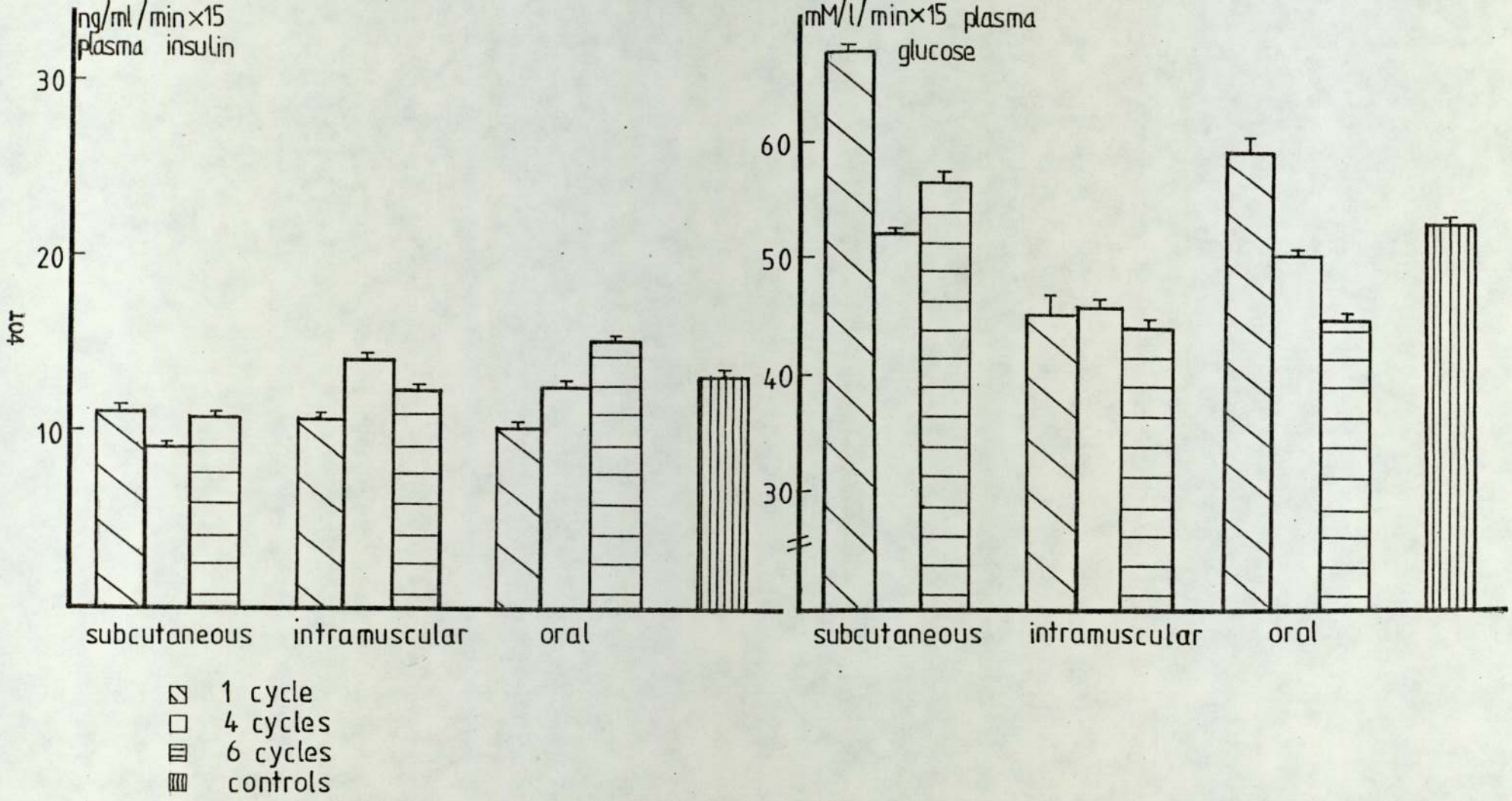


Figure 32

Areas under the GTT curves at 1, 4 and 6 cycles during EE and N treatment (5ug +1mg/kg/day) in 24 hr. fasted mice (mean ± SEM for 5 determinations).

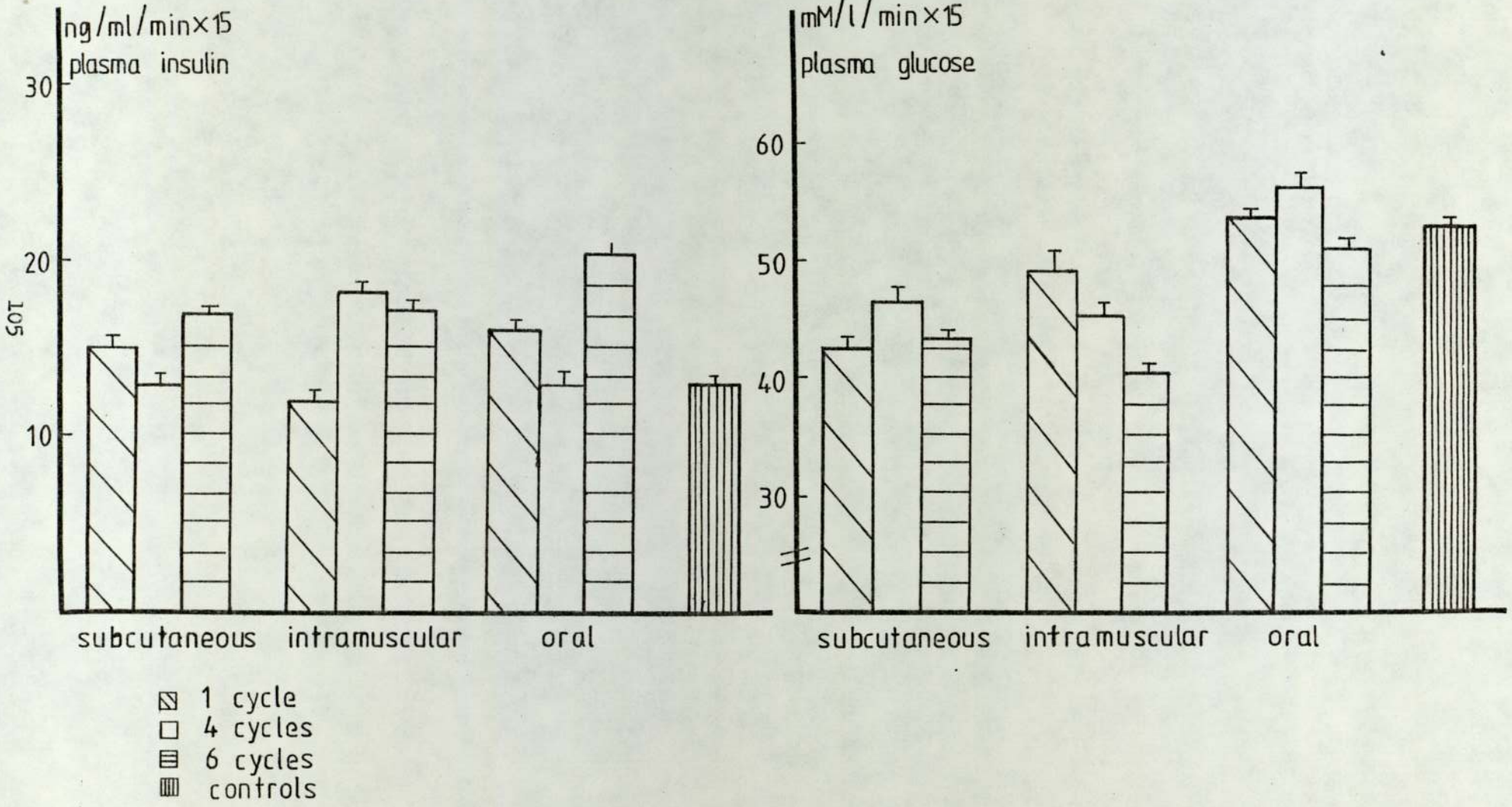


Figure 33

Areas under the GTT curves at 1, 4 and 6 cycles during EE and NA treatment (5ug + 1mg/kg/day) in 24hr. fasted mice (mean ± SEM for 5 determinations).

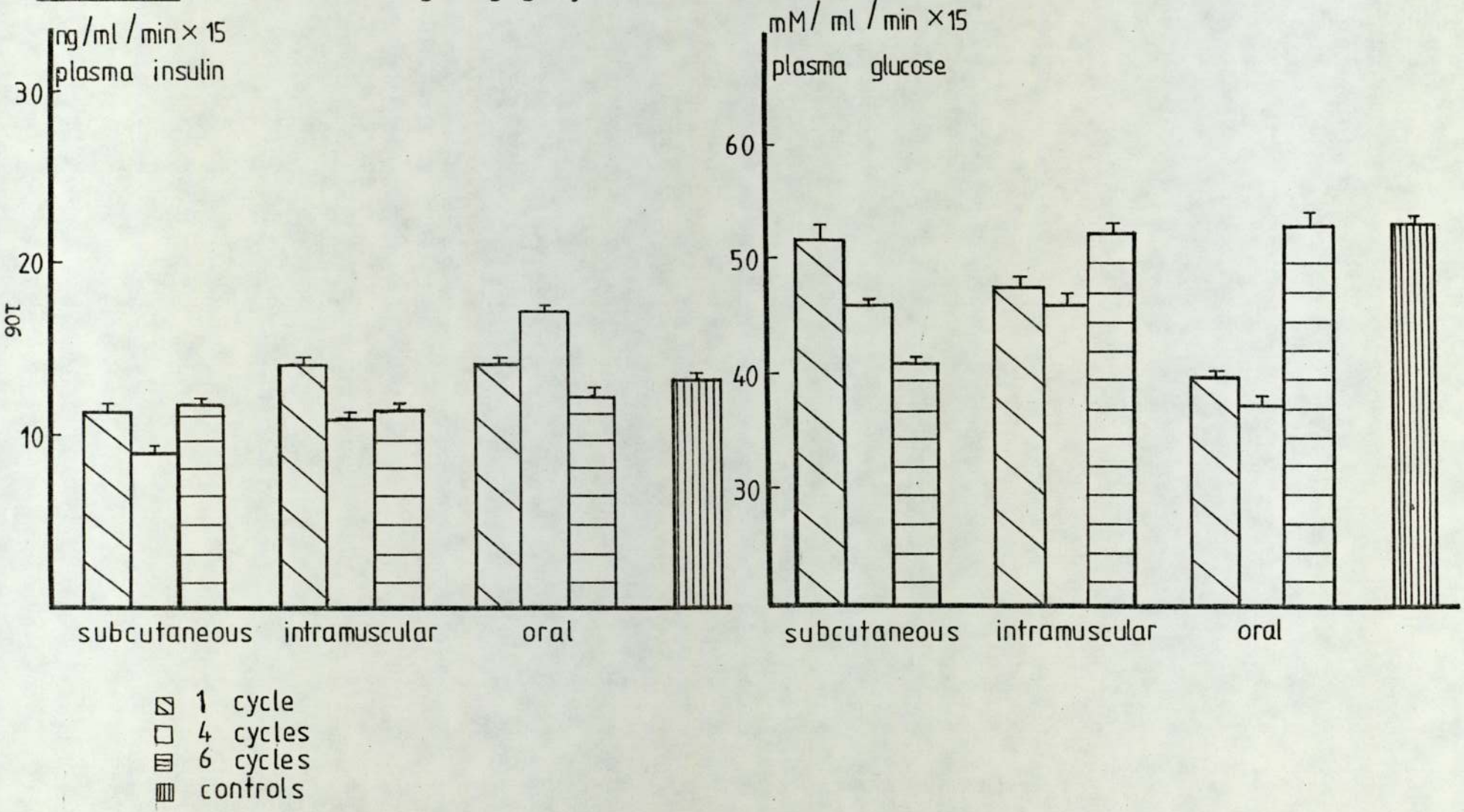
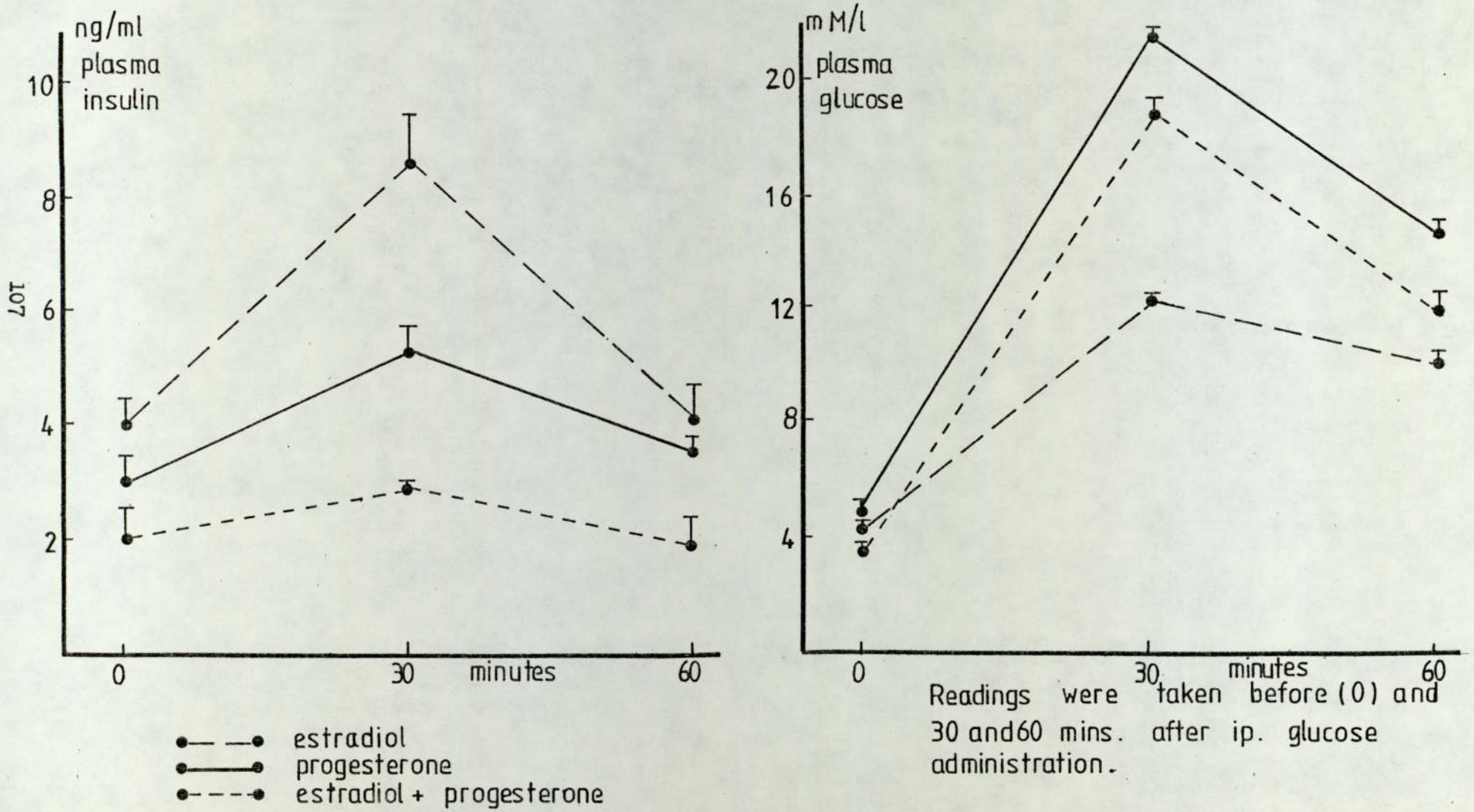


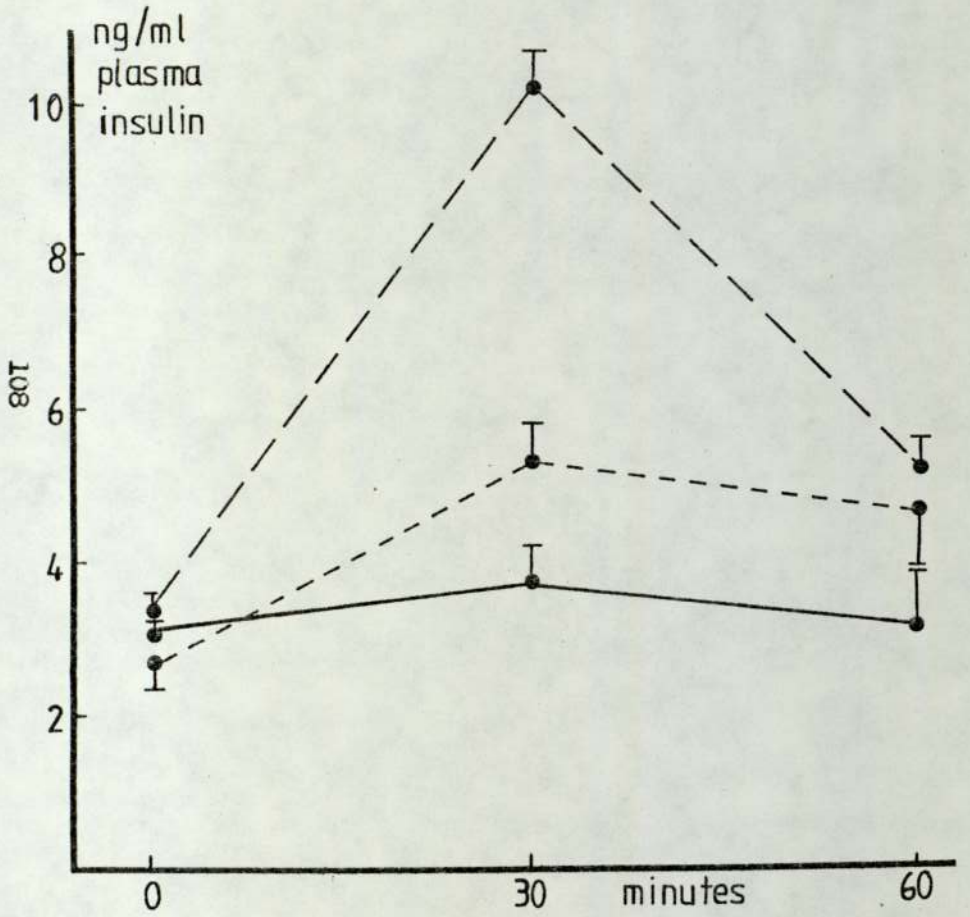
Figure 34 Comparison of GTT's in 24 hr. fasted mice treated with subcutaneous single and combined steroid preparations for 4 cycles (mean ± SEM for 5 determinations).

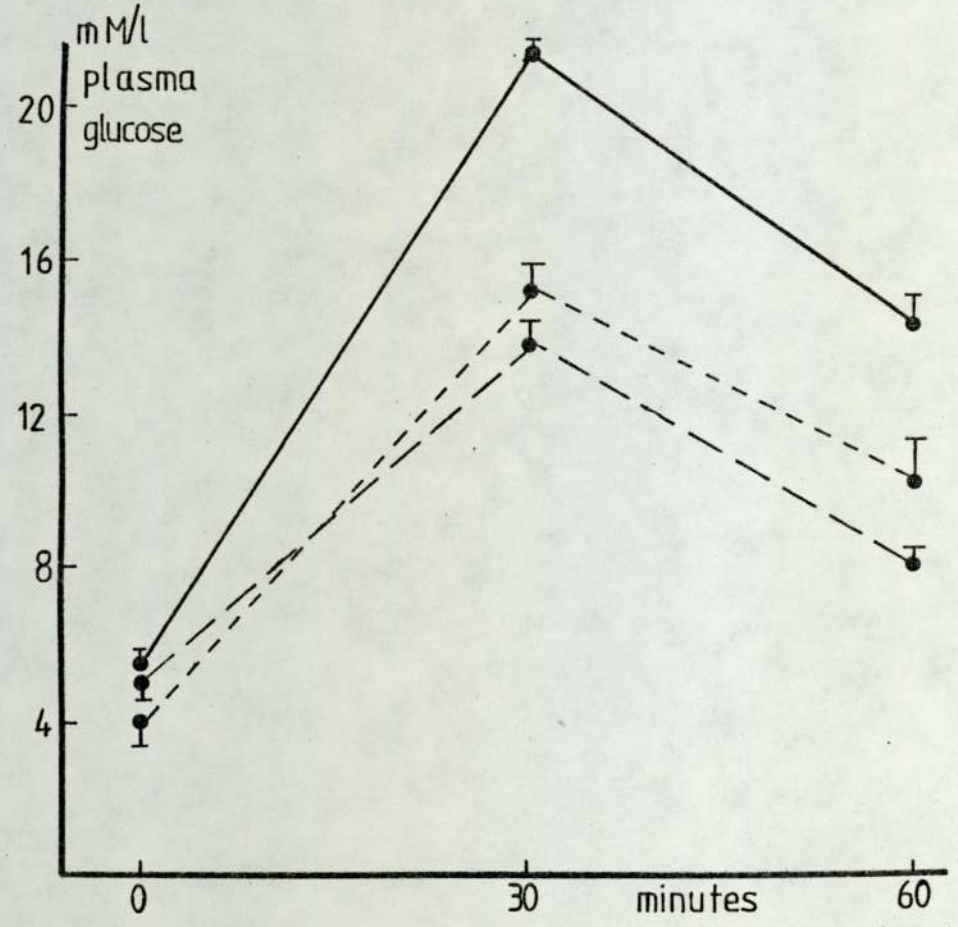


Readings were taken before (0) and 30 and 60 mins. after ip. glucose administration.

Figure 35

Comparison of GTT's in 24hr. fasted mice treated with intramuscular single and combined steroid preparations for 4 cycles (mean ± SEM for 5 determinations).



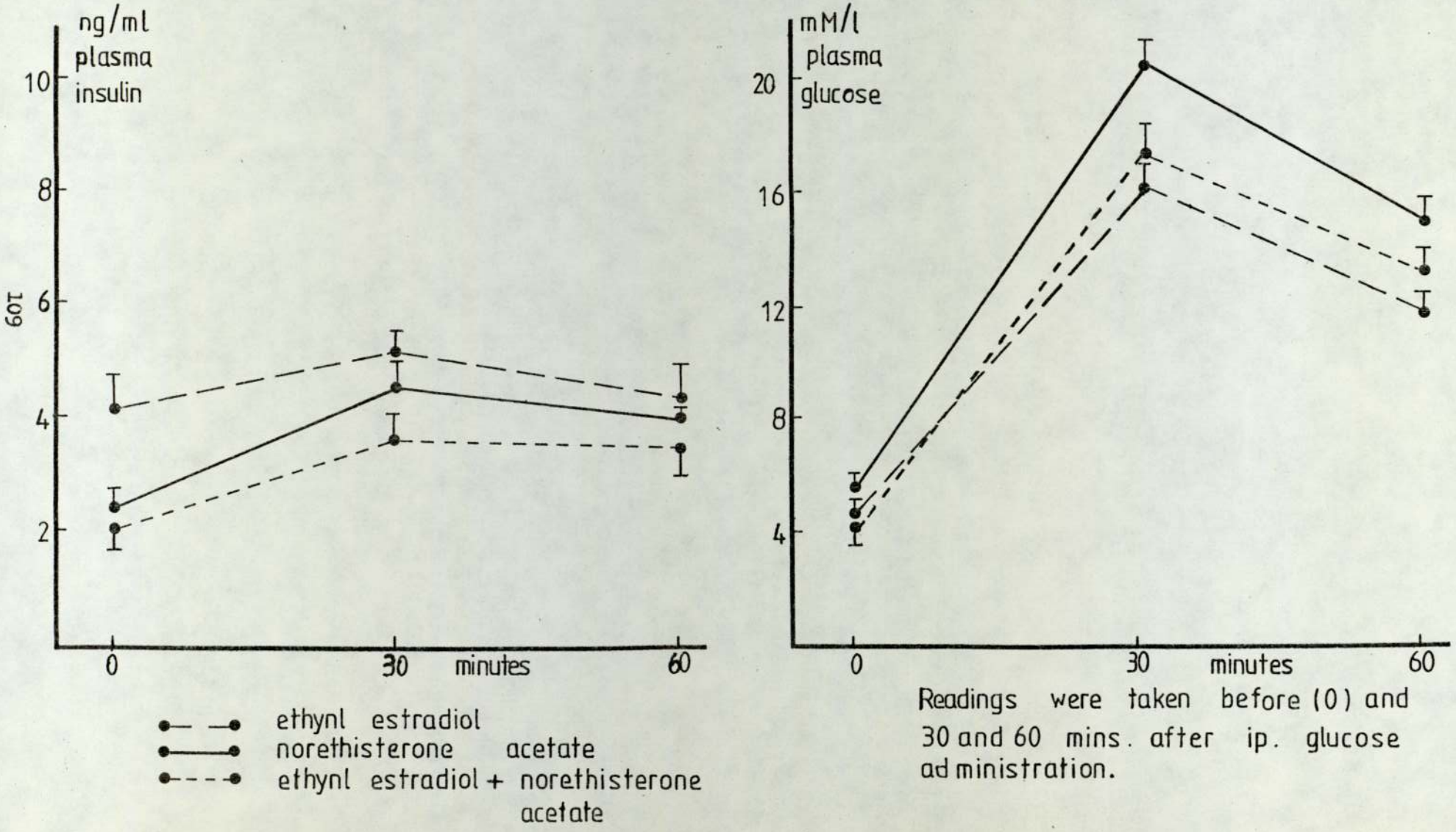


ethynl estradiol
norethnodrel
ethynl estradiol + norethnodrel

Readings were taken before (0) and 30 and 60 mins. after ip. glucose administration.

Figure 36

Comparison of GTT's in 24hr. fasted mice treated with oral single and combined steroid preparations for 6 cycles (mean ± SEM for 5 determinations).



Readings were taken before (0) and 30 and 60 mins. after ip. glucose administration.

Table 3 GLUCOSE TOLERANCE TESTS (n = 5) - CHAPTER 3

	Plasma Insulin (ng/ml)			Plasma Glucose (mM/l)		
	0	30	60	0	30	60
CONTROLS						
subcutaneous						
1 cycle						
mean	2.34	3.44	3.72	5.01	18.6	14.46
SEM	0.3	0.4	0.3	0.42	2.47	1.6
4cycles						
mean	2.32	3.42	3.66	5.05	18.36	14.24
SEM	0.3	0.2	0.35	0.42	1.3	1.86
6 cycles						
mean	2.32	3.74	3.86	5.05	18.61	14.1
SEM	0.2	0.62	0.9	0.5	1.6	2.1
intramuscular						
1 cycle						
mean	2.36	3.48	3.78	5.02	18.1	14.22
SEM	0.4	0.7	0.5	0.53	1.56	0.7
4 cycles						
mean	2.38	3.54	3.8	5.02	18.15	14.3
SEM	0.33	0.53	0.37	0.24	0.57	1.27
6 cycles						
mean	2.32	3.52	3.8	4.98	18.1	14.19
SEM	0.32	0.39	0.4	0.42	0.97	0.89
oral						
1 cycle						
mean	2.34	3.54	3.78	5.01	18.61	14.25
SEM	0.37	0.3	0.73	0.3	1.47	1.56
4 cycles						
mean	2.34	3.56	3.72	5.01	18.21	14.19
SEM	0.4	0.3	0.6	0.2	1.41	1.31
6 cycles						
mean	2.3	3.56	3.76	5.08	18.05	14.13
SEM	0.28	0.32	0.27	0.43	2.02	1.54
ESTRADIOL-17β						
subcutaneous						
1 cycle						
mean	3.74	4.4	3.86	4.93	15.93	10.67
SEM	0.45	0.71	0.7	0.51	0.5	0.75
4 cycles						
mean	3.98	8.64	4.68	4.54	12.4	9.91
SEM	0.45	0.9	0.6	0.28	0.28	0.51
6 cycles						
mean	3.08	6.68	4.0	4.41	11.26	8.58
SEM	0.7	0.74	0.36	0.39	0.1	0.23
intramuscular						
1 cycle						
mean	2.2	5.38	3.2	3.79	13.14	10.44
SEM	0.34	0.45	0.44	0.12	0.85	0.75
4 cycles						
mean	2.64	6.78	3.74	4.63	14.1	11.14
SEM	0.43	0.22	0.33	0.34	0.53	0.41

Table 3 contn.

	Plasma Insulin (ng/ml)			Plasma Glucose (mM/l)		
	0	30	60	0	30	60
ESTRADIOL-17β						
intramuscular						
6cycles						
mean	2.96	7.98	6.34	3.42	15.68	9.67
SEM	0.23	0.43	0.3	0.17	0.59	0.21
oral						
1 cycle						
mean	2.86	4.82	4.46	4.32	20.06	11.87
SEM	0.69	0.48	0.29	0.58	0.57	0.45
4 cycle						
mean	3.56	5.76	5.52	4.9	12.84	9.41
SEM	0.56	0.57	0.32	0.27	0.54	0.2
6 cycles						
mean	4.3	7.34	6.3	3.6	17.02	11.67
SEM	0.22	0.47	0.28	0.01	0.69	0.11
ETHYNYL ESTRADIOL						
subcutaneous						
1 cycle						
mean	2.62	3.04	2.24	4.78	14.6	12.42
SEM	0.23	0.6	0.15	0.38	1.1	0.68
4 cycles						
mean	3.3	4.38	3.32	4.44	14.16	11.14
SEM	0.18	0.39	0.37	0.2	0.64	0.88
6 cycles						
mean	3.26	3.94	2.6	4.17	12.77	9.99
SEM	0.5	0.14	0.16	0.32	1.18	1.1
intramuscular						
1 cycle						
mean	1.42	3.5	2.24	4.58	16.39	11.84
SEM	0.34	0.53	0.25	0.83	0.42	0.82
4 cycles						
mean	3.16	10.28	5.2	4.86	13.76	8.49
SEM	0.23	0.48	0.33	0.42	0.52	0.34
6 cycles						
mean	2.86	8.58	4.88	4.76	19.42	13.64
SEM	0.2	1.09	0.55	0.41	0.96	0.88
oral						
1 cycle						
mean	3.44	4.54	3.46	5.74	20.15	12.91
SEM	0.12	0.48	0.34	0.2	0.12	0.32
4 cycle						
mean	3.7	7.46	4.38	4.76	14.93	10.9
SEM	0.34	1.1	0.6	0.47	0.77	0.64
6 cycles						
mean	4.04	5.06	4.04	4.88	16.56	11.81
SEM	0.59	0.36	0.65	0.5	0.83	0.61

Table 3 contn.

	Plasma Insulin (ng/ml)			Plasma Glucose (mM/l)		
	0	30	60	0	30	60
PROGESTERONE						
subcutaneous						
1 cycle						
mean	1.52	4.56	2.98	4.02	16.07	12.02
SEM	0.16	0.66	0.7	0.36	0.64	0.58
4 cycles						
mean	3.02	5.3	3.44	4.91	21.13	14.71
SEM	0.41	0.49	0.29	0.41	0.19	0.5
6 cycles						
mean	3.04	4.86	3.42	4.44	19.3	12.38
SEM	0.38	0.45	0.34	0.38	0.42	0.68
intramuscular						
1 cycle						
mean	2.54	3.2	2.18	4.76	23.24	15.94
SEM	0.21	0.46	0.19	0.52	0.96	0.51
4 cycles						
mean	3.38	3.74	2.5	4.52	17.53	12.39
SEM	0.45	0.45	0.35	0.45	0.45	0.48
6 cycles						
mean	3.88	5.32	3.82	4.3	16.94	12.26
SEM	0.3	0.33	0.34	0.29	0.6	0.78
oral						
1 cycle						
mean	2.34	3.52	2.42	4.3	15.4	11.64
SEM	0.4	0.31	0.46	0.33	0.9	0.61
4 cycles						
mean	4.24	5.04	3.4	4.65	17.67	12.91
SEM	0.29	0.23	0.19	0.27	1.1	0.4
6 cycles						
mean	4.0	5.56	4.7	4.65	21.03	14.69
SEM	0.5	0.25	0.34	0.57	0.58	0.74
NORETHNODREL						
subcutaneous						
1 cycle						
mean	1.42	3.54	2.72	5.26	18.87	13.85
SEM	0.2	0.37	0.54	0.37	0.5	0.97
4 cycles						
mean	2.52	3.72	3.36	5.26	21.62	13.29
SEM	0.23	0.13	0.26	0.26	0.76	0.13
6 cycles						
mean	3.1	4.08	3.5	5.35	21.17	15.59
SEM	0.3	0.51	0.49	0.42	0.81	0.65
intramuscular						
1 cycle						
mean	1.9	3.4	2.2	5.05	22.66	17.17
SEM	0.52	0.66	0.25	0.46	0.69	0.67
4 cycles						
mean	3.08	3.72	3.22	4.92	21.61	14.83
SEM	0.15	0.43	0.7	0.25	0.23	0.54
6 cycles						
mean	3.22	4.5	4.64	5.04	22.01	14.95
SEM	0.32	0.3	0.44	0.54	1.27	0.53

Table 3 contn.

	Plasma Insulin (ng/ml)			Plasma Glucose (mM/l)		
	0	30	60	0	30	60
NORETHNODREL						
oral						
1 cycle						
mean	3.94	6.0	4.06	5.74	21.39	15.62
SEM	0.42	0.66	0.5	0.57	0.71	1.0
4 cycles						
mean	2.88	5.04	4.76	5.08	15.2	10.39
SEM	0.51	0.53	0.71	0.78	0.8	0.56
6 cycles						
mean	1.84	4.12	4.52	5.1	15.3	10.76
SEM	0.26	0.48	0.27	0.87	0.71	1.08
NORETHISTERONE ACETATE						
subcutaneous						
1 cycle						
mean	1.52	2.6	1.68	3.98	16.03	12.51
SEM	0.35	0.36	0.26	0.84	0.94	1.26
4 cycles						
mean	1.56	2.98	2.04	4.77	15.98	12.55
SEM	0.4	0.38	0.43	0.89	1.5	0.78
6 cycles						
mean	2.84	4.12	2.98	5.59	17.37	15.14
SEM	0.4	0.5	0.48	0.56	1.08	0.75
intramuscular						
1 cycle						
mean	3.82	4.9	2.6	4.29	12.7	9.48
SEM	0.59	0.64	0.3	0.56	1.27	0.49
4 cycles						
mean	2.46	3.18	2.54	4.39	15.96	10.19
SEM	0.27	0.26	0.37	0.56	0.39	0.65
6 cycles						
mean	1.9	4.44	2.66	4.85	15.67	10.58
SEM	0.37	0.46	0.25	0.52	0.33	0.86
oral						
1 cycle						
mean	2.3	3.72	3.02	4.15	14.47	10.79
SEM	0.34	0.5	0.59	0.25	0.49	1.14
4 cycles						
mean	1.58	3.96	3.94	4.52	19.93	13.87
SEM	0.4	0.6	0.2	0.43	0.72	0.56
6 cycle						
mean	2.22	4.4	4.0	5.52	20.87	15.19
SEM	0.31	0.43	0.16	0.46	0.97	0.81
ESTRADIOL + PROGESTERONE						
subcutaneous						
1 cycle						
mean	1.44	3.76	2.24	4.29	23.09	16.77
SEM	0.16	0.39	0.31	0.17	0.57	0.43
4 cycles						
mean	1.96	2.7	1.88	3.88	18.31	11.43
SEM	0.56	0.13	0.53	0.13	0.45	0.47

Table 3 contn.

	Plasma Insulin (ng/ml)			Plasma Glucose (mM/l)		
	0	30	60	0	30	60
ESTRADIOL + PROGESTERONE						
subcutaneous						
6 cycles						
mean	1.82	3.3	2.42	3.84	19.63	13.41
SEM	0.4	0.46	0.32	0.14	0.57	0.49
intramuscular						
1 cycle						
mean	2.18	2.74	3.0	4.35	15.51	9.96
SEM	0.35	0.17	0.51	0.71	1.48	1.14
4 cycles						
mean	3.3	4.12	2.54	3.84	15.42	11.49
SEM	0.39	0.51	0.16	0.16	0.46	0.4
6 cycles						
mean	2.64	3.14	3.3	3.49	14.36	12.39
SEM	0.25	0.28	0.38	0.35	0.37	0.29
oral						
1 cycle						
mean	1.54	2.64	3.1	4.39	19.07	16.22
SEM	0.08	0.29	0.48	0.42	1.28	0.7
4 cycles						
mean	1.5	3.5	3.8	4.23	16.71	12.48
SEM	0.25	0.39	0.17	0.22	0.79	0.57
6 cycles						
mean	3.42	3.84	3.88	3.63	15.01	10.63
SEM	0.41	0.56	0.23	0.38	0.31	0.39
ETHYNYL ESTRADIOL + NORETHNODREL						
subcutaneous						
1 cycle						
mean	1.86	4.4	4.18	4.47	13.66	10.86
SEM	0.4	0.35	0.59	0.41	0.69	0.57
4 cycles						
mean	2.36	3.94	2.52	3.73	16.4	10.07
SEM	0.5	0.47	0.43	0.5	0.93	0.68
6 cycles						
mean	2.56	4.98	3.84	4.38	14.21	10.86
SEM	0.23	0.26	0.33	0.24	0.9	0.51
intramuscular						
1 cycle						
mean	1.7	3.64	3.2	4.31	17.08	10.74
SEM	0.27	0.64	0.4	0.37	1.03	1.06
4 cycles						
mean	2.44	5.28	4.7	4.05	15.29	10.13
SEM	0.3	0.41	0.75	0.51	0.64	1.0
6 cycles						
mean	2.45	5.74	3.36	3.38	13.91	9.37
SEM	0.4	0.2	0.22	0.23	0.67	0.91

Table 3 contn.

	Plasma Insulin (ng/ml)			Plasma Glucose (mM/l)		
	0	30	60	0	30	60
ETHYNYL ESTRADIOL + NORETHNODREL						
oral						
1 cycle						
mean	2.06	4.8	4.24	3.86	18.83	13.08
SEM	0.4	0.62	0.62	0.48	1.31	0.52
4 cycles						
mean	1.58	4.54	2.72	4.15	19.55	13.34
SEM	0.28	0.69	0.33	0.66	1.26	0.67
6 cycles						
mean	2.5	6.86	4.32	4.56	17.63	11.43
SEM	0.3	1.02	0.32	0.4	0.86	0.12
ETHYNYL ESTRADIOL + NORETHISTERONE ACETATE						
subcutaneous						
1 cycle						
mean	2.68	3.04	2.2	4.16	18.05	11.24
SEM	0.35	0.49	0.42	0.35	1.04	0.6
4 cycles						
mean	1.48	2.68	2.24	3.96	15.87	10.81
SEM	0.29	0.23	0.41	0.2	0.61	0.68
6 cycles						
mean	2.3	3.1	2.74	3.9	13.48	9.89
SEM	0.25	0.58	0.25	0.64	0.12	1.0
intramuscular						
1 cycle						
mean	3.24	3.96	3.04	4.03	16.66	10.45
SEM	0.21	0.44	0.34	0.6	0.79	0.55
4 cycles						
mean	2.06	3.08	2.5	3.69	15.9	10.26
6 cycles						
mean	1.62	4.04	2.7	4.17	17.81	12.38
SEM	0.42	0.52	0.35	0.27	0.89	1.02
oral						
1 cycle						
mean	2.9	4.86	2.1	4.01	12.94	9.69
SEM	0.44	0.17	0.42	0.55	0.39	0.69
4 cycles						
mean	2.46	5.88	2.88	3.24	12.49	8.9
SEM	0.46	0.15	0.64	0.34	1.21	0.48
6 cycles						
mean	2.1	3.56	3.36	4.15	17.49	13.48
SEM	0.33	0.46	0.46	0.5	1.0	0.7

Table 4 GLUCOSE TOLERANCE TEST - STATISTICAL COMPARISON WITH DIESTRUS CONTROLS - CHAPTER 3

	Plasma Insulin (ng/ml)			Plasma Glucose (mM/l)		
	0	30	60	0	30	60
ESTRADIOL-17β						
subcutaneous						
1 cycle	$p < 0.001$	NS	NS	NS	NS	$p < 0.01$
4 cycles	$p < 0.001$	$p < 0.001$	$p < 0.02$	NS	$p < 0.001$	$p < 0.01$
6 cycles	NS	$p < 0.001$	NS	NS	$p < 0.001$	$p < 0.001$
intramuscular						
1 cycle	NS	$p < 0.01$	NS	$p < 0.002$	$p < 0.001$	$p < 0.001$
4 cycles	NS	$p < 0.001$	NS	NS	$p < 0.001$	$p < 0.002$
6 cycles	$p < 0.02$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.01$	$p < 0.001$
oral						
1 cycle	NS	$p < 0.01$	NS	NS	NS	$p < 0.02$
4 cycles	$p < 0.01$	$p < 0.001$	$p < 0.001$	NS	$p < 0.001$	$p < 0.001$
6 cycles	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	NS	$p < 0.02$
ETHYNYL ESTRADIOL						
subcutaneous						
1 cycle	NS	NS	$p < 0.001$	NS	$p < 0.02$	$p < 0.05$
4 cycles	$p < 0.001$	$p < 0.01$	NS	$p < 0.05$	$p < 0.001$	$p < 0.02$
6 cycles	$p < 0.01$	NS	$p < 0.05$	$p < 0.05$	$p < 0.001$	$p < 0.01$
intramuscular						
1 cycle	$p < 0.01$	NS	$p < 0.001$	NS	NS	$p < 0.01$
4 cycles	NS	$p < 0.001$	$p < 0.001$	NS	$p < 0.001$	$p < 0.001$
6 cycles	p 0.05	$p < 0.001$	$p < 0.02$	NS	NS	NS
oral						
1 cycle	$p < 0.001$	$p < 0.01$	NS	$p < 0.01$	NS	NS
4 cycles	$p < 0.001$	$p < 0.001$	NS	NS	$p < 0.01$	$p < 0.002$
6 cycles	$p < 0.001$	$p < 0.001$	NS	NS	NS	$p < 0.02$
PROGESTERONE						
subcutaneous						
1 cycle	$p < 0.01$	$p < 0.05$	NS	$p < 0.01$	NS	$p < 0.05$
4 cycles	$p < 0.05$	$p < 0.001$	NS	NS	$p < 0.01$	NS
6 cycles	$p < 0.01$	$p < 0.02$	NS	NS	NS	NS
intramuscular						
1 cycle	NS	NS	$p < 0.001$	NS	$p < 0.001$	$p < 0.01$
4 cycles	$p < 0.01$	NS	$p < 0.001$	NS	NS	$p < 0.05$
6 cycles	$p < 0.001$	$p < 0.001$	NS	$p < 0.05$	NS	$p < 0.02$
oral						
1 cycle	NS	NS	$p < 0.02$	$p < 0.02$	$p < 0.01$	$p < 0.02$
4 cycles	$p < 0.001$	$p < 0.001$	NS	NS	NS	NS
6 cycles	$p < 0.001$	$p < 0.001$	$p < 0.01$	NS	p 0.05	NS
NORETHNODREL						
subcutaneous						
1 cycle	$p < 0.01$	NS	$p < 0.02$	NS	NS	NS
4 cycles	NS	NS	NS	NS	$p < 0.01$	NS
6 cycles	$p < 0.01$	NS	NS	NS	$p < 0.05$	NS
intramuscular						
1 cycle	NS	NS	$p < 0.001$	NS	$p < 0.001$	$p < 0.001$
4 cycles	$p < 0.01$	NS	NS	NS	$p < 0.001$	NS
6 cycles	$p < 0.01$	$p < 0.01$	$p < 0.05$	NS	$p < 0.002$	NS

Table 4 contn.

	Plasma Insulin (ng/ml)			Plasma Glucose (mM/l)		
	0	30	60	0	30	60
NORETHNODREL						
oral						
1 cycle	$p < 0.001$	$p < 0.001$	NS	NS	$p < 0.01$	NS
4 cycles	NS	$p < 0.001$	$p < 0.05$	NS	$p < 0.05$	$p < 0.01$
6 cycles	$p < 0.05$	NS	$p < 0.001$	NS	$p < 0.05$	$p < 0.01$
NORETHISTERONE ACETATE						
subcutaneous						
1 cycle	$p < 0.02$	$p < 0.02$	$p < 0.001$	NS	NS	NS
4 cycles	$p < 0.02$	NS	$p < 0.001$	NS	$p < 0.05$	NS
6 cycles	$p < 0.05$	NS	NS	NS	NS	NS
intramuscular						
1 cycle	NS	NS	$p < 0.002$	NS	$p < 0.001$	$p < 0.001$
4 cycles	NS	NS	$p < 0.002$	NS	$p < 0.001$	$p < 0.001$
6 cycles	NS	$p < 0.02$	$p < 0.002$	NS	$p < 0.002$	$p < 0.001$
oral						
1 cycle	NS	NS	NS	$p < 0.01$	$p < 0.001$	$p < 0.01$
4 cycles	$p < 0.05$	NS	NS	NS	$p < 0.002$	NS
6 cycles	NS	$p < 0.01$	NS	NS	$p < 0.05$	NS
ESTRADIOL + PROGESTERONE						
subcutaneous						
1 cycle	$p < 0.002$	NS	$p < 0.001$	$p < 0.02$	$p < 0.01$	$p < 0.05$
4 cycles	NS	$p < 0.002$	$p < 0.001$	$p < 0.001$	NS	$p < 0.02$
6 cycles	$p < 0.05$	NS	$p < 0.02$	$p < 0.01$	NS	NS
intramuscular						
1 cycle	NS	NS	NS	NS	$p < 0.05$	$p < 0.001$
4 cycles	$p < 0.01$	NS	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.01$
6 cycles	NS	NS	NS	NS	$p < 0.001$	$p < 0.01$
oral						
1 cycle	$p < 0.01$	$p < 0.01$	NS	$p < 0.05$	NS	NS
4 cycles	$p < 0.01$	NS	NS	$p < 0.001$	NS	$p < 0.05$
6 cycles	$p < 0.01$	NS	NS	$p < 0.002$	$p < 0.02$	$p < 0.01$
ETHYNYL ESTRADIOL + NORETHNODREL						
subcutaneous						
1 cycle	NS	$p < 0.01$	NS	NS	$p < 0.01$	$p < 0.01$
4 cycles	NS	NS	$p < 0.01$	$p < 0.01$	$p < 0.05$	$p < 0.01$
6 cycles	NS	$p < 0.01$	NS	NS	$p < 0.002$	$p < 0.02$
intramuscular						
1 cycle	$p < 0.05$	NS	NS	NS	NS	$p < 0.001$
4 cycles	NS	$p < 0.001$	$p < 0.05$	$p < 0.01$	$p < 0.001$	$p < 0.001$
6 cycles	NS	$p < 0.001$	NS	$p < 0.001$	$p < 0.001$	$p < 0.001$
oral						
1 cycle	NS	$p < 0.01$	NS	$p < 0.01$	NS	NS
4 cycles	$p < 0.02$	$p < 0.05$	$p < 0.02$	$p < 0.05$	NS	NS
6 cycles	NS	$p < 0.001$	$p < 0.05$	NS	NS	$p < 0.01$

Table 4 contn.

	Plasma Insulin (ng/ml)			Plasma Glucose (mM/l)		
	0	30	60	0	30	60
ETHYNYL ESTRADIOL + NORETHISTERONE ACETATE						
subcutaneous						
1 cycle	NS	NS	$p < 0.001$	$p < 0.02$	NS	$p < 0.01$
4 cycles	$p < 0.01$	$p < 0.01$	$p < 0.001$	$p < 0.002$	$p < 0.01$	$p < 0.01$
6 cycles	NS	NS	$p < 0.05$	$p < 0.05$	$p < 0.001$	$p < 0.01$
intramuscular						
1 cycle	$p < 0.01$	NS	$p < 0.05$	$p < 0.05$	NS	$p < 0.001$
4 cycles	NS	NS	$p < 0.001$	$p < 0.01$	$p < 0.01$	$p < 0.001$
6 cycles	$p < 0.05$	NS	$p < 0.01$	NS	NS	$p < 0.05$
oral						
1 cycle	NS	$p < 0.001$	$p < 0.01$	$p < 0.02$	$p < 0.001$	$p < 0.001$
4 cycles	NS	$p < 0.001$	NS	$p < 0.001$	$p < 0.001$	$p < 0.001$
6 cycles	NS	NS	NS	$p < 0.05$	NS	NS

Where diestrus controls, n = 5

steroid-treated mice, n = 5

$p < 0.05$ is significant

$p < 0.02$ & $p < 0.01$ are highly significant

$p < 0.002$ & $p < 0.001$ are very highly significant

Chapter 4

EFFECT OF SEX STEROIDS ON TISSUE GLYCOGEN LEVELS

CHAPTER 4

TISSUE GLYCOGEN LEVELS

INTRODUCTION

Carbohydrate fuel stores in mammals mainly take the form of glycogen. Glycogen is a highly branched glucose-based polymer that provides a high concentration of breakdown points. The major glycogen depots are located in liver and muscle. Liver glycogen is subject to rapid and marked diurnal variations which are largely dependent on the physiological condition (41). In addition to supplying hepatic requirements, liver glycogen is liberated for use by other tissues during fasting (47). Muscle glycogen undergoes slower, more shallow variations which reflect activity. Very little glycogen is used in the resting state but during muscular activity it provides energy when glucose or oxygen is scarce (162, 410 - 413).

Normal glycaemia is maintained within strict limits, and glycogen metabolism plays an important role. The regulation of glycogen metabolism involves both homeostatic and hormonal control mechanisms, with the availability of glucose being an important controlling influence (47). Endogenous ovarian hormones and contraceptive sex steroids have been reported to alter glycogen homeostasis. It is important to clarify the situation in view of the role glycogen metabolism plays in maintaining normal glycaemia. Indeed, it has been suggested that sex steroid-induced alterations of carbohydrate metabolism are attributable to changes in tissue glycogen deposition (162).

Variations in hepatic and uterine glycogen levels were observed in the rat during the estrous cycle, and a tentative correlation was made with changes of circulating ovarian hormone titres (123). It has been reported that tissue glycogen levels fall following ovariectomy in the mouse (126), thus further supporting the link between

ovarian hormones levels and glycogen deposition. Estrogens have been shown on many occasions to increase both hepatic (168, 414 - 417) and uterine (2 & 168) glycogen concentrations. Few studies have been performed on other tissues, but it appears that estrogens have a similar effect on skeletal muscle and fat (169, 175 & 418). These alterations are dependent on dose and treatment duration (167 & 415).

The influence of progestogens on tissue glycogen concentrations has not been clearly determined and few studies have been performed. Short term progesterone treatment was shown to elevate hepatic glycogen (162 & 179), but this was not confirmed in other experiments although a correlation was noted between circulating progesterone titres and the glycogen content of the endometrium (419). In pregnant rats, liver and uterine glycogen levels are elevated and these changes have been correlated with changes in maternal progesterone titres (168 & 420).

The effects on glycogen homeostasis produced following combined therapy with either natural or synthetic contraceptive preparations have received scant attention. Administration of combined E_2 and P was reported to reduce E_2-induced glycolysis (122, 421 & 422). Whereas E_2 and P treated ovariectomised mice had tissue glycogen levels that were in the normal range (126). Little is known about the effects of oral contraceptive preparations on glycogen homeostasis. It has been reported that Enovid, mestranol and norethynodrel, treatment was associated with reduced hepatic glycogen deposition (216 & 233).

The present study was undertaken to examine the effects of natural and synthetic sex steroids on the glycogen content of liver, uterus, femoral skeletal muscle, cardiac muscle and abdominal muscle. The influence of the administration route, treatment duration and of single and combined preparations has been considered.

METHODS

Intact adult female mice were divided into nine groups and treated as follows -

Groups 1 Controls - arachis oil

2 Estradiol-17β - 5µg/kg (body wt.) / day

3 Ethynyl estradiol - 5µg/kg (body wt.) / day

4 Progesterone - 1mg/kg (body wt.) / day

5 Norethnodrel - 1mg/kg (body wt.) / day

6 Norethisterone acetate - 1mg/kg (body wt.) / day

7 E_2 & P - 5µg & 1mg/kg (body wt.) / day

8 EE & N - 5µg & 1mg/kg (body wt.) / day

9 EE & NA - 5µg & 1mg/kg (body wt.) / day

Within each group the Sc, Im and oral routes of administration were examined. Glycogen determinations were performed on freely fed mice after one, four and six cycles of steroid administration. The glycogen content of liver, uterus, femoral, cardiac and abdominal muscle was assayed using a modified phenol-sulphuric acid method, described in detail in Chapter 2.

RESULTS

(i) CONTROLS

Diestrus control mice were treated with the vehicle, arachis oil, only. Tissue glycogen levels in this group of mice are shown in figure 37. Neither the administration route nor the duration of treatment had a significant influence on tissue glycogen levels in the control group. Therefore for convenience, it was decided to use a mean calculated from all the control data, including all three administration routes and time periods, for each tissue. Thus in all subsequent figures in this chapter, this is the control mean $\pm$ SEM used for comparison with the steroid treated mice. The statistics, however, were calculated for each route at each time and compared as such with the other groups.

(ii) ESTROGEN TREATMENT

Tissue glycogen levels observed after natural estrogen (E_2) administration are shown in figure 38, and after synthetic estrogen (EE) administration in figure 39. These data show that estrogen treatment produced significant elevations of glycogen above control levels in all five tissues. E_2 had a greater influence on glycogen deposition than the synthetic estrogen (see figure 40). A time-dependent increase in glycogen deposition was seen with both estrogens, maximum levels occured at six cycles. EE was generally slower in eliciting this response, as illustrated in uterine tissue (figure 41). Both estrogens markedly raised hepatic glycogen after only one cycle of treatment, whereas in other tissues, elevations were not apparent until four or six cycles (figures 38 & 39).

The administration route affected both the absolute amount of glycogen and also the time-dependency of the deposition process. Differences were most prevalent at four and six cycles, and were

characteristic for the two estrogens. During E_2 administration for example, the Sc route produced the highest glycogen levels in all tissues except liver (figure 38). In this particular tissue the oral administration route induced the greatest deposition of glycogen. Since the liver would be exposed to the highest concentrations of E_2 and its metabolites following oral administration, one would expect this administration route to produce greater glycogen deposition in liver. In the case of EE treatment, in the long term the Im route produced the largest glycogen concentrations (figure 39), whereas Im E_2 usually produced the smallest elevations of glycogen (figure 38). The smallest increased glycogenesis during EE treatment occurred following oral administration in all tissues (figure 39). Orally administered EE would be metabolised more quickly, and probably to a greater extent than either Sc or Im EE treatment. These results suggest that it is the administered form of EE, and not a metabolite that is responsible for inducing glycogenesis in the tissues tested. It is also interesting to note that EE is more resistant to hepatic degradation than the natural hormone, E_2, which is rapidly metabolised. Thus the peripheral tissues would be subjected to higher concentrations of EE for longer periods than E_2. Yet E_2 still exerted a stronger influence on glycogenesis than the synthetic steroid. It appears that the tissues are much more responsive to the natural estrogen and/or that a metabolite of E_2 is responsible for inducing glycogenesis.

Comparison of the average elevations of glycogen at the end of the study showed that both estrogens affected liver and uterus to a similar extent. Muscle glycogenesis was stimulated to a greater extent, E_2 produced a 4 - 5 fold and EE a 3 - 5 fold increase in glycogen deposition.

(iii) PROGESTOGEN TREATMENT

The tissue glycogen levels following progestogen treatment are illustrated in figures 42 - 44. Progestogen administration usually produced tissue glycogen concentrations significantly higher than the diestrus controls. P and N treatment, however, also produced significant decreases below the control levels at one and four cycles (figures 42 & 43). Invariably levels were raised at six cycles. The synthetic progestogens, particularly NA, had a greater stimulatory effect on glycogen than P (figures 45). This may be interpreted as evidence that the synthetic estrogens were metabolised less rapidly than the natural hormone, P. Synthetic progestogens are also metabolised to EE as part of the conversion and degradation process (484 - 486). It was shown in the previous section that EE is a strong glycogenic agent (see figure 39). Thus the glycogenic influence of synthetic progestogens may be attributed to peripheral formation of EE which is infact the active agent.

The time-dependent alterations of glycogen deposition varied according to the tissue examined. Glycogen levels increased with the length of treatment in both liver and uterus under the influence of all three steroids. Hence maximum glycogen concentrations were seen at six cycles in these tissues (figure 45). The converse effect was apparent in cardiac muscle. In this tissue glycogen levels at six cycles, although still higher than control levels, were decreasing (figure 45). In skeletal and abdominal muscle P and N -induced glycogenesis increased, whereas NA-induced glycogenesis decreased with treatment duration (figures 42 - 44). NA elicited a faster and greater elevation of glycogen than other progestogens. This is evident from the higher glycogen levels in all tissues after one cycle of treatment and at subsequent test periods (figure 45).

The administration route also affected glycogen deposition.

Differences were predominant in the NA treated mice which showed higher glycogen values after Sc administration in each of the tissues examined (figure 44). A similar, but less obvious trend was seen following P treatment (figure 42). In mice treated with N, the oral route produced the greatest glycogen deposition, but changes associated with the different administration routes were not marked. The diverse effects of the administration route are further complicated by the observation that the smallest glycogen deposition differed in each group. The lowest value for P was oral; Sc for N; and Im for NA. It might be expected that orally administered P would produce the least effect as the hormone is metabolised more rapidly, and to a greater extent when given by this route than when given either by the Sc and Im routes. These results suggest glycolysis is stimulated by the unmetabolised P molecule as opposed to a P metabolite.

(iv) COMPARISON OF ESTROGEN AND PROGESTOGEN TREATMENT

In this section the changes of glycogen homeostasis produced by estrogen and progestogen administration are compared. Following estrogen treatment, the natural hormone produced the greatest stimulation, whereas following progestogen treatment, the natural hormone produced the smallest stimulation of glycogen deposition. The ability to increase tissue glycogen deposition was found to be E_2 > EE > NA > N > P > controls (figure 46). Thus it appears that in the tissues tested the extent of glycolysis was a function of estrogenicity. Comparison of the data indicates NA acted more like an estrogen than a progestogen with respect to glycogen deposition. For example, at no time were glycogen concentrations significantly lower than control levels (figure 44), whereas such decreases were often seen during both P and N therapy (figure 46). NA administration produced a more rapid and marked elevation of glycogenesis than either E_2 or EE

(figure 46). This result contrasted with the other progestogens which produced slow increases in glycogen deposition, usually after an initial lag or decline (figure 46 & 47).

Time-dependent alterations of glycogen metabolism were more consistent during estrogen than during progestogen treatment. At all times following estrogen administration, increases in glycogenesis parallel increases in the duration of treatment. This effect was observed after progestogen use only in liver and uterus (figure 46). In muscle, progestogen use frequently induced greatest glycogenesis four cycles (figure 47). It is of interest to note that the natural and synthetic estrogens produced similar time-dependent alterations of glycogen, as did the natural and synthetic progestogens, P and N (figures 46 & 47). The response seen during NA treatment was different, this steroid produced a marked and rapid response followed by a gradual fall as treatment progressed.

Few consistent trends were apparent from a comparison of the routes of administration. Generally the Sc route produced the greatest and the oral route the smallest glycogen deposition.

(v) COMBINED ESTROGEN AND PROGESTOGEN TREATMENT

This section describes the effects on glycogen metabolism produced by giving an estrogen and a progestogen in combination. The natural combination (E_2 and P) is shown in figure 48 and the synthetic combinations, EE and N; and EE and NA, are illustrated in figures 49 & 50 respectivly.

As previouly observed in the estrogen and progestogen treated mice, combined steroid treatment predominately raised glycogenesis above the controls. There were instances when significant elevations were not observed, but on no occasion did levels fall below the control levels. The natural preparation had a stronger glycogenic effect than either of

the synthetic preparations (figure 51) in most tissues. However, in femoral and cardiac muscle the highest levels were seen in the mice receiving EE and NA (figure 51). EE and N treatment had the smallest stimulatory influence on glycogen deposition and was also slowest to promote this response (figure 49). Liver exhibited a greater response to both the natural and synthetic combinations than the other tissues examined (figures 48 - 50). E_2 and P treatment produced a 3-fold, EE and N a 2.3-fold and EE and NA a 2.8-fold elevation of hepatic glycogen.

Glycogen deposition was influenced by treatment duration. During EE and NA administration glycogenesis increased as the duration of treatment increased in all tissues (figure 50). The same trend was evident in femoral and abdominal muscle following E_2 and P administration (figure 48). During EE and N treatment, however, maximum deposition occurred at four cycles, and this trend was also seen in liver and muscle after E_2 and P use (figure 48 & 49). In all groups, significant elevations of glycogen were produced at all times in liver, but levels varied considerably in other tissues.

The administration route also affected glycogenesis. In both the E_2 and P, and EE and NA groups these differences were most clearly evident after six cycles of treatment. The converse effect was seen during EE and N use where differences were most obvious at one cycle. There were additional similarities in the E_2 and P, and EE and NA groups. For example, the oral route produced the highest glycogen levels and the Sc route the lowest levels. As noted previously, EE and N administration was different. Oral EE and N treatment produced the smallest deposition of glycogen. Differences were more variable in this group, the Im route produced the greatest glycogen levels in most tissues, but the Sc route produced the highest levels in liver and uterus (figure 49).

(vi) COMPARISON OF SINGLE AND COMBINED STEROID TREATMENTS

This section discusses a comparison of the effect of estrogens and progestogens on glycogen metabolism when given alone or together in combined preparations.

A comparison of the natural hormones is illustrated in figure 52. E_2 administered alone produced the highest elevations of glycogen in all tissues, and the smallest rises were produced by P. The preparation containing both hormones elicited an intermediate response (figure 52). Thus estrogen-induced glycogenesis was still present in the combined preparation, but to a lesser extent. In previous sections it was noted that P exerted a glycogenic effect (see figure 42). Thus it appears that in the E_2 and P regime, P partially antagonises the glycogenic action of E_2, but not sufficiently to completly abolish the effect of the estrogen.

The time-dependent alterations of glycogen metabolism were similar in all three treatment regimes. Glycogen deposition generally increased as treatment duration increased (figure 52), but there were exceptions to this trend following combined E_2 and P administration (figure 48). The length of treatment required to elicit a significant elevation above the control levels varied in the three groups. The quickest response occurred during combined therapy and the slowest during P administration. These results suggest that initially there was little antagonism between the two hormones and that it developed as treatment length extended.

The influence of the administration route on glycogenesis was altered when the steroids were given in combination. After single E_2 and P treatment, the Sc route produced the greatest glycogen deposition. In the combined preparation, the oral route was associated with the highest glycogen concentration and the Sc route the lowest. These results may be interpreted as being due to alterations in the

rate of steroid degradation by the liver. The greater load presented to the liver by giving the two hormones together may result in peripheral hormone concentrations that are considerably higher than those produced by the single steroid treatments.

Comparison of glycogen concentrations in EE and N administered alone or together is shown in figure 53. It shows that EE produced the highest levels of glycogen in all tissues. The lowest deposition was observed following combined EE and N treatment. Since both EE and N stimulate glycogenesis, and N is converted to EE in vivo (484 - 486), one might expect the combined treatment to produce glycogen levels that are considerably higher than those seen in the present study (see figure 49). Thus it appears that N partially antagonises the glycogenic action of EE, but does not completely abolish the effect of the estrogen.

During EE and N treatment glycogenesis increased as treatment duration increased. The reverse effect, however, was seen following combined EE and N administration. In these mice glycogen levels declined at six cycles to values which were not significantly different to the controls (figure 49). The time required to stimulate glycogenesis varied in the three regimes. The quickest response was produced by combined EE and N administration, and the slowest in the N treated mice. As previously seen with the natural steroids, it appears that initially there was little antagonism between the steroids in the combined preparation and that it developed as treatment progressed.

The influence of the administration route was similar in the EE and combined EE and N groups. Both treatments produced the highest glycogen levels during Im and the lowest during oral administration. The converse situation applied to N treatment, with the oral route producing the greatest elevation of glycogen and the Sc route the

smallest increases.

Comparison of glycogen deposition in EE, NA and combined EE and NA treated mice is shown in figure 54. This shows that all three treatment regimes produced glycogen levels that were significantly higher than the controls. The estrogen produced the greatest glycogen deposition in all tissues except liver. In this organ NA produced the highest levels (figure 54). The combined preparation, EE and NA, provoked the smallest response, and for reasons previously discussed it appears that partial antagonism occurs in these mice reducing, but not obliterating the response.

In both the EE and combined EE and NA groups glycogenesis increased with the duration of treatment (figure 54). This occurred in liver and uterus during NA administration. However, in the muscles NA treatment produced higher glycogen levels at four cycles than at six cycles. At six cycles nevertheless, glycogen levels were still significantly higher than the controls (figure 44). The time required to elicit a significant elevation was smallest following progestogen treatment (figure 54).

No consistent trends were apparent when the influence of the administration route was compared in these three treatment regimes.

Figure 37 Tissue glycogen levels at 1, 4 and 6 cycles in diestrus control mice (mean ± SEM, n = 5).

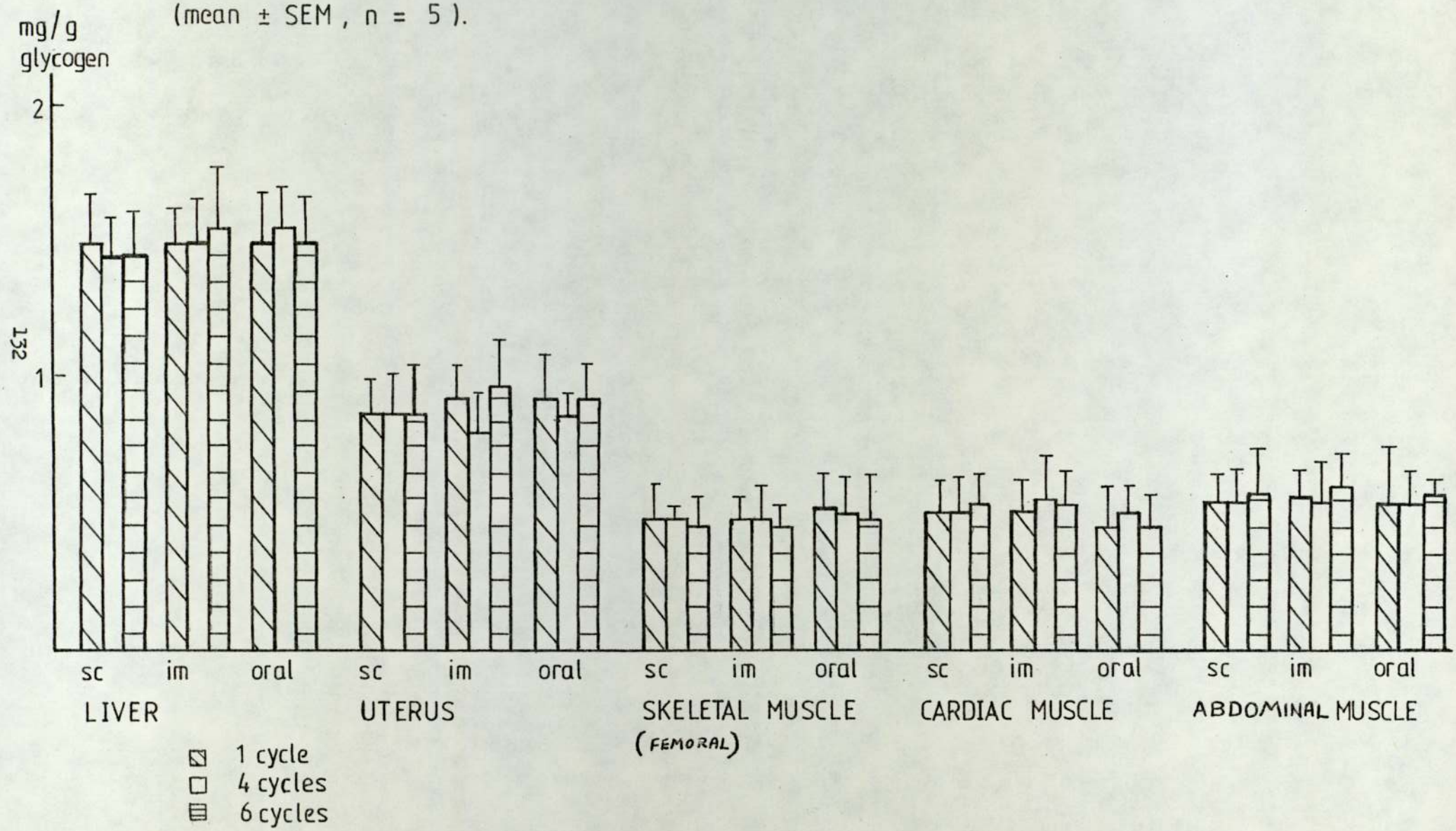


Figure 38 Effect of subcutaneous, intramuscular & oral estradiol (5ug/kg/day) treatment for 1, 4 & 6 cycles on tissue glycogen levels (mean ± SEM, n = 5).

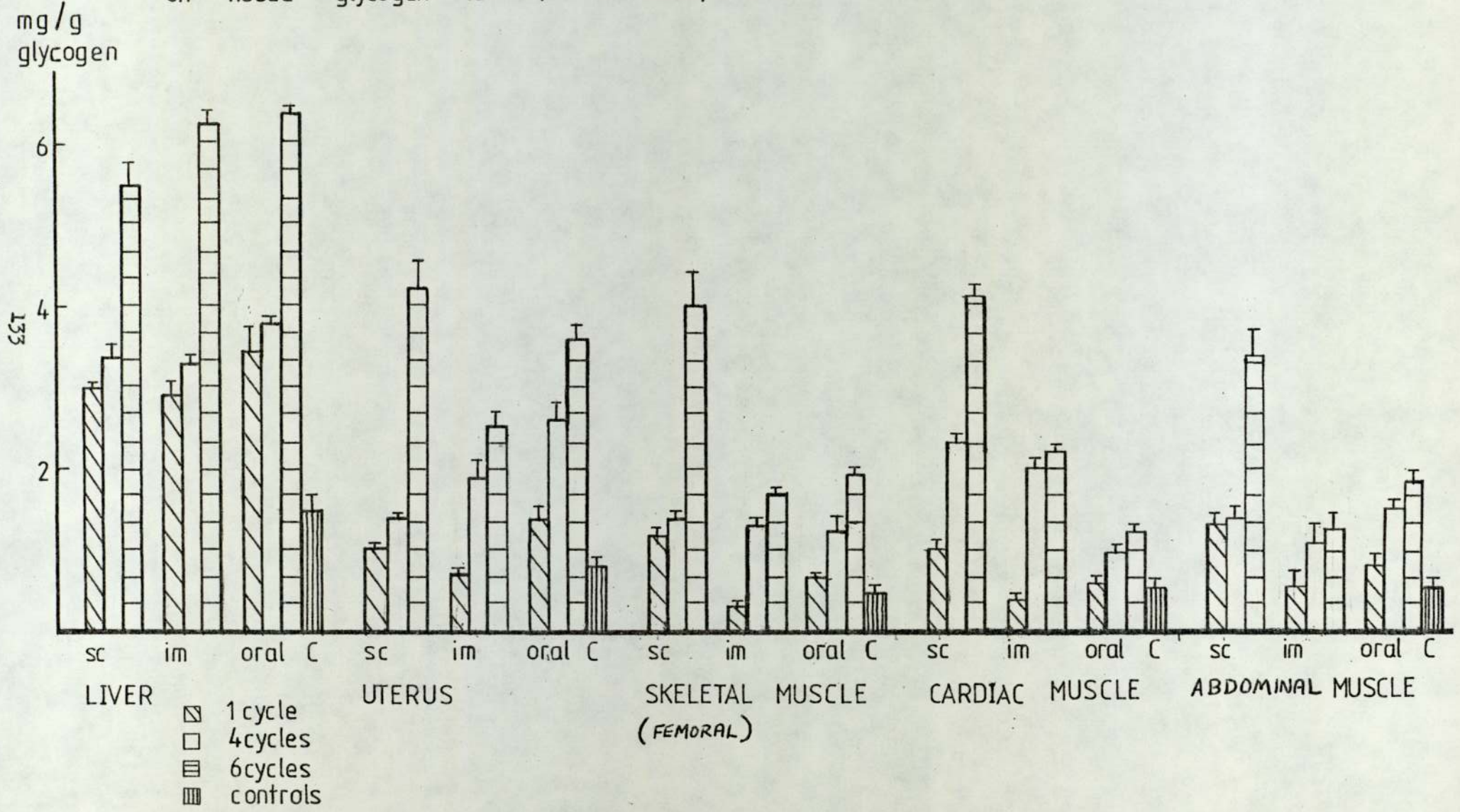


Figure 39 Effect of subcutaneous, intramuscular & oral ethynyl estradiol (5ug/kg/day) for 1, 4 & 6 cycles on tissue glycogen levels (mean ± SEM, n = 5).

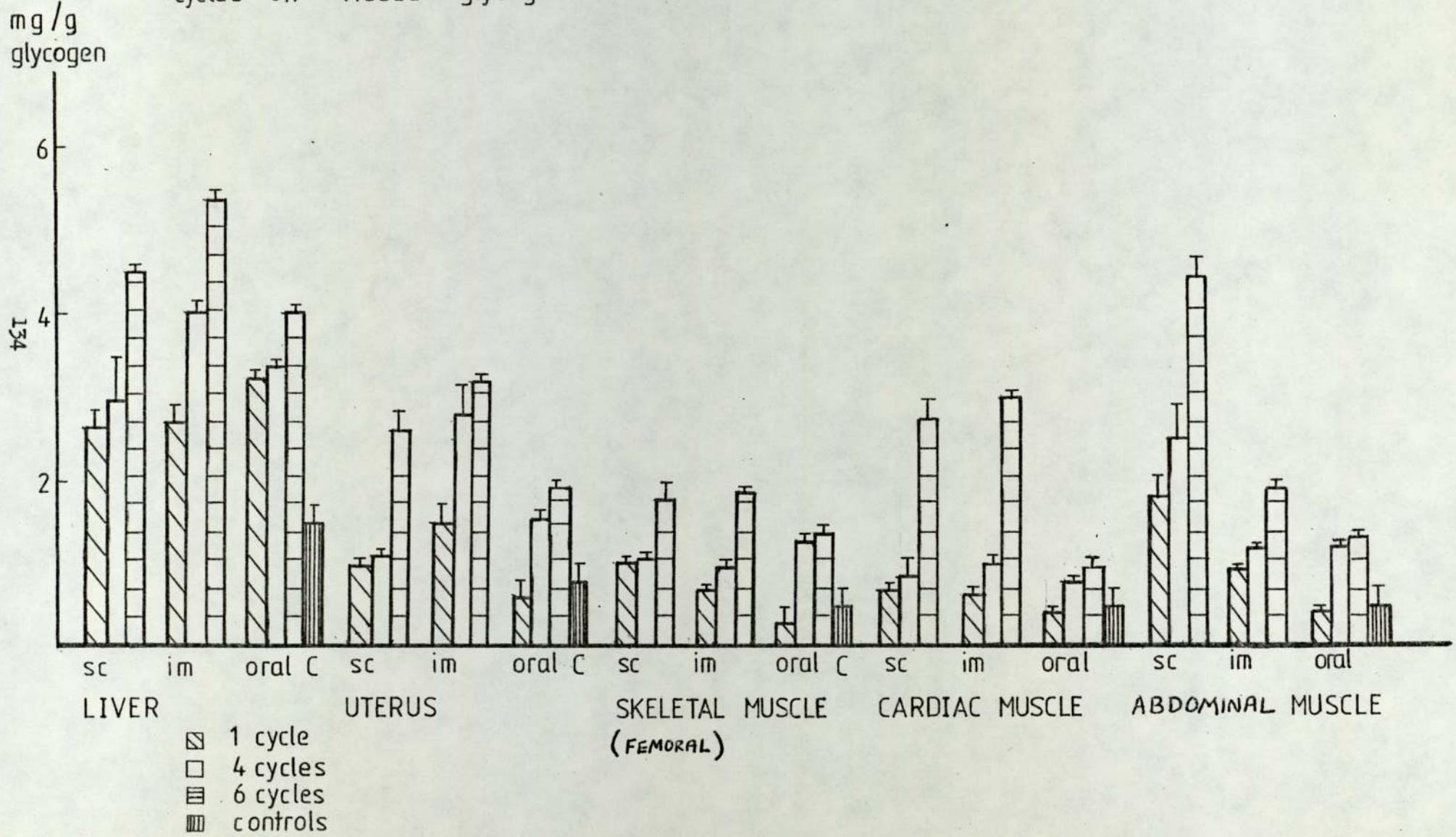


Figure 40 Comparison of liver glycogen levels during estradiol (5ug/kg/day) & ethynyl estradiol (5ug/kg/day) treatment at 1, 4 & 6 cycles (mean ± SEM, n = 5).

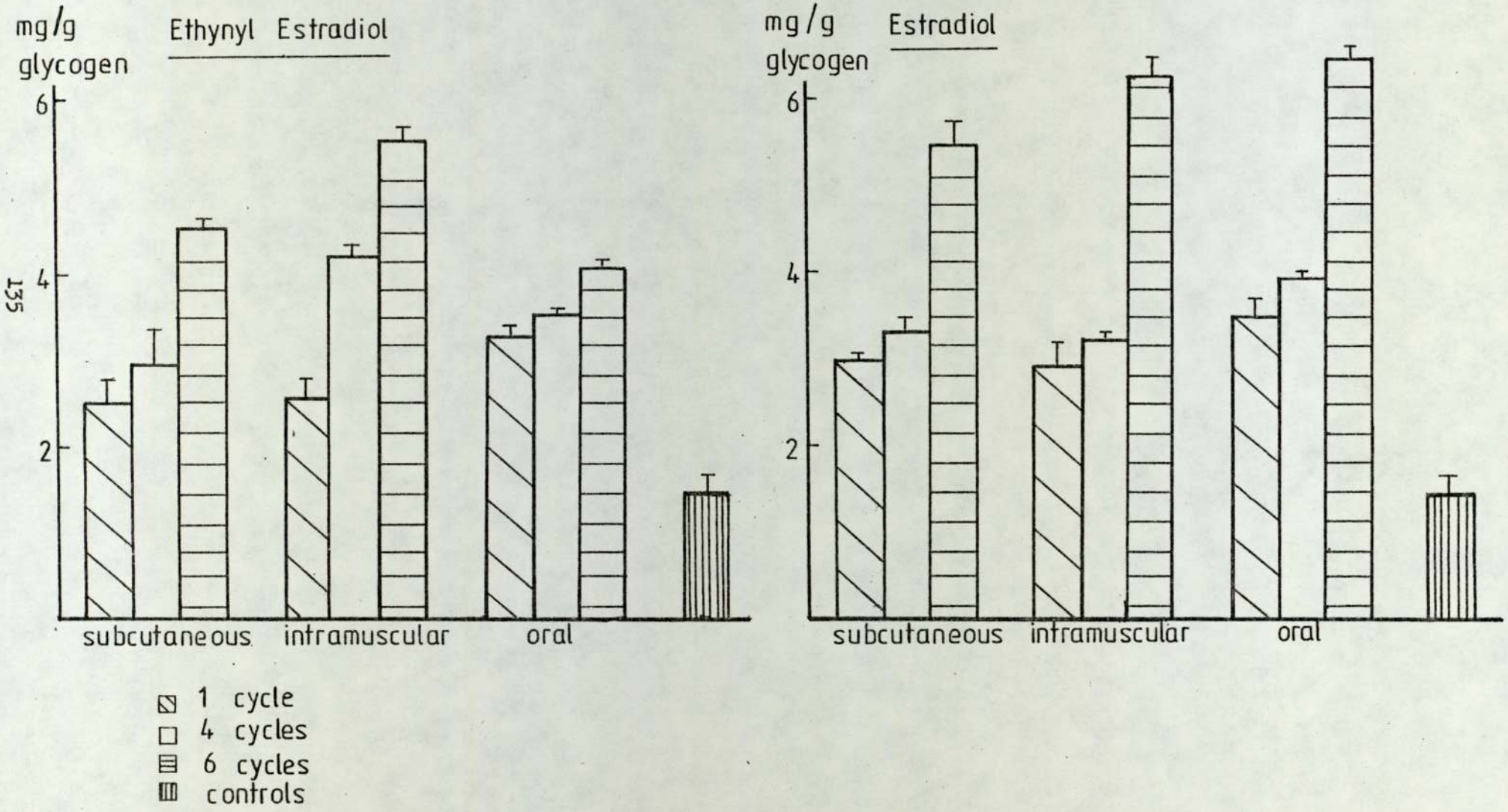


Figure 41 Comparison of uterine glycogen levels during estradiol (5ug/kg/day) & ethynyl estradiol (5ug/kg/day) treatment at 1, 4 & 6 cycles (mean ± SEM, n = 5).

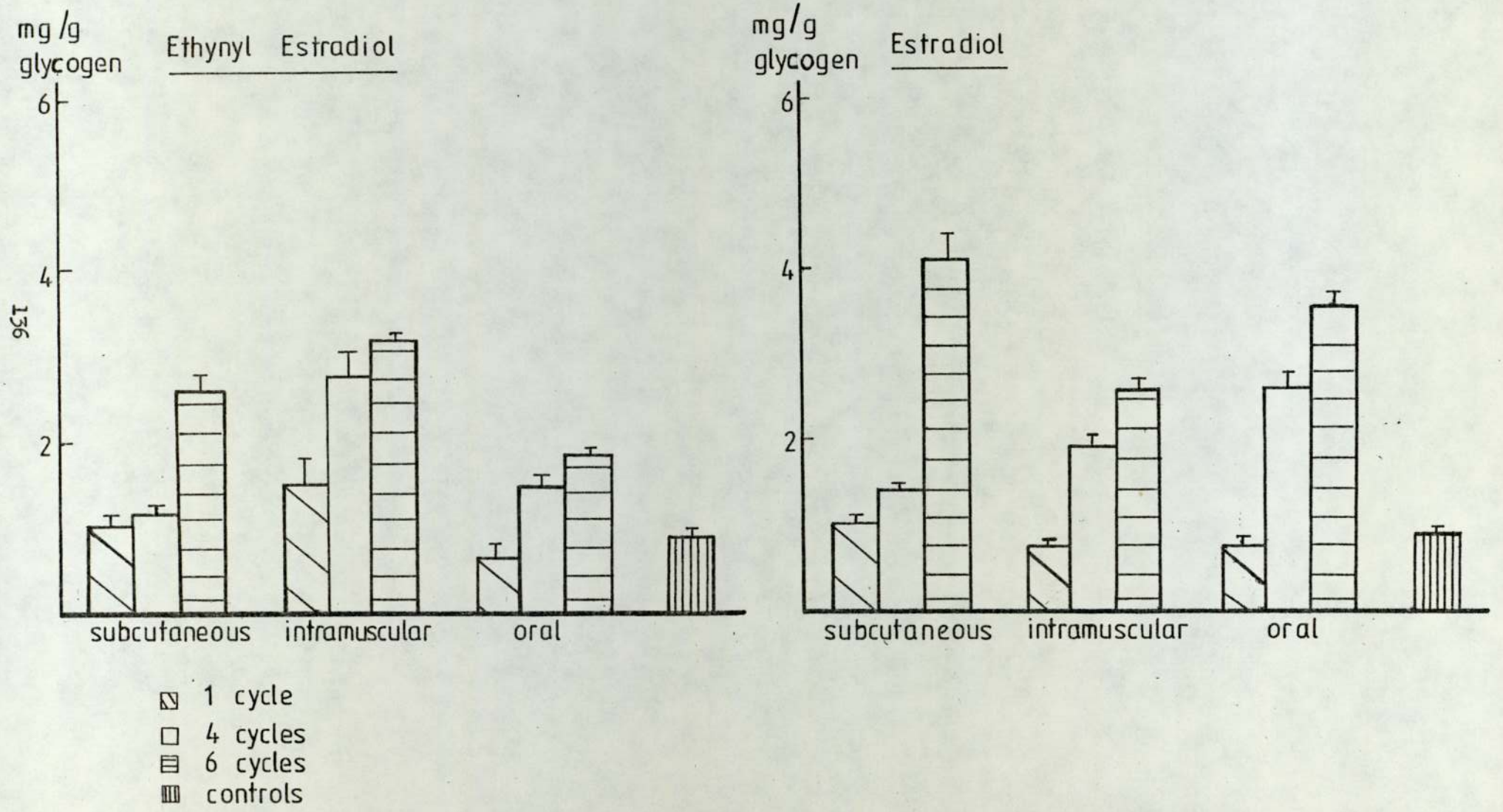


Figure 42 Effect of subcutaneous, intramuscular & oral progesterone (1mg/kg/day) treatment for 1, 4 & 6 cycles on tissue glycogen levels (mean ± SEM, n = 5).

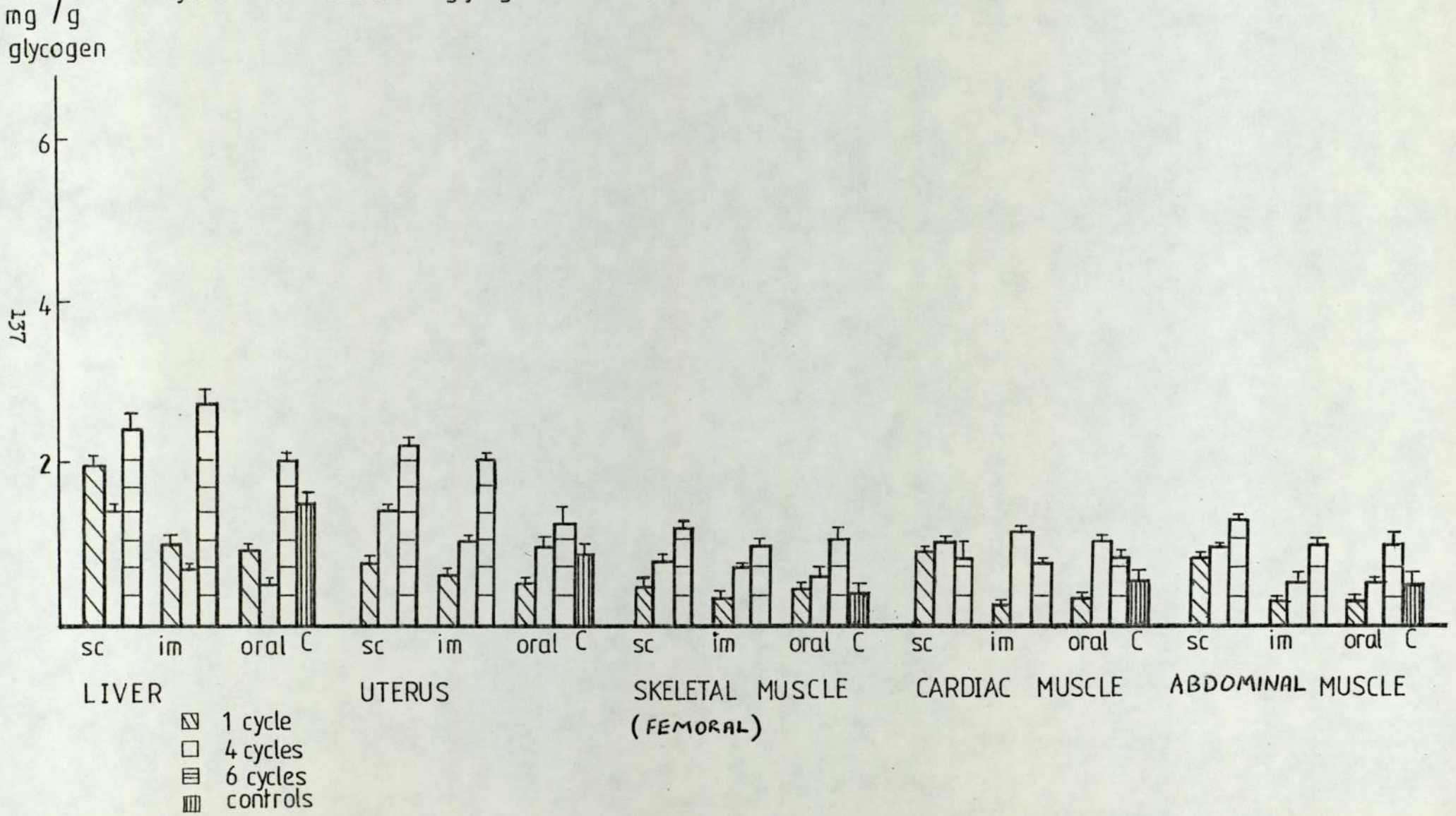


Figure 43 Effect of subcutaneous, intramuscular & oral norethnodrel (1mg / kg/day) treatment for 1, 4 & 6 cycles on tissue glycogen levels (mean ± SEM , n = 5).

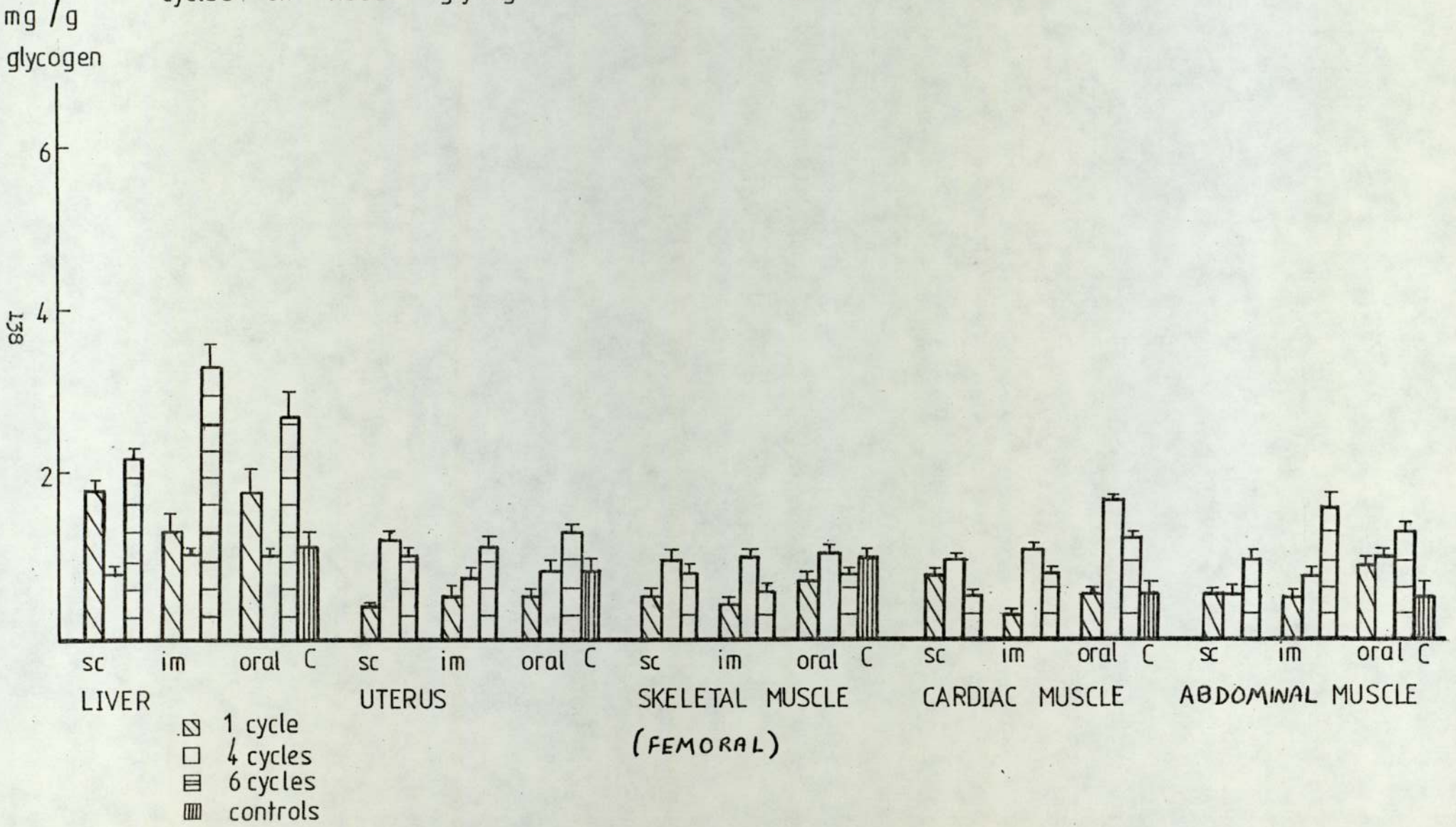


Figure 44 Effect of subcutaneous, intramuscular & oral norethisterone acetate (1mg/kg/day) for 1, 4 & 6 cycles on tissue glycogen levels (mean ± SEM, n = 5).

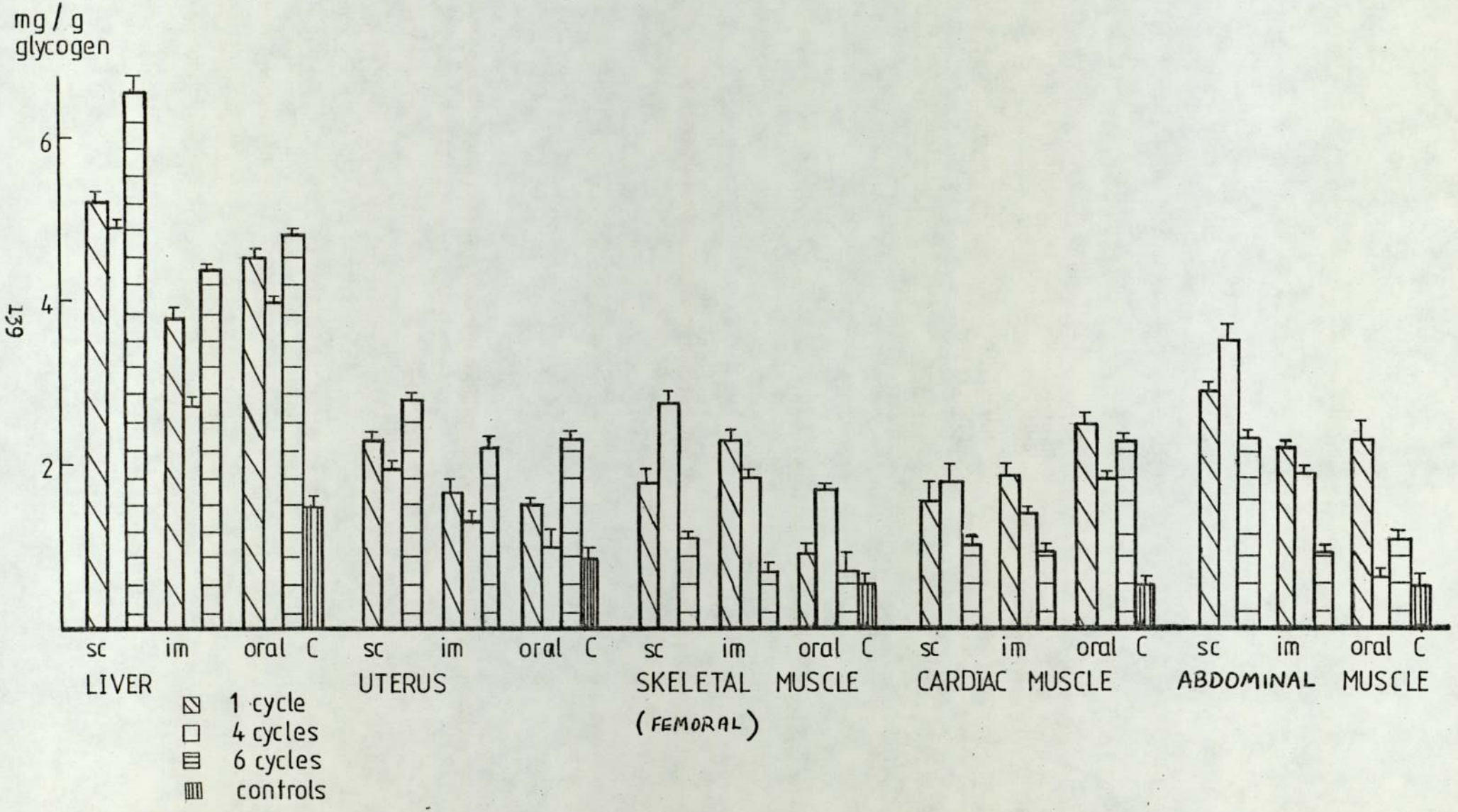


Figure 45 Comparison of tissue glycogen levels during progestogen (1mg/kg/day) treatments at 1, 4 & 6 cycles (mean ± SEM , n = 5).

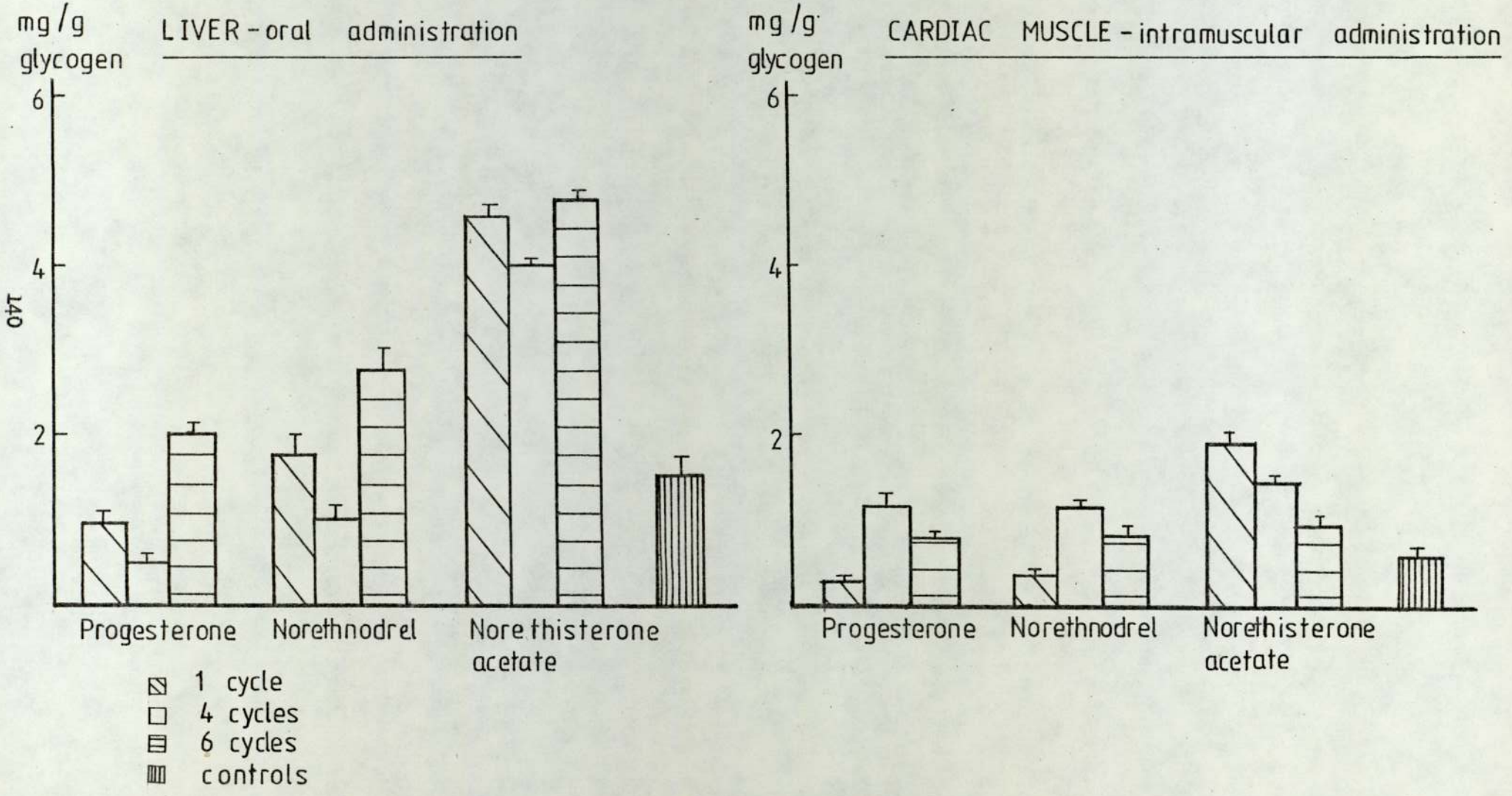


Figure 46 Comparison of oral estrogen (5ug /kg /day) & progestogen (1mg /kg /day) treatments on hepatic glycogen levels at 1, 4 & 6 cycles (mean ± SEM ,n = 5).

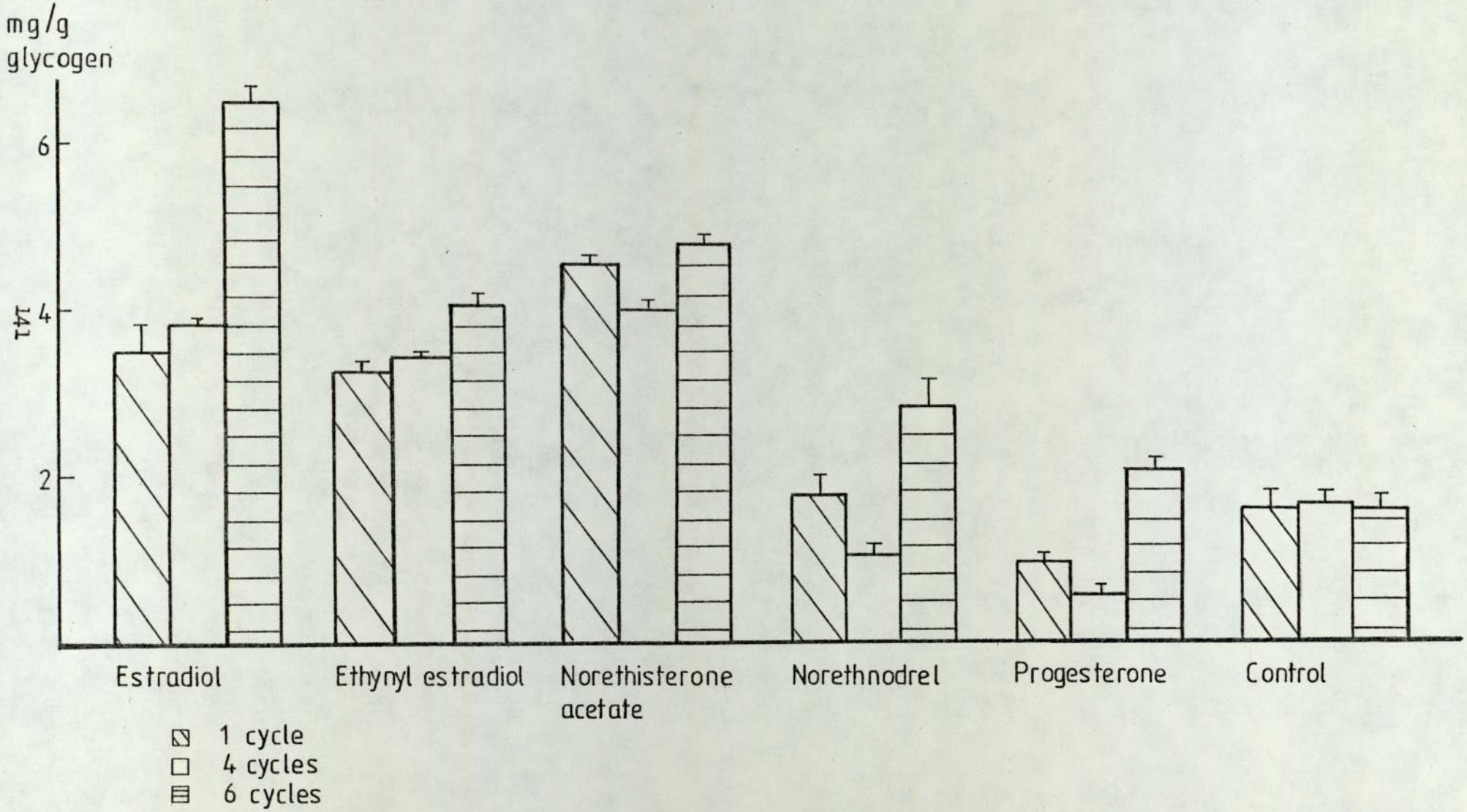


Figure 47 Comparison of intramuscular estrogen (5ug/kg/day) & progestogen (1mg/kg/day) treatments on cardiac muscle glycogen levels at 1, 4 & 6 cycles (mean ± SEM, n= 5).

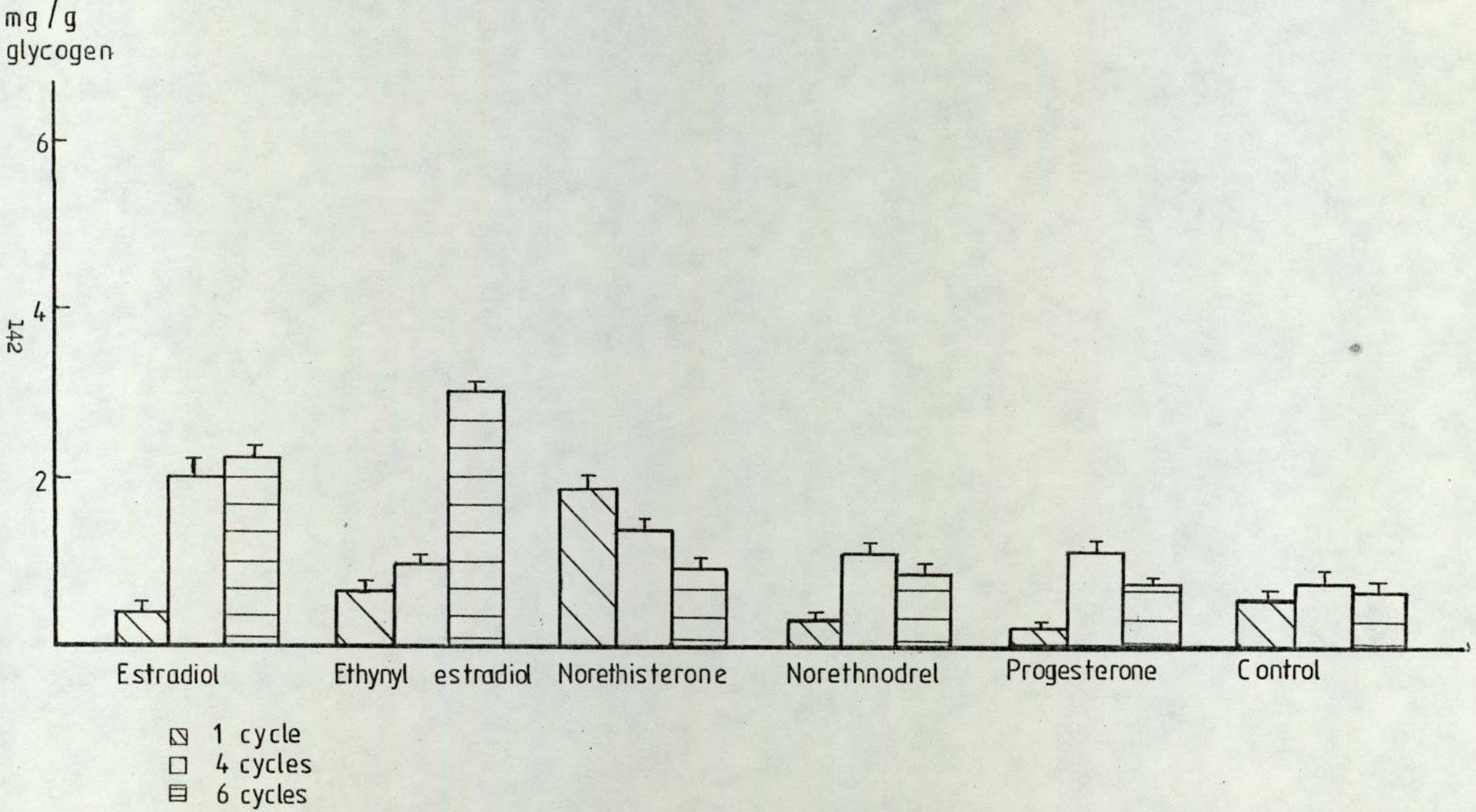


Figure 48 Effect of subcutaneous, intramuscular & oral estradiol + progesterone (5ug +1mg /kg/ day) treatment for 1, 4 & 6 cycles on tissue glycogen levels (mean ± SEM, n = 5).

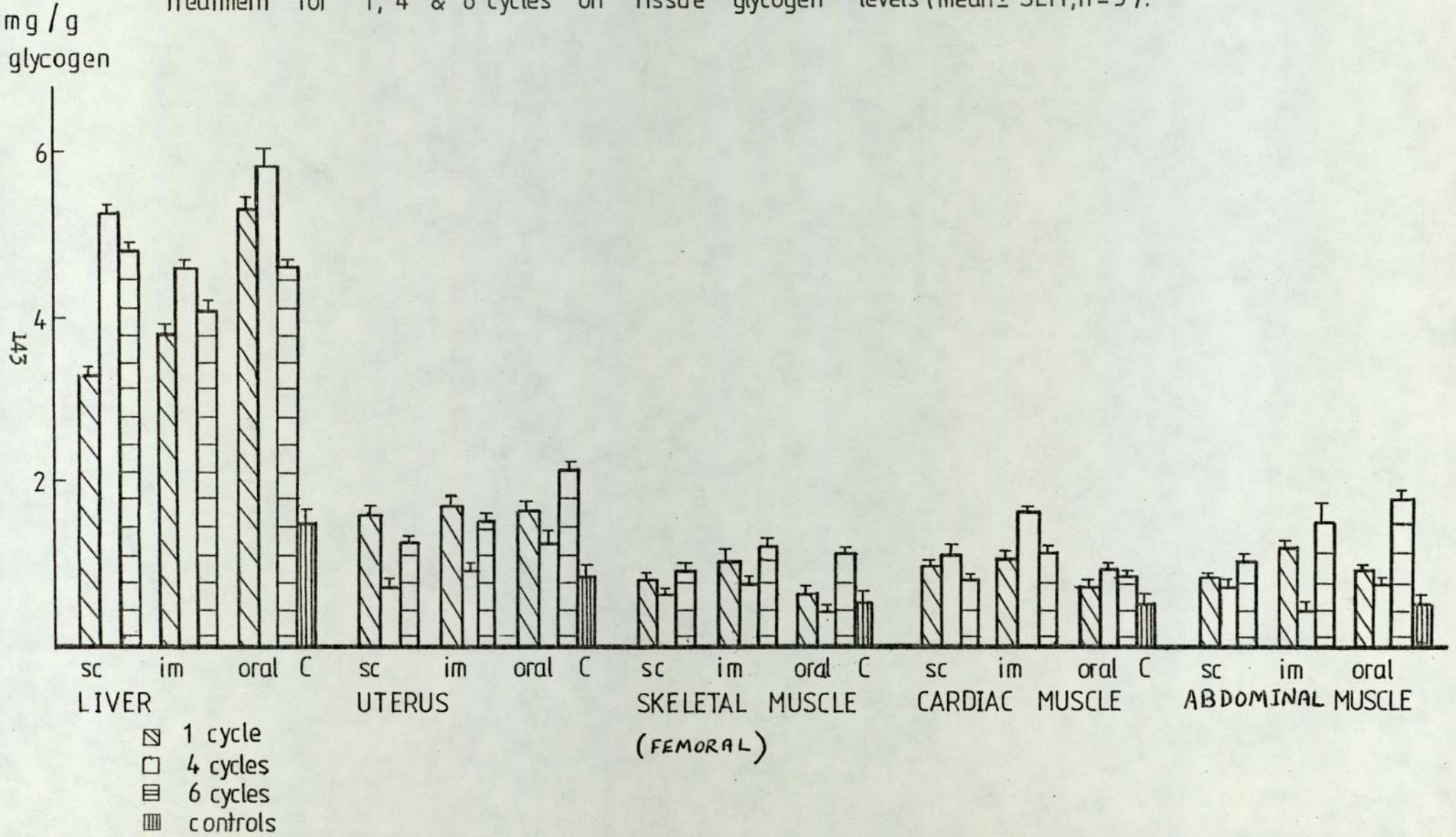


Figure 49 Effect of subcutaneous, intramuscular & oral ethynyl estradiol + norethnodrel (5ug+1mg/kg/day) treatment for 1,4 & 6 cycles on tissue glycogen levels (mean ± SEM, n = 5).

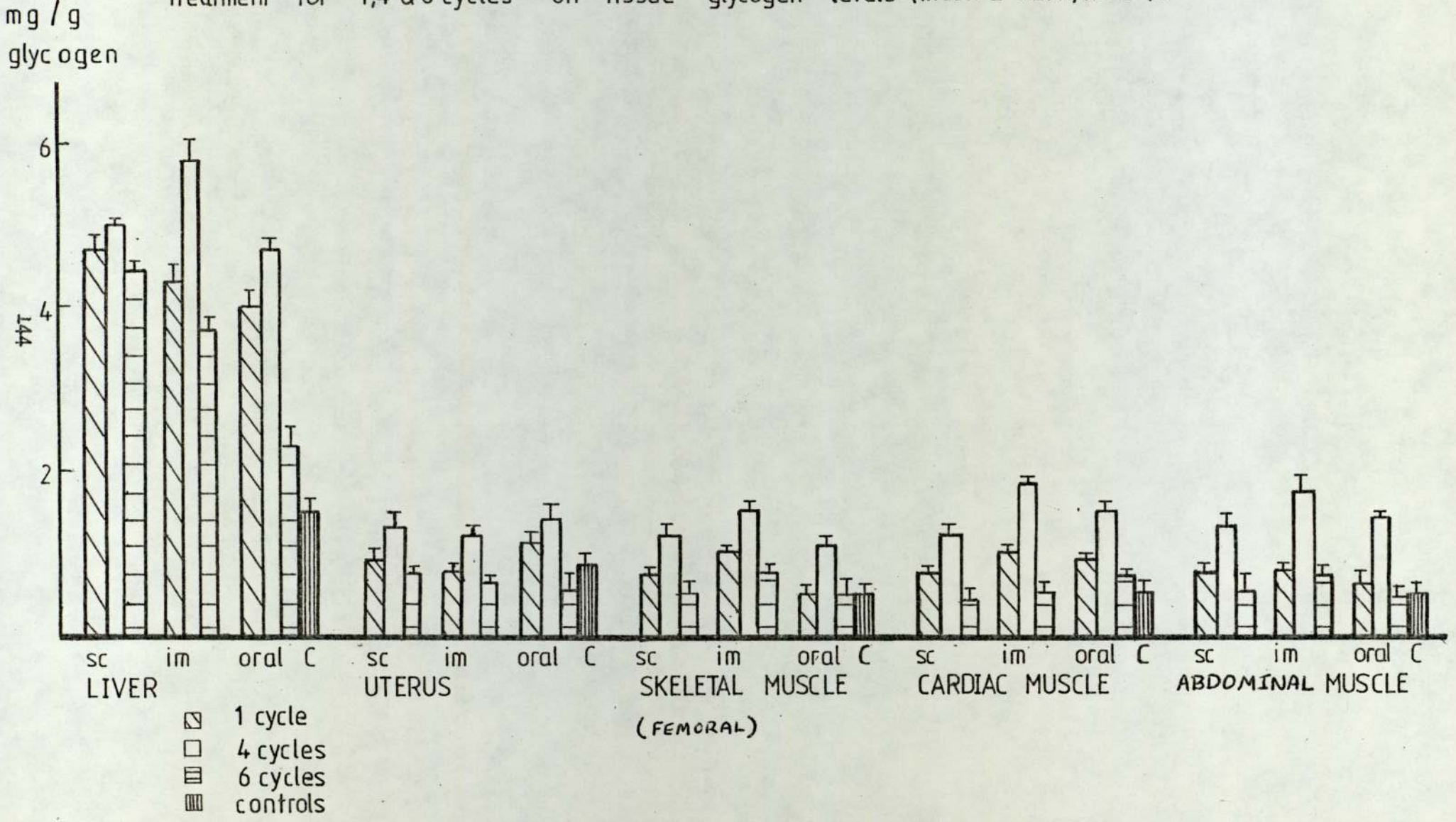


Figure 50 Effect of subcutaneous, intramuscular & oral ethynyl estradiol + norethisterone acetate (5ug+1mg/kg/day) treatment on tissue glycogen levels (mean ± SEM, n= 5).

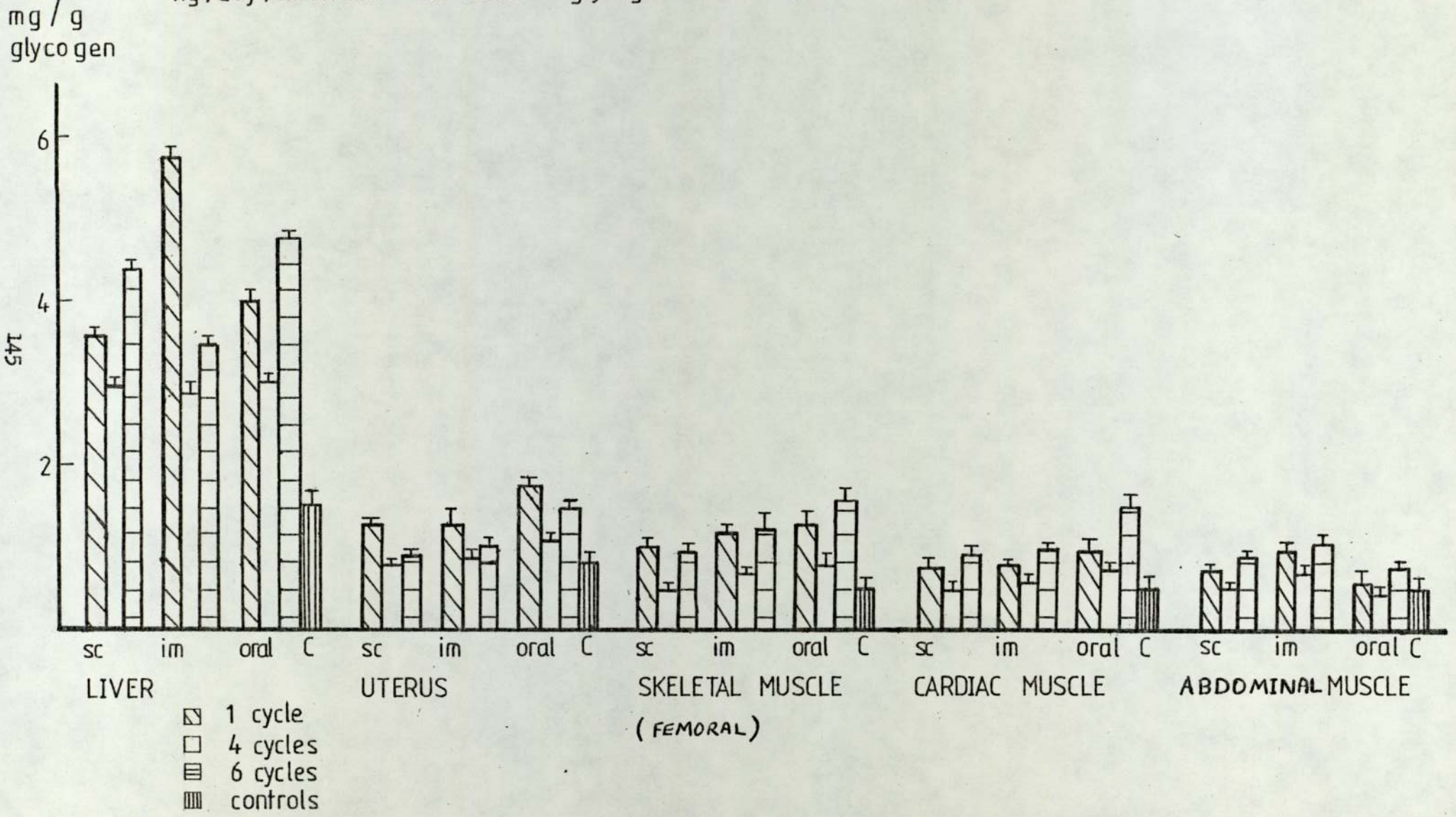


Figure 51 Comparison of oral combined preparation treatments on tissue glycogen levels at 1, 4 & 6 cycles (mean ± SEM, n = 5).

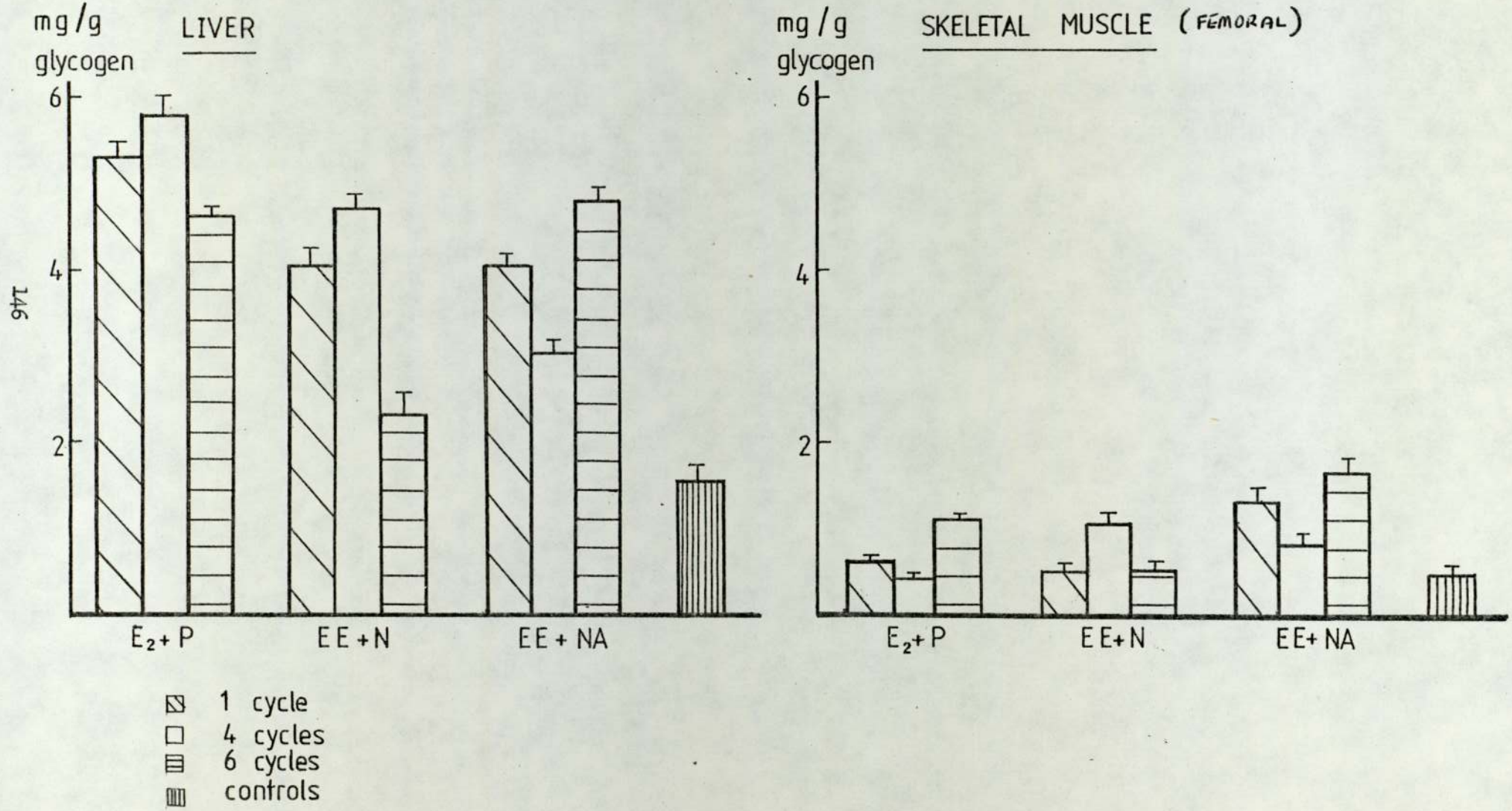


Figure 52 Comparison of hepatic glycogen levels during intramuscular single & combined sex steroid treatments at 1, 4 & 6 cycles (mean ± SEM, n = 5).

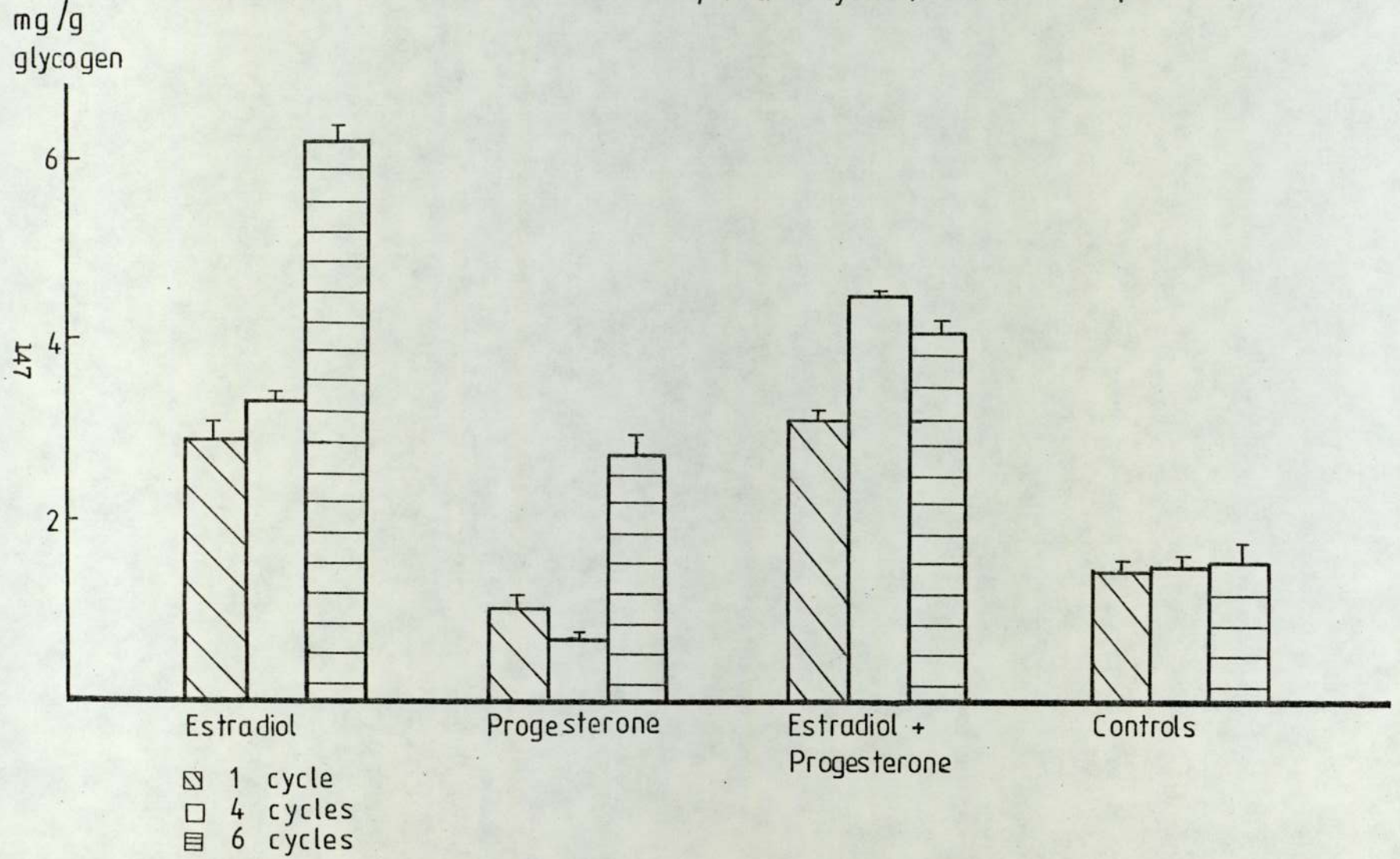


Figure 53 Comparison of uterine glycogen levels during subcutaneous single & combined sex steroid treatments at 1, 4 & 6 cycles (mean ± SEM, n = 5).

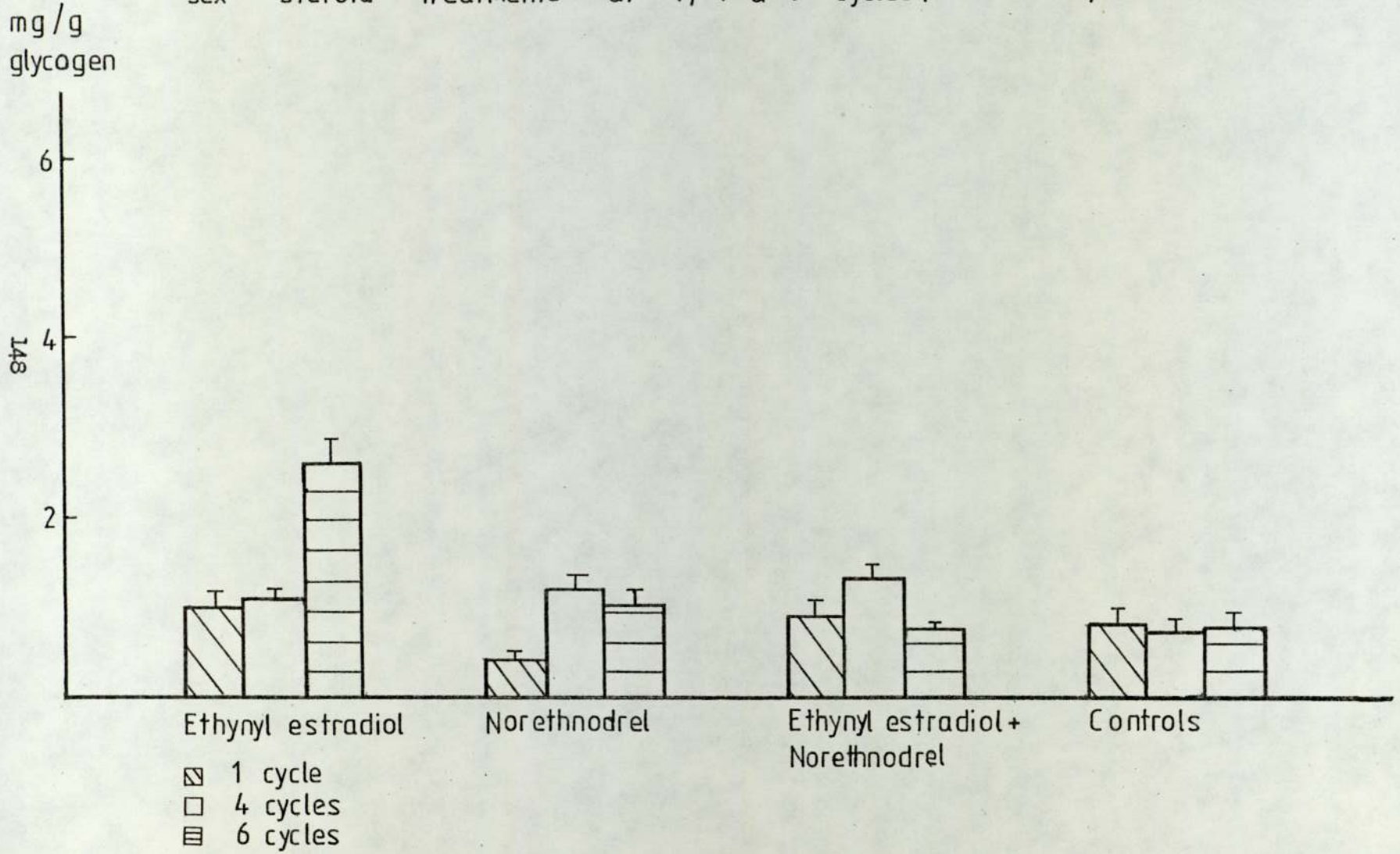


Figure 54 Comparison of glycogen levels during subcutaneous single & combined sex steroid treatments at 1, 4 & 6 cycles (mean ± SEM, n = 5).

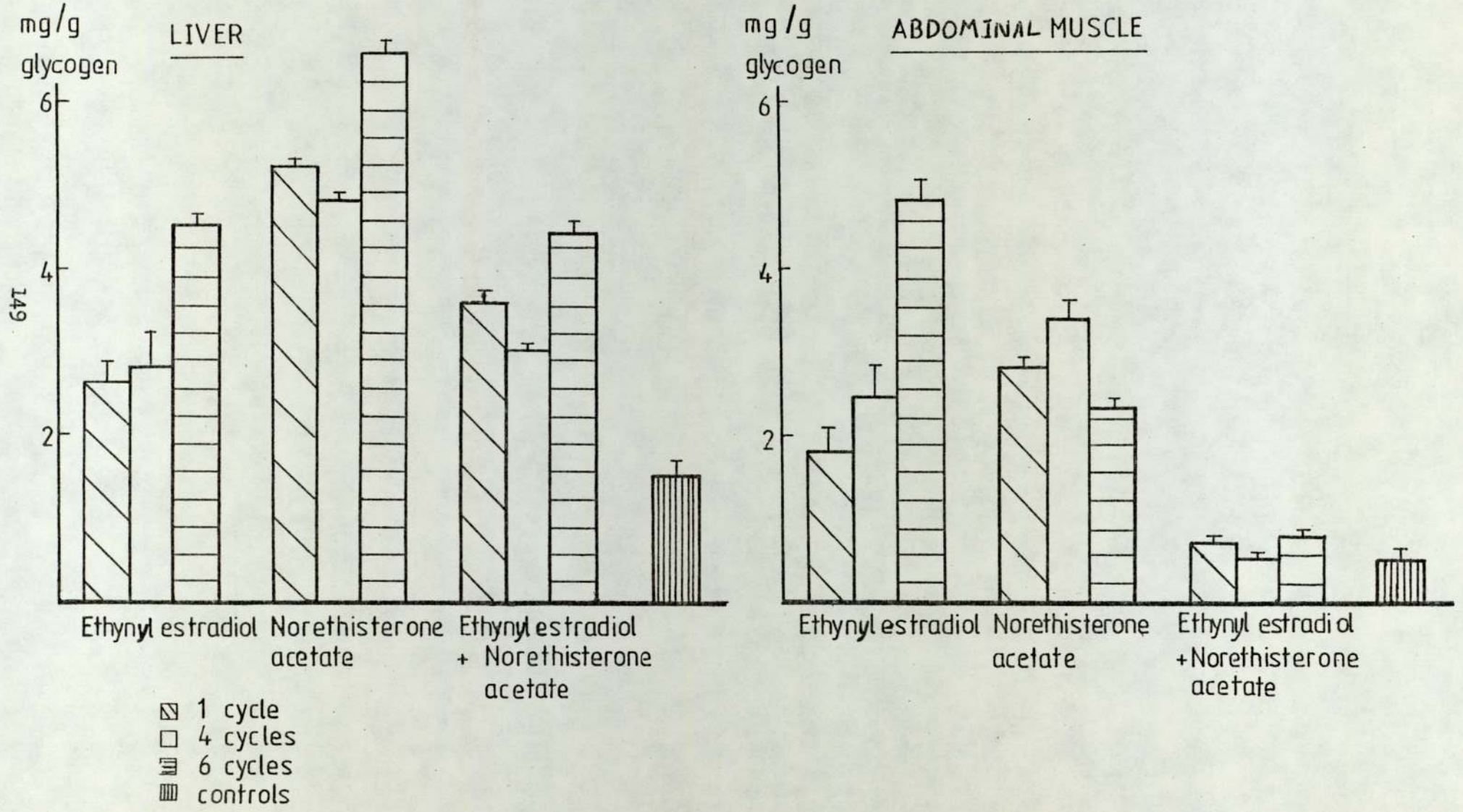


Table 5 TISSUE GLYCOGEN CONCENTRATIONS (n = 5) - CHAPTER 4

	GLYCOGEN (mg/g)				
	LIVER	UTERUS	FEMORAL MUSCLE	CARDIAC MUSCLE	ABDOMINAL MUSCLE
DIESTRUS CONTROLS					
subcutaneous					
1 cycle					
mean	1.5	0.84	0.48	0.5	0.52
SEM	0.18	0.12	0.12	0.11	0.1
4 cycles					
mean	1.4	0.84	0.48	0.5	0.52
SEM	0.14	0.14	0.04	0.13	0.12
6 cycles					
mean	1.48	0.84	0.46	0.52	0.56
SEM	0.16	0.21	0.1	0.1	0.16
intramuscular					
1 cycle					
mean	1.5	0.86	0.48	0.5	0.54
SEM	0.14	0.12	0.08	0.11	0.1
4 cycles					
mean	1.52	0.8	0.48	0.54	0.52
SEM	0.16	0.14	0.12	0.16	0.15
6 cycles					
mean	1.56	0.9	0.46	0.52	0.6
SEM	0.23	0.17	0.08	0.12	0.12
oral					
1 cycle					
mean	1.52	0.86	0.52	0.48	0.52
SEM	0.18	0.16	0.12	0.15	0.2
4 cycles					
mean	1.54	0.84	0.5	0.5	0.52
SEM	0.14	0.08	0.13	0.09	0.12
6 cycles					
mean	1.52	0.86	0.48	0.48	0.56
SEM	0.17	0.12	0.16	0.12	0.05
ESTRADIOL-17β					
subcutaneous					
1 cycle					
mean	2.98	0.98	1.24	1.02	1.3
SEM	0.08	0.08	0.14	0.12	0.13
4 cycles					
mean	3.38	1.4	1.48	2.34	1.36
SEM	0.15	0.06	0.08	0.1	0.14
6 cycles					
mean	5.46	4.2	3.92	4.12	3.4
SEM	0.33	0.39	0.43	0.17	0.36
intramuscular					
1 cycle					
mean	2.9	0.68	0.32	0.44	0.6
SEM	0.26	0.1	0.08	0.1	0.2

Table 5 contn.

	GLYCOGEN (mg/g)				
	LIVER	UTERUS	FEMORAL MUSCLE	CARDIAC MUSCLE	ABDOMINAL MUSCLE
ESTRADIOL-17β					
intramuscular					
4 cycles					
mean	3.34	1.92	1.28	2.04	1.08
SEM	0.1	0.2	0.15	0.19	0.21
6 cycles					
mean	6.24	2.54	1.74	2.24	1.16
SEM	0.24	0.27	0.12	0.16	0.22
oral					
1 cycle					
mean	3.52	0.7	0.7	0.58	0.92
SEM	0.33	0.14	0.11	0.08	0.12
4 cycles					
mean	3.82	2.58	1.16	1.02	1.54
SEM	0.04	0.29	0.17	0.08	0.1
6 cycles					
mean	6.42	3.6	1.9	1.2	1.92
SEM	0.16	0.19	0.14	0.11	0.17
ETHYNYL ESTRADIOL					
subcutaneous					
1 cycle					
mean	2.64	0.98	0.94	0.72	1.84
SEM	0.23	0.1	0.14	0.12	0.29
4 cycles					
mean	2.94	1.1	0.96	0.86	2.48
SEM	0.57	0.09	0.14	0.25	0.4
6 cycles					
mean	4.52	2.62	1.78	2.72	4.44
SEM	0.12	0.29	0.29	0.28	0.27
intramuscular					
1 cycle					
mean	2.7	1.52	0.74	0.64	0.92
SEM	0.28	0.27	0.04	0.1	0.08
4 cycles					
mean	4.18	2.74	0.96	1.0	1.04
SEM	0.17	0.29	0.16	0.09	0.08
6 cycles					
mean	5.42	3.22	1.94	3.0	1.88
SEM	0.18	0.08	0.05	0.09	0.12
oral					
1 cycle					
mean	3.22	0.6	0.34	0.44	0.42
SEM	0.12	0.17	0.12	0.05	0.08
4 cycles					
mean	3.4	1.54	1.32	0.92	1.18
SEM	0.06	0.1	0.08	0.08	0.13
6 cycles					
mean	4.04	1.84	1.36	1.0	1.3
SEM	0.1	0.1	0.08	0.09	0.09

Table 5 contn.

	GLYCOGEN (mg/g)				
	LIVER	UTERUS	FEMORAL MUSCLE	CARDIAC MUSCLE	ABDOMINAL MUSCLE
PROGESTERONE					
subcutaneous					
1 cycle					
mean	1.98	0.76	0.46	0.92	0.82
SEM	0.19	0.16	0.14	0.08	0.04
4 cycles					
mean	1.4	1.42	0.74	0.96	0.96
SEM	0.11	0.12	0.1	0.08	0.05
6 cycles					
mean	2.42	2.2	1.16	0.82	1.3
SEM	0.18	0.18	0.17	0.16	0.06
intramuscular					
1 cycle					
mean	0.96	0.66	0.36	0.22	0.32
SEM	0.16	0.1	0.1	0.04	0.12
4 cycles					
mean	0.7	1.08	0.7	1.12	0.52
SEM	0.06	0.1	0.06	0.13	0.1
6 cycles					
mean	2.73	2.04	0.98	0.74	0.98
SEM	0.21	0.1	0.08	0.05	0.08
oral					
1 cycle					
mean	0.94	0.48	0.4	0.32	0.34
SEM	0.1	0.12	0.09	0.08	0.05
4 cycles					
mean	0.48	0.94	0.58	1.02	0.48
SEM	0.12	0.1	0.08	0.08	0.08
6 cycles					
mean	2.0	1.26	1.06	0.82	0.96
SEM	0.11	0.21	0.16	0.08	0.16
NORETHNODREL					
subcutaneous					
1 cycle					
mean	1.82	0.4	0.52	0.8	0.54
SEM	0.19	0.06	0.08	0.06	0.05
4 cycles					
mean	0.84	1.2	0.9	0.98	0.52
SEM	0.1	0.14	0.11	0.08	0.08
6 cycles					
mean	2.18	1.04	0.82	0.48	0.92
SEM	0.13	0.14	0.08	0.04	0.08
intramuscular					
1 cycle					
mean	1.34	0.52	0.42	0.3	0.52
SEM	0.27	0.12	0.16	0.06	0.08
4 cycles					
mean	0.92	0.78	1.0	1.08	0.74
SEM	0.1	0.08	0.06	0.08	0.05
6 cycles					
mean	3.32	1.2	0.56	0.84	1.6
SEM	0.2	0.11	0.05	0.12	0.11

Table 5 contn.

	GLYCOGEN (mg/g)				
	LIVER	UTERUS	FEMORAL MUSCLE	CARDIAC MUSCLE	ABDOMINAL MUSCLE
NORETHNODREL					
oral					
1 cycle					
mean	1.74	0.5	0.68	0.5	0.88
SEM	0.29	0.11	0.08	0.13	0.08
4 cycles					
mean	1.04	0.84	1.06	1.7	1.0
SEM	0.15	0.1	0.14	0.07	0.09
6 cycles					
mean	2.74	1.26	0.84	1.16	1.32
SEM	0.31	0.1	0.05	0.05	0.08
NORETHISTERONE ACETATE					
subcutaneous					
1 cycle					
mean	5.24	2.34	1.8	1.58	2.9
SEM	0.15	0.15	0.16	0.25	0.14
4 cycles					
mean	4.95	1.94	2.72	1.82	3.46
SEM	0.1	0.12	0.17	0.21	0.2
6 cycles					
mean	6.58	2.82	1.1	1.02	2.34
SEM	0.2	0.08	0.13	0.12	0.1
intramuscular					
1 cycle					
mean	3.8	1.68	2.24	1.86	2.2
SEM	0.11	0.21	0.16	0.17	0.14
4 cycles					
mean	2.72	1.38	1.86	1.44	1.82
SEM	0.17	0.12	0.1	0.08	0.08
6 cycles					
mean	4.34	2.16	0.74	0.9	0.94
SEM	0.08	0.1	0.12	0.09	0.14
oral					
1 cycle					
mean	4.56	1.54	1.42	2.52	2.3
SEM	0.1	0.12	0.17	0.19	0.21
4 cycles					
mean	3.98	0.96	1.7	1.84	0.66
SEM	0.08	0.21	0.11	0.1	0.1
6 cycles					
mean	4.76	2.32	0.74	2.3	1.12
SEM	0.1	0.16	0.28	0.14	0.13

Table 5 contn.

	GLYCOGEN (mg/g)				
	LIVER	UTERUS	FEMORAL MUSCLE	CARDIAC MUSCLE	ABDOMINAL MUSCLE
ESTRADIOL + PROGESTERONE					
subcutaneous					
1 cycle					
mean	3.32	1.64	0.84	0.96	0.84
SEM	0.12	0.1	0.05	0.08	0.05
4 cycles					
mean	5.24	0.7	0.68	1.08	0.72
SEM	0.1	0.11	0.1	0.1	0.08
6 cycles					
mean	4.84	1.26	0.94	0.82	0.98
SEM	0.17	0.05	0.1	0.08	0.08
intramuscular					
1 cycle					
mean	3.8	1.68	1.02	1.06	1.12
SEM	0.13	0.08	0.15	0.1	0.08
4 cycles					
mean	4.56	0.92	0.74	1.6	0.42
SEM	0.08	0.12	0.08	0.06	0.08
6 cycles					
mean	4.12	1.52	1.2	1.12	1.54
SEM	0.19	0.12	0.09	0.08	0.21
oral					
1 cycle					
mean	5.32	1.66	0.62	0.68	0.88
SEM	0.19	0.1	0.08	0.08	0.08
4 cycles					
mean	5.82	1.28	0.4	0.9	0.7
SEM	0.21	0.2	0.06	0.09	0.06
6 cycles					
mean	4.64	2.08	1.1	0.8	1.72
SEM	0.1	0.12	0.06	0.06	0.1
ETHYNYL ESTRADIOL + NORETHNODREL					
subcutaneous					
1 cycle					
mean	4.68	0.92	0.76	0.72	0.7
SEM	0.21	0.15	0.05	0.08	0.09
4 cycles					
mean	5.0	1.34	1.2	1.2	1.32
SEM	0.09	0.17	0.13	0.14	0.12
6 cycles					
mean	4.4	0.78	0.54	0.42	0.52
SEM	0.14	0.08	0.14	0.15	0.15
intramuscular					
1 cycle					
mean	4.3	0.82	0.98	1.0	0.78
SEM	0.21	0.08	0.08	0.09	0.08

Table 5 contn.

	GLYCOGEN (mg/g)				
	LIVER	UTERUS	FEMORAL MUSCLE	CARDIAC MUSCLE	ABDOMINAL MUSCLE
ETHYNYL ESTRADIOL + NORETHNODREL					
intramuscular					
4 cycles					
mean	5.78	1.22	1.46	1.82	1.68
SEM	0.23	0.08	0.1	0.08	0.15
6 cycles					
mean	3.72	0.6	0.76	0.52	0.86
SEM	0.12	0.06	0.1	0.12	0.08
oral					
1 cycle					
mean	4.06	1.14	0.52	0.86	0.64
SEM	0.19	0.1	0.12	0.1	0.12
4 cycles					
mean	4.74	1.4	1.08	1.5	1.4
SEM	0.14	0.09	0.12	0.09	0.06
6 cycles					
mean	2.32	0.56	0.5	0.72	0.46
SEM	0.23	0.14	0.14	0.08	0.1
ETHYNYL ESTRADIOL + NORETHISTERONE ACETATE					
subcutaneous					
1 cycle					
mean	3.58	1.26	1.02	0.78	0.74
SEM	0.13	0.08	0.13	0.08	0.08
4 cycles					
mean	2.96	0.72	0.46	0.52	0.54
SEM	0.1	0.1	0.08	0.1	0.08
6 cycles					
mean	4.42	0.88	0.94	0.9	0.92
SEM	0.17	0.08	0.08	0.09	0.08
intramuscular					
1 cycle					
mean	5.8	1.3	1.08	0.86	0.92
SEM	0.14	0.18	0.08	0.05	0.02
4 cycles					
mean	2.92	0.9	0.66	0.6	0.74
SEM	0.12	0.09	0.05	0.09	0.08
6 cycles					
mean	3.5	1.08	1.22	1.0	1.06
SEM	0.14	0.12	0.15	0.09	0.1
oral					
1 cycle					
mean	4.06	1.74	1.32	0.98	0.6
SEM	0.14	0.1	0.12	0.12	0.11

Table 5 contn.

	GLYCOGEN (mg/g)				
	LIVER	UTERUS	FEMORAL MUSCLE	CARDIAC MUSCLE	ABDOMINAL MUSCLE
ETHYNYL ESTRADIOL + NORETHISTERONE ACETATE oral					
4 cycles					
mean	3.08	1.12	0.82	0.76	0.48
SEM	0.12	0.12	0.12	0.1	0.08
6 cycles					
mean	4.8	1.5	1.56	1.48	0.78
SEM	0.14	0.11	0.16	0.12	0.08

Table 6 TISSUE GLYCOGEN CONCENTRATIONS - STATISTICAL COMPARISON WITH DIESTRUS CONTROLS - CHAPTER 4

	GLYCOGEN (mg/g)				
	LIVER	UTERUS	FEMORAL MUSCLE	CARDIAC MUSCLE	ABDOMINAL MUSCLE
ESTRADIOL-17β					
subcutaneous					
1 cycle	$p < 0.001$	NS	$p < 0.001$	$p < 0.001$	$p < 0.001$
4 cycles	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$
6 cycles	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$
intramuscular					
1 cycle	$p < 0.001$	$p < 0.05$	$p < 0.05$	NS	NS
4 cycles	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.01$
6 cycles	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.002$
oral					
1 cycle	$p < 0.001$	NS	NS	NS	$p < 0.01$
4 cycles	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$
6 cycles	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$
ETHYNYL ESTRADIOL					
subcutaneous					
1 cycle	$p < 0.001$	NS	$p < 0.001$	$p < 0.05$	$p < 0.001$
4 cycles	$p < 0.001$	$p < 0.02$	$p < 0.001$	$p < 0.05$	$p < 0.001$
6 cycles	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$
intramuscular					
1 cycle	$p < 0.001$	$p < 0.002$	$p < 0.001$	NS	$p < 0.001$
4 cycles	$p < 0.001$	$p < 0.001$	$p < 0.002$	$p < 0.002$	$p < 0.001$
6 cycles	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$
oral					
1 cycle	$p < 0.001$	NS	NS	NS	NS
4 cycles	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$
6 cycles	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$
PROGESTERONE					
subcutaneous					
1 cycle	$p < 0.01$	NS	NS	$p < 0.001$	$p < 0.001$
4 cycles	NS	$p < 0.001$	$p < 0.002$	$p < 0.001$	$p < 0.001$
6 cycles	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.02$	$p < 0.001$
intramuscular					
1 cycle	$p < 0.002$	$p < 0.05$	NS	$p < 0.002$	$p < 0.05$
4 cycles	$p < 0.001$	$p < 0.02$	$p < 0.02$	$p < 0.001$	NS
6 cycles	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.01$	$p < 0.001$
oral					
1 cycle	$p < 0.001$	$p < 0.01$	NS	NS	NS
4 cycles	NS	NS	NS	$p < 0.001$	NS
6 cycles	$p < 0.002$	$p < 0.01$	$p < 0.001$	$p < 0.002$	$p < 0.01$

Table 6 contn.

	GLYCOGEN (mg/g)				
	LIVER	UTERUS	FEMORAL MUSCLE	CARDIAC MUSCLE	ABDOMINAL MUSCLE
NORETHNODREL					
subcutaneous					
1 cycle	$p < 0.05$	$p < 0.001$	NS	$p < 0.002$	NS
4 cycles	$p < 0.001$	$p < 0.01$	$p < 0.001$	$p < 0.001$	NS
6 cycles	$p < 0.001$	NS	$p < 0.001$	NS	$p < 0.01$
intramuscular					
1 cycle	NS	$p < 0.01$	NS	$p < 0.02$	NS
4 cycles	$p < 0.001$	$p < 0.02$	$p < 0.001$	$p < 0.001$	$p < 0.05$
6 cycles	$p < 0.001$	$p < 0.02$	NS	$p < 0.01$	$p < 0.001$
oral					
1 cycle	NS	$p < 0.01$	$p < 0.05$	NS	$p < 0.02$
4 cycles	$p < 0.002$	NS	$p < 0.001$	$p < 0.001$	$p < 0.001$
6 cycles	$p < 0.001$	$p < 0.001$	$p < 0.01$	$p < 0.001$	$p < 0.001$
NORETHISTERONE ACETATE					
subcutaneous					
1 cycle	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$
4 cycles	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$
6 cycles	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$
intramuscular					
1 cycle	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$
4 cycles	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$
6 cycles	$p < 0.001$	$p < 0.001$	$p < 0.01$	$p < 0.001$	$p < 0.001$
oral					
1 cycle	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$
4 cycles	$p < 0.001$	NS	$p < 0.001$	$p < 0.001$	NS
6 cycles	$p < 0.001$	$p < 0.001$	NS	$p < 0.001$	$p < 0.001$
ESTRADIOL + PROGESTERONE					
subcutaneous					
1 cycle	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$
4 cycles	$p < 0.001$	NS	$p < 0.02$	$p < 0.001$	$p < 0.02$
6 cycles	$p < 0.001$	$p < 0.01$	$p < 0.001$	$p < 0.02$	$p < 0.02$
intramuscular					
1 cycle	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$
4 cycles	$p < 0.001$	NS	$p < 0.01$	$p < 0.001$	NS
6 cycles	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$
oral					
1 cycle	$p < 0.001$	$p < 0.001$	NS	$p < 0.05$	$p < 0.02$
4 cycles	$p < 0.001$	$p < 0.01$	NS	$p < 0.001$	$p < 0.05$
6 cycles	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.002$	$p < 0.001$

Table 6 contn.

	GLYCOGEN (mg/g)				
	LIVER	UTERUS	FEMORAL MUSCLE	CARDIAC MUSCLE	ABDOMINAL MUSCLE
ETHYNYL ESTRADIOL + NORETHNODREL					
subcutaneous					
1 cycle	$p < 0.001$	NS	$p < 0.01$	$p < 0.01$	$p < 0.05$
4 cycles	$p < 0.001$	$p < 0.002$	$p < 0.001$	$p < 0.001$	$p < 0.001$
6 cycles	$p < 0.001$	NS	NS	NS	NS
intramuscular					
1 cycle	$p < 0.001$	NS	$p < 0.001$	$p < 0.001$	$p < 0.01$
4 cycles	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$
6 cycles	$p < 0.001$	$p < 0.02$	$p < 0.002$	NS	$p < 0.01$
oral					
1 cycle	$p < 0.001$	$p < 0.02$	NS	$p < 0.01$	NS
4 cycles	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$
6 cycles	$p < 0.001$	$p < 0.01$	NS	$p < 0.01$	NS
ETHYNYL ESTRADIOL + NORETHISTERONE ACETATE					
subcutaneous					
1 cycle	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.01$	$p < 0.01$
4 cycles	$p < 0.001$	NS	NS	NS	NS
6 cycles	$p < 0.001$	NS	$p < 0.001$	$p < 0.001$	$p < 0.01$
intramuscular					
1 cycle	$p < 0.001$	$p < 0.01$	$p < 0.001$	$p < 0.001$	$p < 0.001$
4 cycles	$p < 0.001$	NS	$p < 0.05$	NS	$p < 0.05$
6 cycles	$p < 0.001$	NS	$p < 0.001$	$p < 0.001$	$p < 0.001$
oral					
1 cycle	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	NS
4 cycles	$p < 0.001$	$p < 0.01$	$p < 0.01$	$p < 0.01$	NS
6 cycles	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.002$

Where diestrus controls, n = 5

steroid-treated mice, n = 5

$p < 0.05$ is significant

$p < 0.02$ & $p < 0.01$ are highly significant

$p < 0.002$ & $p < 0.001$ are very highly significant

Chapter 5

EFFECT OF SEX HORMONES AND OVARIECTOMY ON THE IN VIVO DISTRIBUTION OF ^{14}C-ESTRADIOL AND ^{14}C-PROGESTERONE

CHAPTER 5

IN VIVO DISTRIBUTION OF ^{14}C-ESTRADIOL & ^{14}C-PROGESTERONE

INTRODUCTION

The preceeding chapters have shown that the administration route of steroid hormones markedly affects the action of these agents on glucose tolerance, insulin secretion and glycogen levels in liver, uterus and muscle. There is considerable evidence to suggest that these alterations may be attributed to differences in distribution and metabolism of the steroid (423 & 424). Animal studies have shown that both estradiol (426) and ethynyl estradiol (427) are rapidly and almost completely absorbed from the gastrointestinal tract. This has been confirmed in human subjects (428). However, orally administered steroids are subjected to greater hepatic metabolism than steroids given by other administration routes and are particularly prone to losses from the circulation (32 & 425). The natural preparations are vulnerable in this respect (425 & 426). The situation is less severe with synthetic oral contraceptives which have been modified to increase resistance to liver degradation after oral administration by addition of an ethynyl or ester group at carbon-17 (230). Furthermore, the absorption of steroids from the gastrointestinal tract is influenced by the food material and bacteria present in the gut and also by the physical properties of the steroid, ie. non-polar compounds such as progesterone are more efficiently absorbed when dissolved in oil than when given in tablet form (423).

The liver is regarded as the major organ involved in sex steroid metabolism (424), and it must be traversed by all endogenous steroids regardless of administration route. Indeed, considerable quantities of steroid are withdrawn from the circulation, metabolised by the liver and either returned to the circulation in modified form or excreted.

Hepatic metabolism produces steroid conjugates with glucuronic acid (423, 424 & 426), or with sulphates (429 & 430) which are excreted in the bile (431 - 434) and to a lesser extent in the urine (435).

The oral administration route produces a surge of steroid into the circulation shortly after administration. For example, peak plasma ethynyl estradiol levels after oral administration occur within one hour (436), whereas after intramuscular administration, plasma levels were higher between four and twenty-four hours (437). The high circulating titres rapidly achieved by oral administration quickly subside as the hormone is taken up by target tissues, metabolised and excreted. The subcutaneous and intramuscular routes reduce exposure of inordinate quantities of steroid to the liver, thus producing a slower rate of steroid degradation (430). Hence by avoiding excessive hepatic metabolism, the half-life of the steroid is increased resulting in a more stable circulating titre. Accordingly therefore, the effects of the administration route on the distribution and metabolism of sex steroids will affect the actions of these compounds in various tissues.

The cellular mode of action of sex steroids has been thoroughly reviewed on several occasions (see 425, 438, 441, 454 & 463). Briefly sex steroids enter the cell and bind to cytosol receptors which undergo a temperature-dependent transfer to the nucleus and activate specific nuclear acceptor molecules (438 & 439). Many studies have shown that a close relationship exists between circulating sex hormone titres and the number and affinity of cytosol sex steroid receptors (440 - 442). Variations in the concentration of estradiol and progesterone receptors during the estrous cycle (444 - 448), pregnancy (183), the menstrual cycle (449 & 450) and ovariectomy (450 - 452) have been reported. The circulating level of the hormone appears to modulate the number of available receptors. Low sex steroid titres are associated with low numbers of receptors, but the affinity

of the receptors may be raised (450). When steroid titres are raised, the number of receptors is also raised (441 & 453).

Receptors for sex steroids have been found in most rodent tissues. These include recognised target tissues associated with reproduction, namely uterus (454 - 456), pituitary and hypothalamus (457 & 458); and other tissues such as liver, kidney, thymus, muscle and lung (459 - 462). However, the recognised target tissues contain many more receptors than the other tissues (463). There is evidence to suggest that circulating sex steroids may modify the receptors of other steroids. For example, exogenous estradiol has been shown to increase the number of progesterone receptors in the rat, whereas progesterone administration had the reverse effect on estradiol receptors (464 - 469).

There is also evidence to suggest that circulating steroid titres affect plasma levels of steroid-carrier proteins. A study by Daughaday (470) was the first to show that estradiol binding to plasma steroid globulin increased with the dose of estradiol administered. This relationship has been confirmed (471), futhermore it appears that increased binding capacity occurs during pregnancy and following oral contraceptive treatment when circulating steroid levels are elevated (472).

Thus the prevailing hormonal environment has an important influence on sex steroid binding and retention in plasma and tissues. The modulation of receptors in this manner is probably one of the major factors controlling the target cell response. The present study was undertaken to determine the uptake of estradiol and progesterone by recognised target tissues and by tissues involved in glucose homeostasis. The experiments were designed to assist the interpretation of studies on the influence of the administration route on carbohydrate metabolism. Three administration routes were compared in the present

study, and the prevailing hormonal environment was taken into account by examining both intact and ovariectomised animals.

METHODS

Intact and bilaterally ovariectomised adult female mice were used for the in vivo distribution study. Four weeks were allowed to elapse between ovariectomy and incorporation into the study. Animals were divided into eight groups and treated for four cycles as described below -

Groups 1 Intact - arachis oil

2 Intact pretrated with E_2 - 5µg/kg (body wt.) / day

3 Ovariectomised - arachis oil

4 Ovariectomised pretreated with E_2 - 5µg/kg (body wt.) / day

5 Intact - arachis oil

6 Intact pretreated with P - 1mg/kg (body wt.) / day

7 Ovariectomised - arachis oil

8 Ovariectomised pretreated with P - 1mg/kg (body wt.) / day

Within each group the Sc, Im and oral routes of administration were examined. After four cycles of treatment, each mouse received 5µCi of ^{14}C-estradiol (groups 1 - 4) or ^{14}C-progesterone (groups 5 - 8). The isotope was administered by the same route as the arachis oil or steroid had been given. Two hours after administration of the labelled steroid, mice were killed by cervical dislocation. Blood was collected by decapitation and plasma immediately separated by centrifugation. Tissues were rapidly excised and blotted to remove excess blood. Plasma and tissue radioactivity levels were determined by LSC, as described in Chapter 2. Specific activity was expressed in dpm/mg wet weight for tissues, and dpm/10ul for plasma. The tissues examined were liver(anterior margin of the median lobe), skeletal muscle (femoral quadriceps), cardiac muscle (ventricular apex), uterus (mid-section of one horn), pancreas (splenic region), adipose tissue (parametrial lateral margin) and pituitary (total).

The intact mice treated with arachis oil, as in all previous experiments, were examined during the diestrus stage of the estrous cycle.

RESULTS

Group 1 - Intact diestrus mice, in vivo ^{14}C-estradiol distribution

Considerable variations of plasma (figure 59) and tissue (figure 55) radioactivity levels were observed in these mice after administration of labelled E_2.

Plasma radioactivity levels were marginally higher after Sc than after Im administration. These routes produced much higher plasma levels of radioactivity than the oral route. This suggests that at the two hour period, the Sc and Im routes of administration produced similar rates of entry, degradation and removal of ^{14}C-estradiol from the circulation. The lower plasma levels observed following oral ^{14}C-estradiol administration suggests either incomplete absorption or increased degradation and removal has taken place. The latter is most probable since other studies have shown that oral E_2 is rapidly and almost completely absorbed from the gastrointestinal tract (426 - 428). Consistently lower levels of radioactivity were also observed in the tissues examined following oral labelled E_2 administration.

A characteristic feature of the distribution of labelled E_2 was the high accumulation of activity in liver. In the present group of mice, uterus showed the second highest accumulation of activity, the other tissues; pancreas, fat, pituitary, skeletal muscle and cardiac muscle had similar radioactivity levels. With the exception of liver, the accumulation of ^{14}C-estradiol was consistently highest in the Sc treated and lowest in the orally treated mice.

Group 2 - Intact estradiol pretreated, in vivo ^{14}C-estradiol distribution

The influence of E_2 pretreatment on the distribution and uptake of labelled E_2 in intact mice is shown in figures 56 and 59. In comparison with the untreated intact diestrus mice, E_2 pretreatment considerably increased radioactivity levels in both plasma and tissues.

The increased plasma radioactivity levels seen in these animals suggest either that plasma E_2 binding was increased or that the removal rate of E_2 from the plasma was decreased.

The greatest accumulation of radioactivity was produced in hepatic tissue. However, E_2 pretreatment was associated with a particularly large increase in radioactivity in pancreas and adipose tissue, such that levels in these tissues exceeded those of the uterus. The reason for this disproportionately large elevation is uncertain. It is known that steroids are lipid soluble and may accumulate in adipose tissue (473) particularly following chronic administration. The raised levels of radioactivity in all tissues examined was consistent with the appearance of increased numbers and/or affinity of specific E_2 receptors after E_2 pretreatment. The E_2 pretreatment regime did not alter the differential effects of the route of administration. The highest plasma and tissue levels of activity were produced by Sc, and the lowest levels by oral administration of E_2 (compare figures 55 & 56).

Group 3 - Ovariectomised, in vivo ^{14}C-estradiol distribution

Tissue (figure 57) and plasma (figure 59) levels of radioactivity in ovariectomised mice not pretreated with E_2 are discussed in this section. In comparison with the intact mice, both untreated (figure 55) and pretreated (figure 56), the levels of radioactivity in plasma and tissues of the ovariectomised mice were considerably lower. This group of animals also showed a characteristic difference from the diestrus mice with regard to uterine E_2 accumulation. In intact mice, uterine radioactivity levels were significantly higher than most other tissue levels (see figures 55 & 56). This was not apparent in ovariectomised mice where uterine tissue showed similar radioactivity levels to other tissues. These results provide evidence to support the view that endogenous ovarian

sex steroids facilitate higher accumulation of E_2 in various tissues, particularly the target tissues such as uterus. E_2 treated intact mice (figure 56) did not show increased uterine uptake to a greater extent than other tissues. Thus it appears that supra-physiological estrogen concentrations affect the availability of specific hormone receptors to only a limited extent.

The differential effects produced by the administration route were not apparent in the ovariectomised mice. In groups 1 and 2, the Sc route produced the highest and the oral route the lowest levels of radioactivity (figures 55 & 56), whereas no consistent alterations were seen in the ovariectomised group. Why the effect of the administration route should differ in the ovariectomised mice compared with the intact mice, cannot be readily explained. It is evident, however, that these alterations are associated with the presence of estrogens because, as shown in the following section, in ovariectomised mice pretreated with E_2 the differential effects of the administration route are restored.

Group 4 - Ovariectomised estradiol pretreated, ^{14}C-estradiol distribution

E_2 pretreated ovariectomised mice displayed tissue and plasma radioactivity levels as shown in figures 58 and 59 respectively. Plasma levels of radioactivity were consistently higher than those seen in the untreated ovariectomised mice. Although levels were higher than the intact diestrus levels, they did not at any time in the study reach the very high levels shown by the pretreated intact mice. This may be taken as further support for the view that exposure to high circulating estrogen titres may increase the plasma binding capacity of estrogens.

The tissue uptake of labelled E_2 was higher than that seen in either ovariectomised or diestrus untreated mice. In some tissues viz liver (Im), fat (Sc & Im) and cardiac muscle (Im & oral),

the radioactivity levels reached those observed in the intact pretreated mice (figure 56). These results substantiate evidence for a close positive relationship between the estrogen titre and estrogen accumulation in tissues (440 - 443, 446 & 448). Thus the results are consistent with the view that raised E_2 titres increase the number and/or affinity of tissue E_2 receptors.

Compared with the untreated ovariectomised mice, E_2 pretreatment increased the accumulation of radioactivity by pancreas and adipose tissue to a much greater extent than other tissues. A similarly disproportionate elevation was noted in the E_2 pretreated intact mice. As discussed earlier, why estrogen administration should preferentially promote binding in these tissues is unknown. As noted in previous sections of this chapter, the Sc route produced the highest levels and the oral route the lowest levels of radioactivity.

Group 5 - Intact diestrus mice, in vivo ^{14}C-progesterone distribution

The distribution and uptake of labelled P in plasma (figure 64) and in tissues (figure 60) is discussed in this section. The greatest radioactivity concentration was observed in the plasma. Plasma radioactivity levels were highest after Sc amd lowest after Im labelled P administration. This is in contrast to labelled E_2 treatment, where the lowest radioactivity levels occurred following oral administration. The overall lower levels of plasma P suggests that the plasma binding capacity for this hormone was lower than that for E_2. This may be coupled with an increased rate of degradation and removal of P from the circulation. Indeed, it has been suggested that only natural estrogens bind specifically to plasma binding globulin and that other sex steroids bind in a non-specific manner with an albumin fraction (474). The metabolic clearance rate of P (475) is higher than that of E_2 (476), thus removing P more rapidly from the body

than E_2.

Consonant with the overall lower activity levels in plasma, tissue radioactivity levels were also lower in the P treated mice. A characteristic feature of P administration was the high accumulation of radioactivity in the liver. However, the consistently high levels of label observed in the uterus during ^{14}C-estradiol administration were not evident during ^{14}C-progesterone administration throughout the study, regardless of the treatment regime utilised. The effect of the administration route was the same for both plasma and tissues. The highest radioactivity levels were seen following Sc and the lowest after Im treatment.

Group 6 - Intact progesterone pretreated, ^{14}C-progesterone distribution

The influence of P pretreatment on tissue uptake of labelled P is shown in figure 61, and plasma uptake is shown in figure 64. Plasma radioactivity levels were markedly increased in the orally treated mice, and increased to a lesser extent following Sc administration. The Im levels were not significantly altered. Thus in P pretreated mice, the highest plasma radioactivity levels were produced by oral treatment, and Im treatment produced the lowest levels. It appears that oral P pretreatment enhanced P entry into the circulation. This may arise from either an increase in the amount and the rate of absorption from the gastrointestinal tract, or it may result from a decreased rate of degradation and removal of P. It is suggested that a surge of P from the intestine would temporarily exceed the capacity of the liver to selectively remove and degrade the hormone. Furthermore, pretreatment will elevate circulating P titres, and a reduced rate of degradation of labelled P may thus be produced by a dilution effect ie. as the hormone levels increase the proportion of label will be relatively reduced.

The different levels of radioactivity produced by the three

administration routes in the plasma were reflected in tissue levels. Tissue radioactivity levels in the Sc and Im treated animals were elevated to a lesser extent by P pretreatment than levels in the oral group. In addition, hepatic and pancreatic accumulation was markedly elevated in comparison with the other tissues. This result is to be expected in liver which would be subjected to high levels of hormone immediately after oral administration. An explanation for pancreatic levels is not evident, but a similar disproportionate elevation was also seen in E_2 treated mice.

Group 7 - Ovariectomised mice, in vivo ^{14}C-progesterone distribution

The effects of ovariectomy on radioactivity levels in plasma and tissues following ^{14}C-progesterone administration are shown in figures 64 and 62 respectively. Mean plasma radioactivity levels in ovariectomised mice were consistently marginally lower than intact diestrus mice. These differences were not statistically significant. This suggests that the endogenous circulating P titre derived from the ovaries of intact mice does not significantly increase or affect plasma P binding capacity. Administration route-induced alterations of plasma radioactivity were the same as those observed in the diestrus mice; namely the highest levels were produced following Sc and the lowest following Im treatment.

Tissue radioactivity levels followed a similar pattern. Mean radioactivity accumulation was lower than that seen in diestrus mice, but again differences were not significant. Generally, levels were highest after Sc and lowest after Im administration. Thus it is evident that ovariectomy does not significantly impair tissue accumulation of P. Hence it can be deduced that ovariectomy does not significantly alter the number and/or affinity of tissue P receptors. These results are not compatible with the E_2 study in which a clear relationship between E_2

concentration and E_2 receptor availability was evident.

Further differences between the E_2 and P regimes were apparent. Ovariectomy did not alter the differential effects of the administration route in the P treated mice. This suggests that P is not required for the differential effects of the administration route to be manifest. This is in contrast to the situation with E_2.

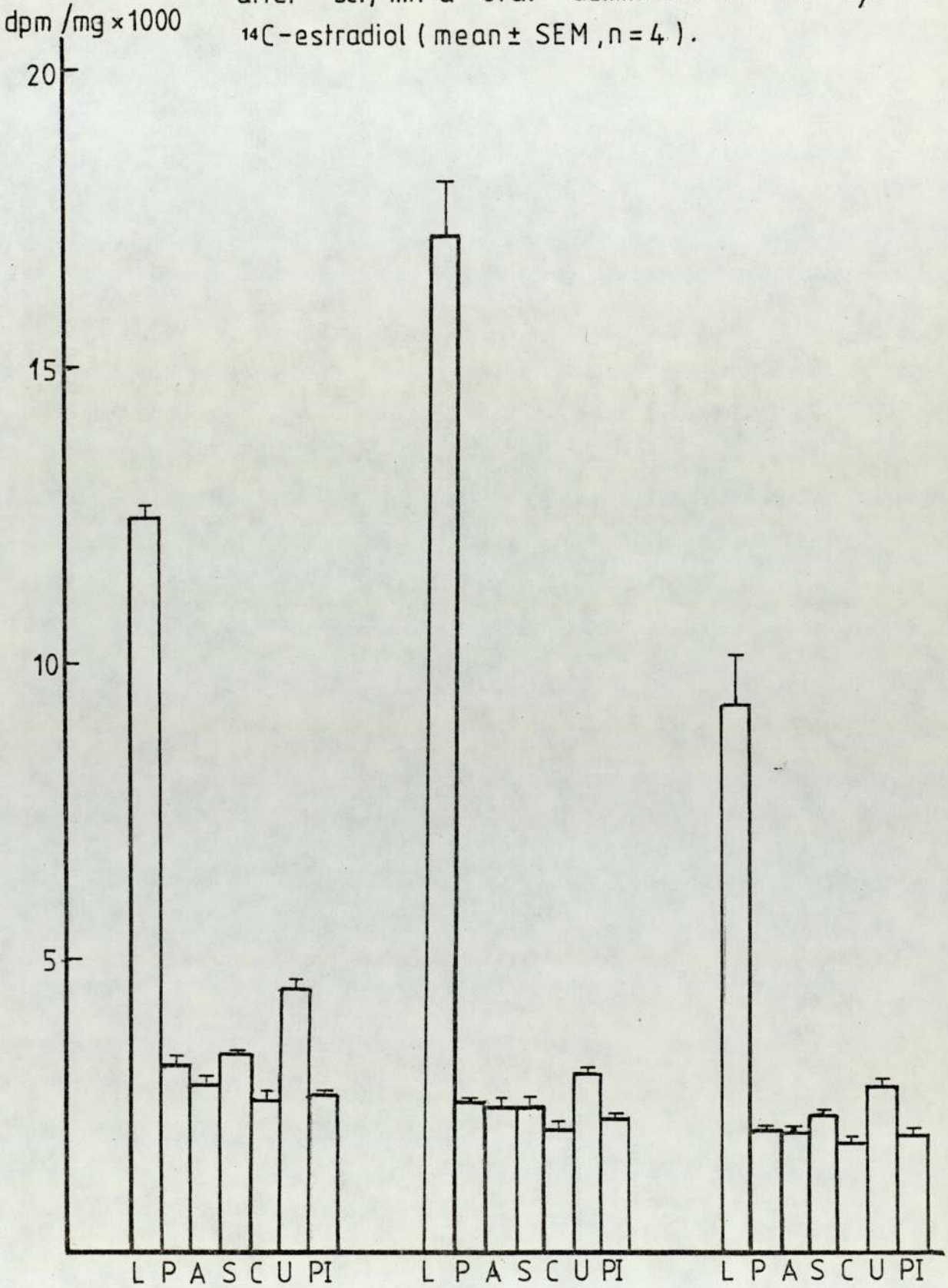
Group 8 - Ovariectomised P pretreated, ^{14}C-progesterone distribution

Radioactivity levels in the tissues (figure 63) and plasma (figure 64) following ovariectomy and P treatment are discussed in this section. The plasma radioactivity levels were consistently higher following P pretreatment than in untreated ovariectomised mice (figure 62). Indeed, the highest levels were produced by this particular treatment regime. The elevation was especially marked after oral administration, as noted in the intact pretreated animals. The possible reasons for this are discussed in section six of this chapter.

A similar situation was observed in the tissues, where radioactivity levels were higher following P administration in the ovariectomised mice. The fact that pretreatment increases sequestration of the labelled hormone is consistent with the view that exposure to higher hormone concentrations elevates both the number and/or availability of specific tissue receptors and the plasma carrying capacity for the hormone. A prominent feature observed after P pretreatment was the increase in radioactivity levels of both plasma and tissues in the ovariectomised mice above those seen in the intact mice. A possible explanation is the absence of endogenous estrogens in the ovariectomised animals which may influence the radioactivity levels as suggested previously (464 - 469). Precisely how estrogens affect P accumulation is uncertain.

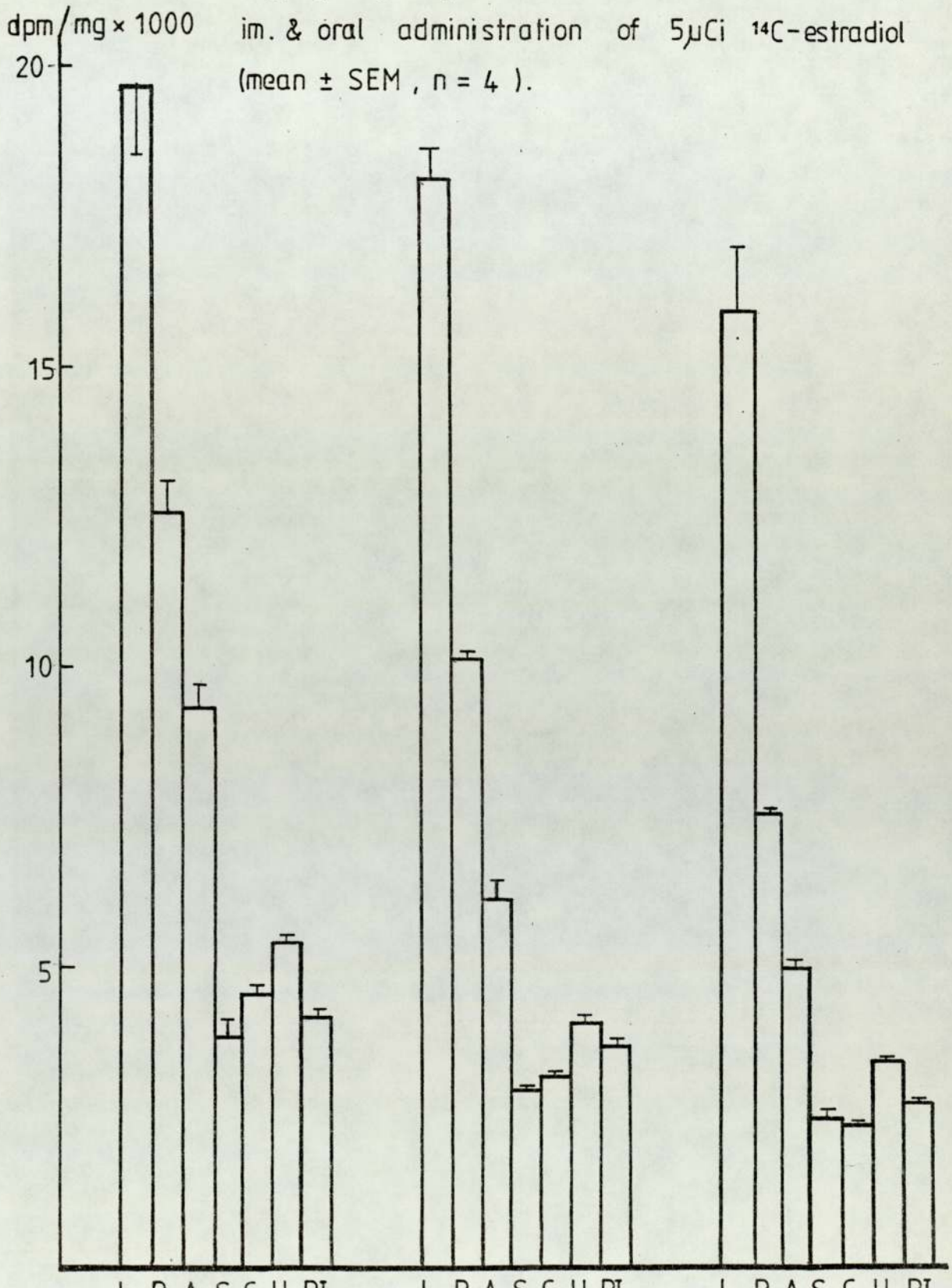
The present distribution studies with labelled E_2 and P have identified the liver as a major site of accumulation of these hormones. In addition, considerable quantities are taken up by adipose tissue and skeletal muscle. These tissues are quantitatively the major sites of glucose metabolism. The present data indicate that estrogens and progestogens may influence carbohydrate homeostasis by directly affecting metabolism in these tissues. Moreover, it has been shown that estrogens and progestogens can alter the activities of enzymes involved in carbohydrate metabolism in the uterus (477 - 479) and liver (169 & 418). In view of the quantitative importance of fat and skeletal muscle in the removal of glucose from the circulation, the effects of estrogens and progestogens on glucose uptake by these tissues in the presence and absence of insulin were studied, as described in the following chapter.

Figure 55 Tissue radioactivity levels in intact mice 2hrs. after sc., im. & oral administration of 5μCi ^{14}C-estradiol (mean ± SEM , n = 4).



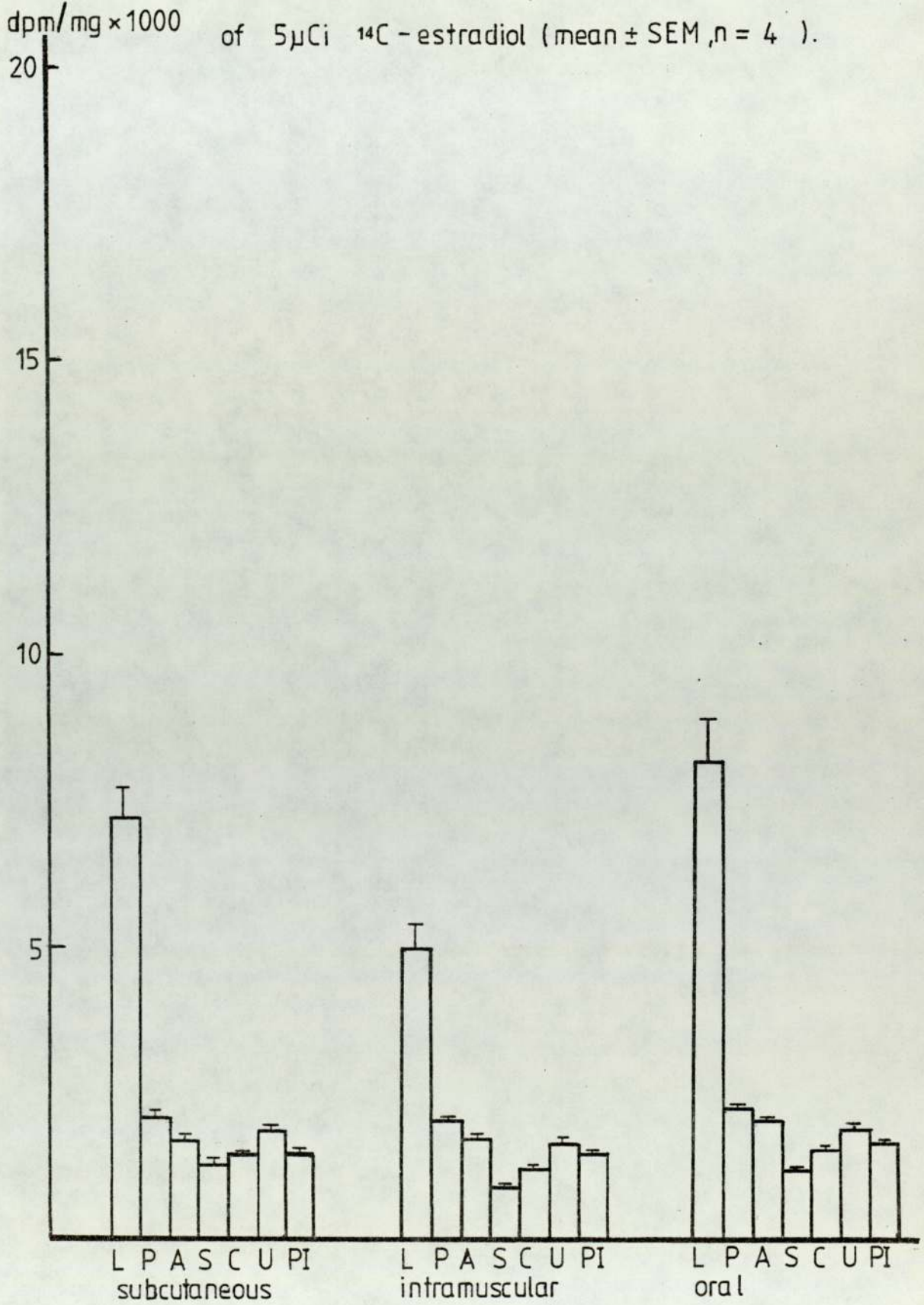
L - liver
P - pancreas
A - adipose tissue
S - skeletal muscle
C - cardiac muscle
U - uterus
PI - pituitary

Figure 56 Tissue radioactivity levels in pretreated (E_2- 5ug/kg/day/4cycles) intact mice 2hrs. after sc., im. & oral administration of 5μCi ^{14}C-estradiol (mean ± SEM , n = 4).



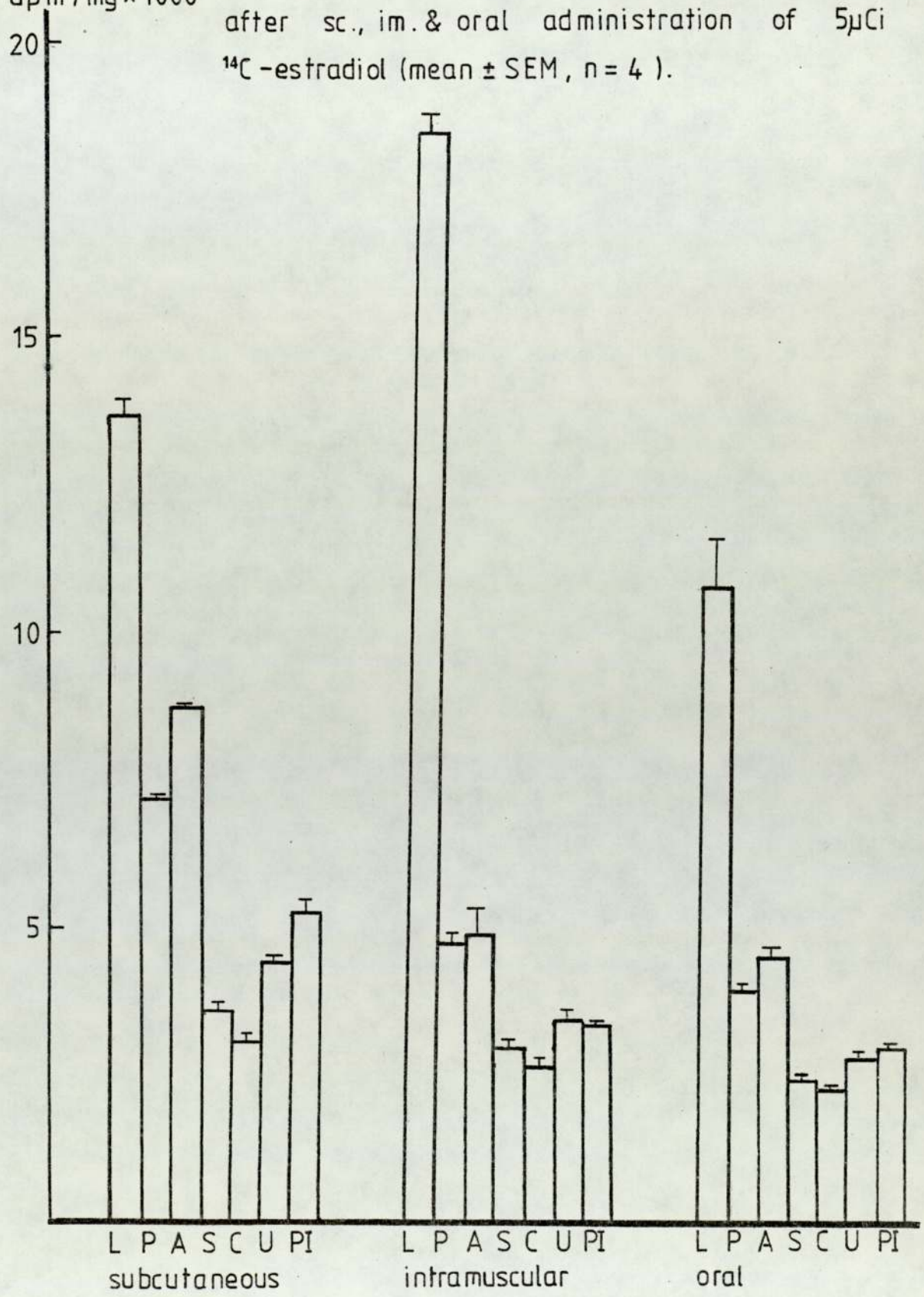
L- liver
P- pancreas
A- adipose tissue
S- skeletal muscle
C- cardiac muscle
U- uterus
PI- pituitary

Figure 57 Tissue radioactivity levels in ovariectomised mice 2hrs. after sc., im. & oral administration of 5µCi ^{14}C - estradiol (mean ± SEM, n = 4).



L - liver
P - pancreas
A - adipose tissue
S - skeletal muscle
C - cardiac muscle
U - uterus
PI - pituitary

Figure 58 Tissue radioactivity levels in pretreated (E_2- 5ug/kg/day / 4 cycles) ovariectomised mice 2hrs. after sc., im. & oral administration of 5µCi ^{14}C-estradiol (mean ± SEM, n = 4).



L - liver
P - pancreas
A - adipose tissue
S - skeletal muscle
C - cardiac muscle
U - uterus
PI - pituitary

Figure 59 Plasma radioactivity levels 2hrs. after subcutaneous, intramuscular & oral administration of 5μCi ^{14}C-estradiol (mean ± SEM, n = 4).

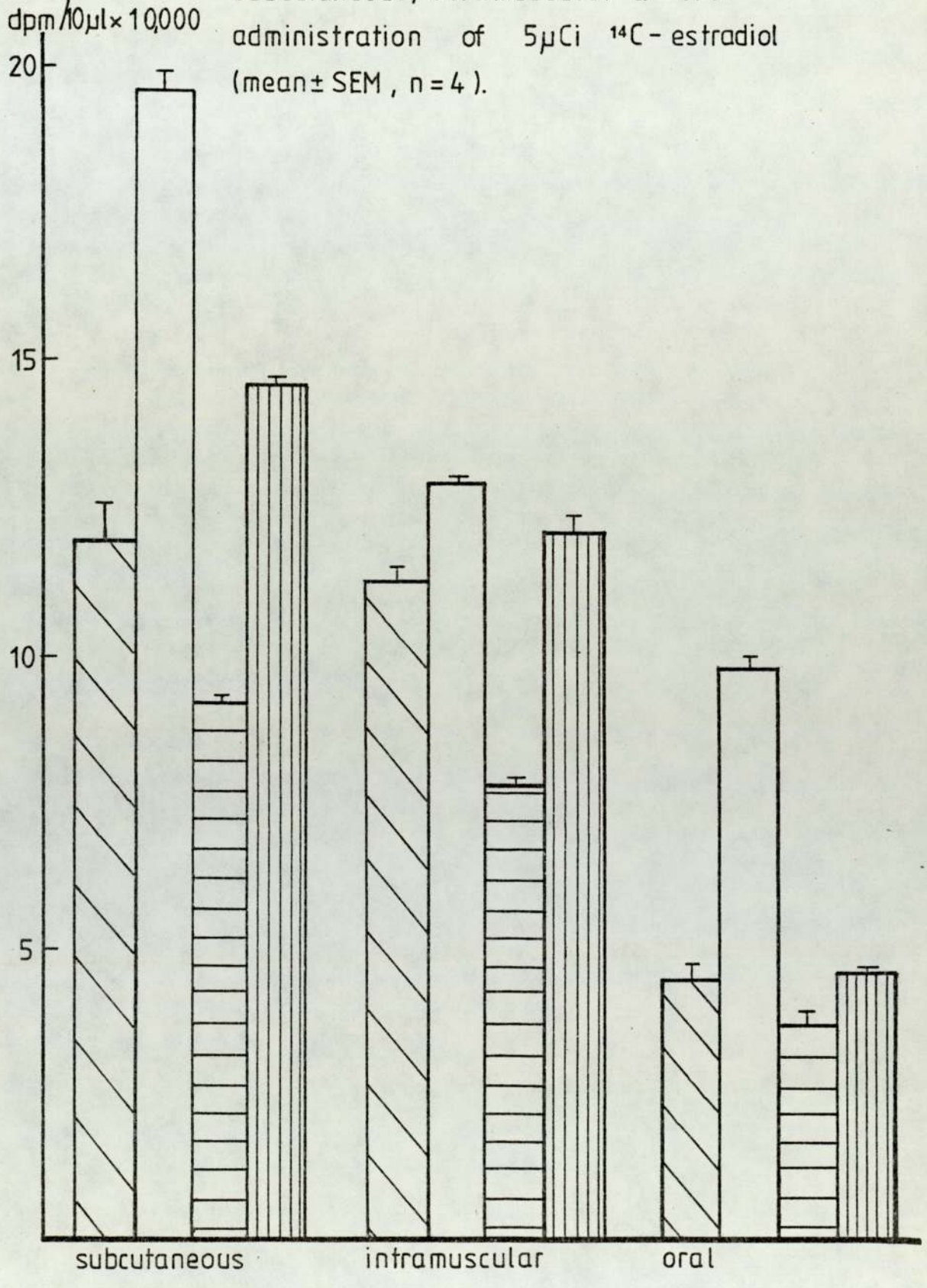
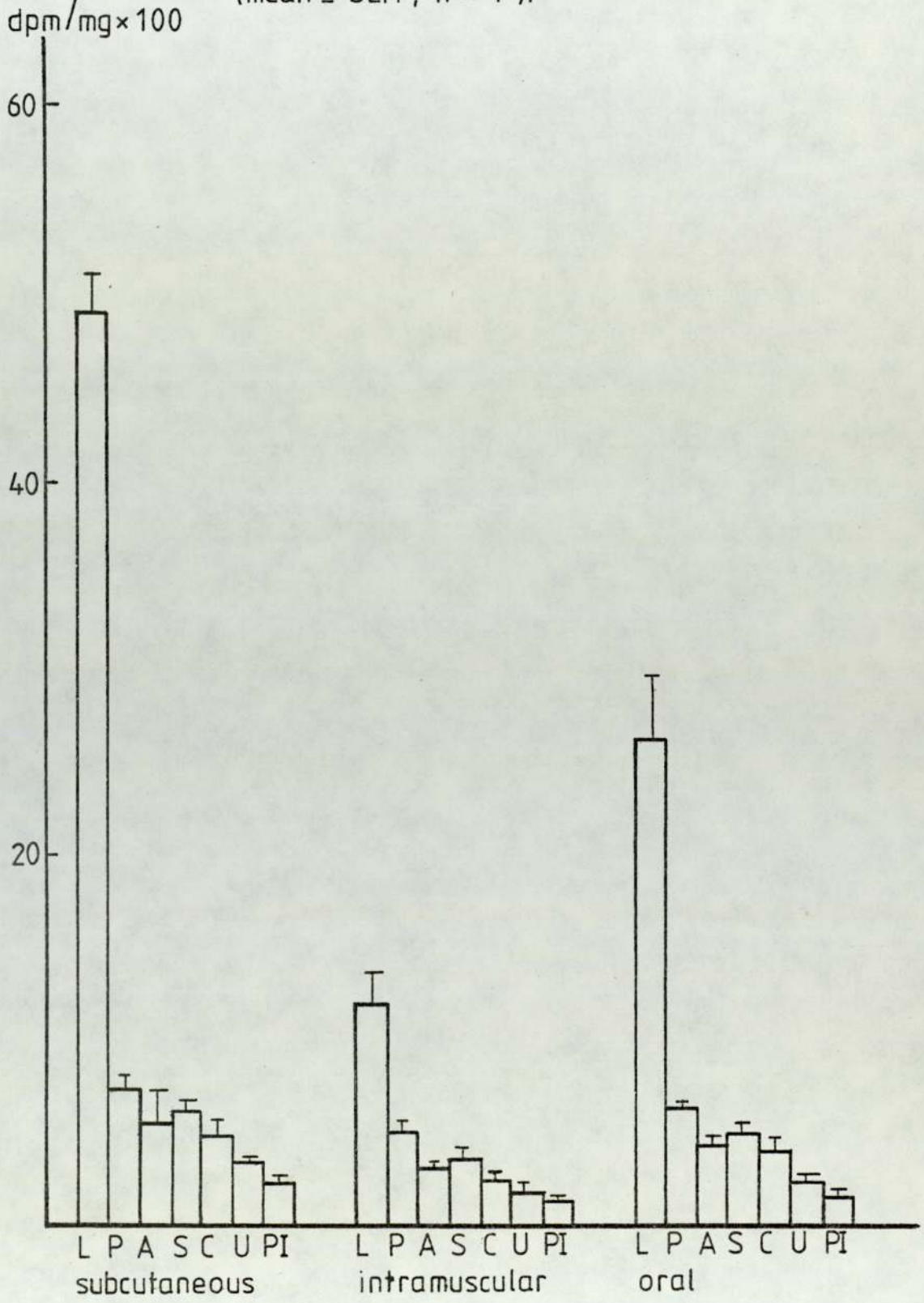
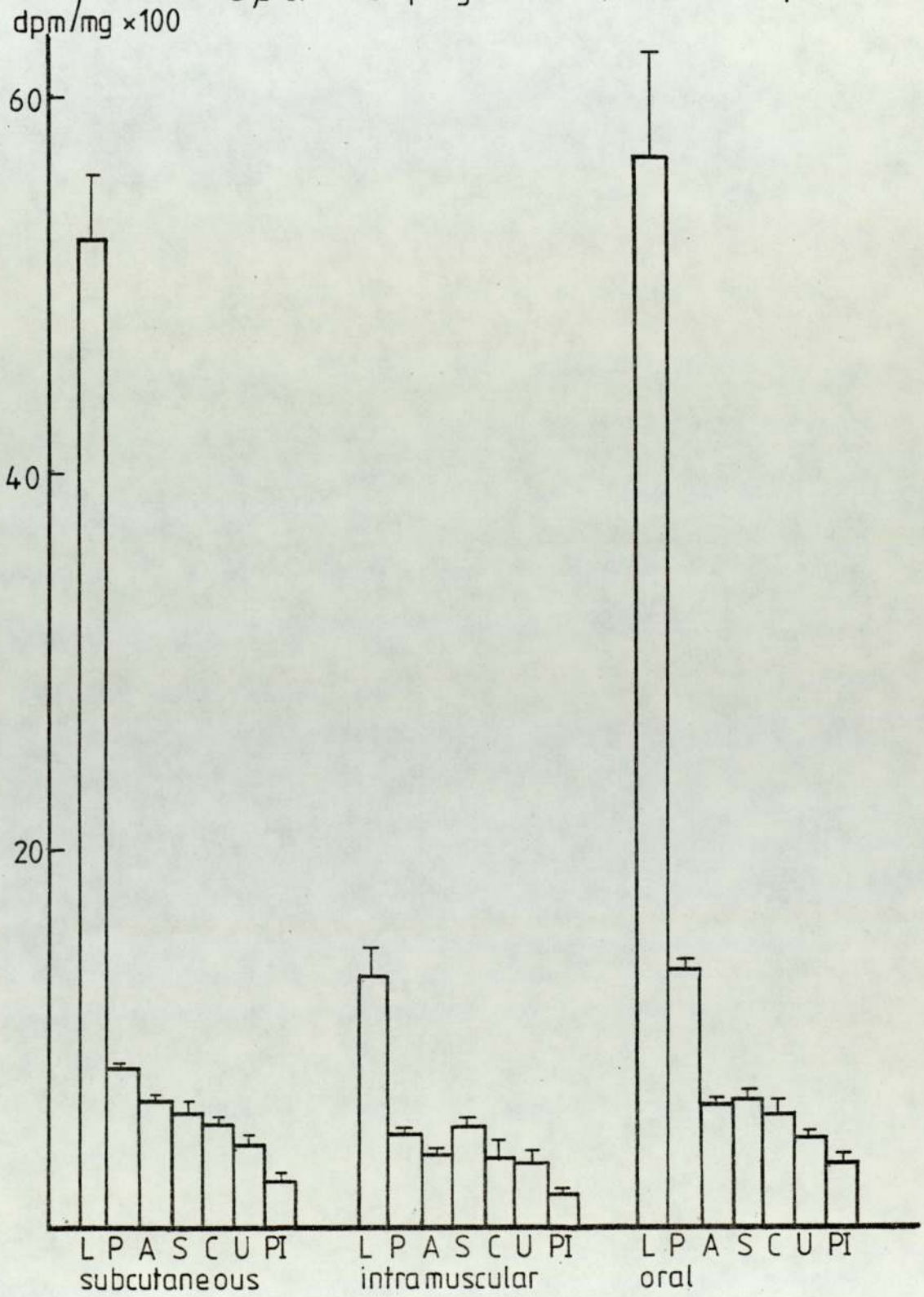


Figure 60 Tissue radioactivity levels in intact mice 2hrs. after sc., im. & oral administration of 5μCi ^{14}C-progesterone (mean ± SEM, n = 4).



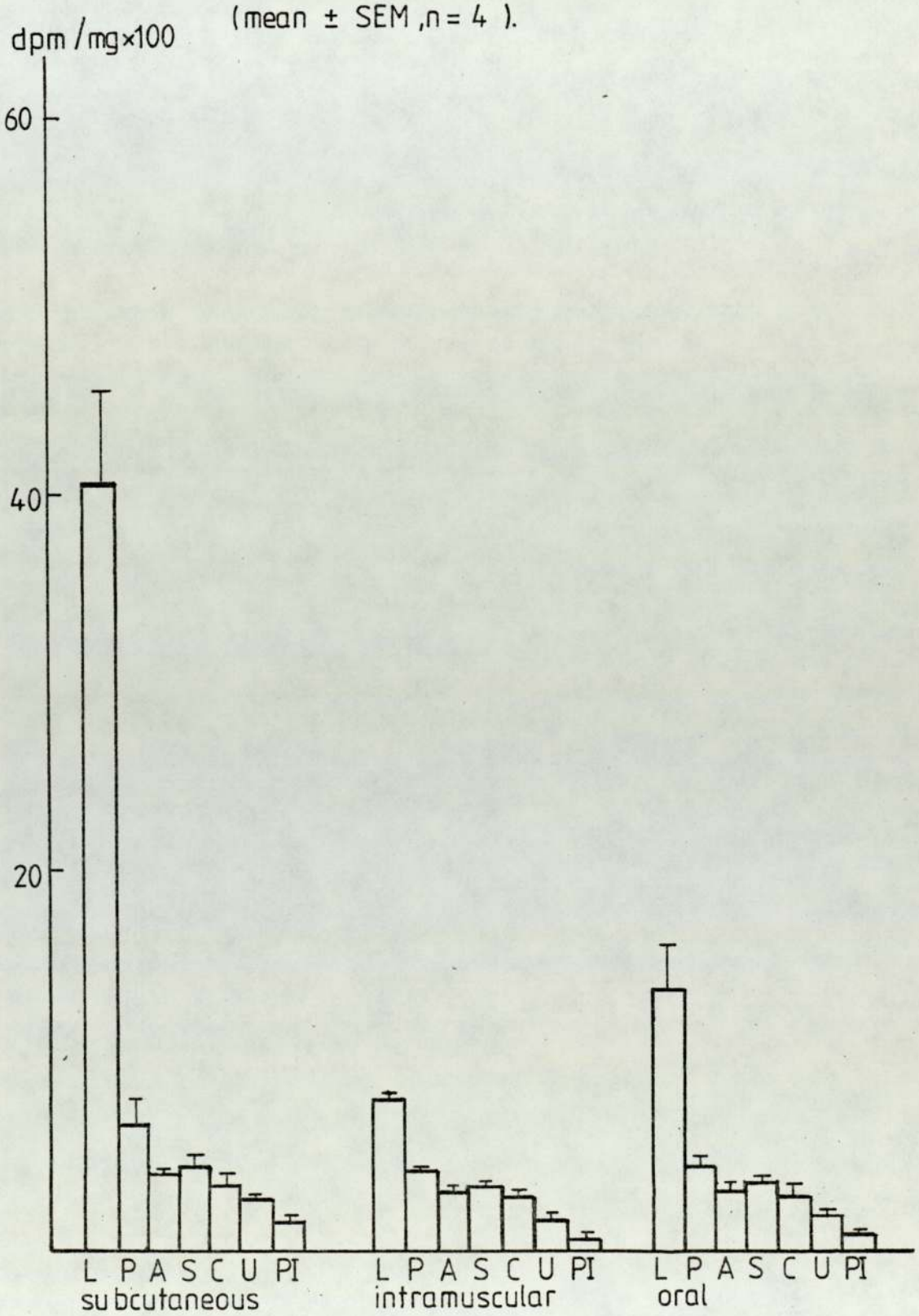
L - liver
P - pancreas
A - adipose tissue
S - skeletal muscle
C - cardiac muscle
U - uterus
PI - pituitary

Figure 61 Tissue radioactivity levels in pretreated (P- 1mg/kg/day/4 cycles) intact mice 2hrs. after sc., im. & oral administration of 5 μ Ci ^{14}C -progesterone (mean ± SEM, n = 4).



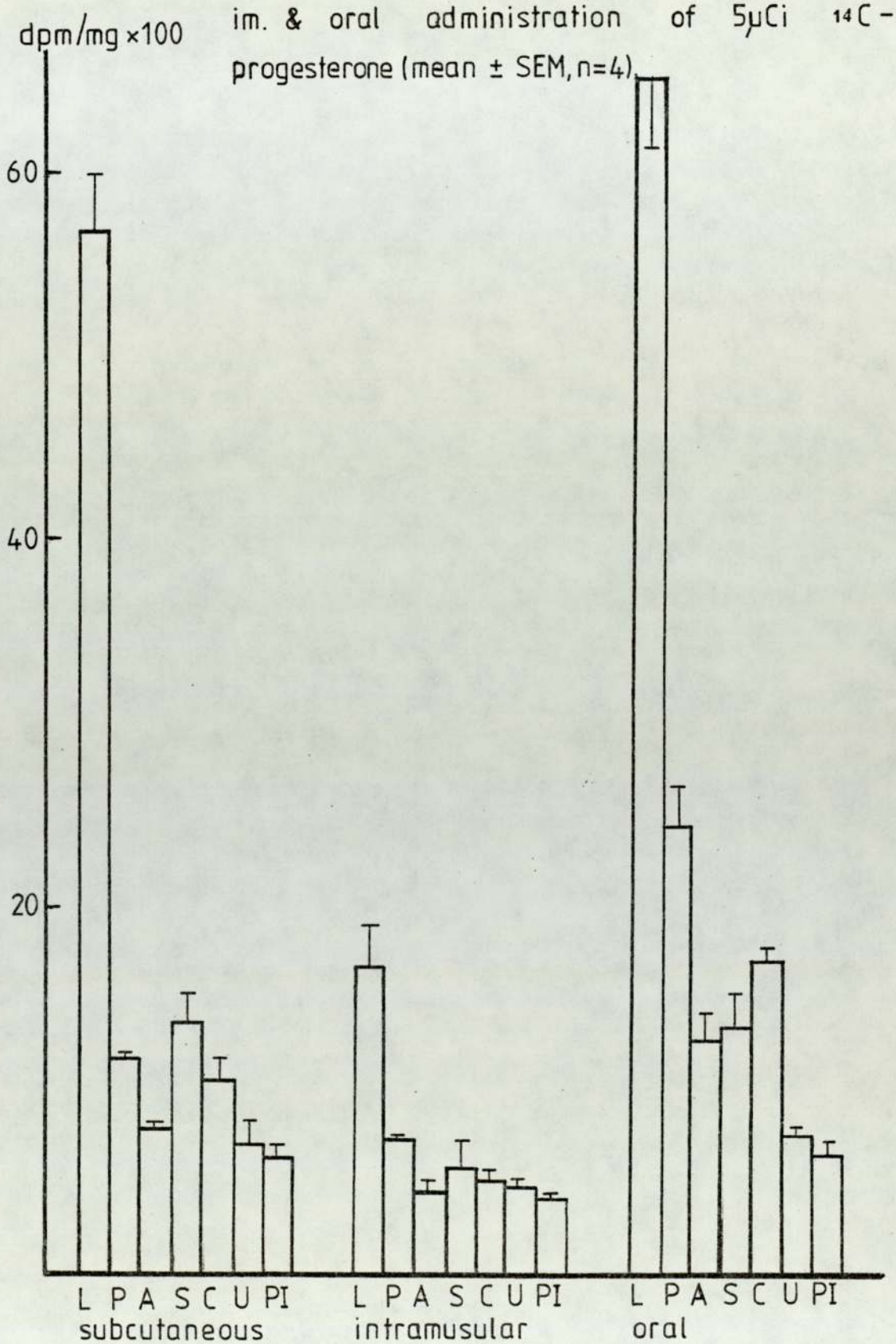
L - liver
P - pancreas
A - adipose tissue
S - skeletal muscle
C - cardiac muscle
U - uterus
PI - pituitary

Figure 62 Tissue radioactivity levels in ovariectomised mice 2hrs. after sc., im. & oral administration of 5μCi ^{14}C-progesterone (mean ± SEM, n = 4).



L - liver
P - pancreas
A - adipose tissue
S - skeletal muscle
C - cardiac muscle
U - uterus
PI - pituitary

Figure 63 Tissue radioactivity levels in ovariectomised (P-1mg/kg/day/4cycles) mice 2hrs. after sc., im. & oral administration of 5μCi ^{14}C-progesterone (mean ± SEM, n=4).



L - liver
P - pancreas
A - adipose tissue
S - skeletal muscle
C - cardiac muscle
U - uterus
PI - pituitary

Figure 64 Plasma radioactivity levels 2hrs. after subcutaneous, intramuscular & oral administration of 5μCi ^{14}C-progesterone (mean± SEM, n = 4).

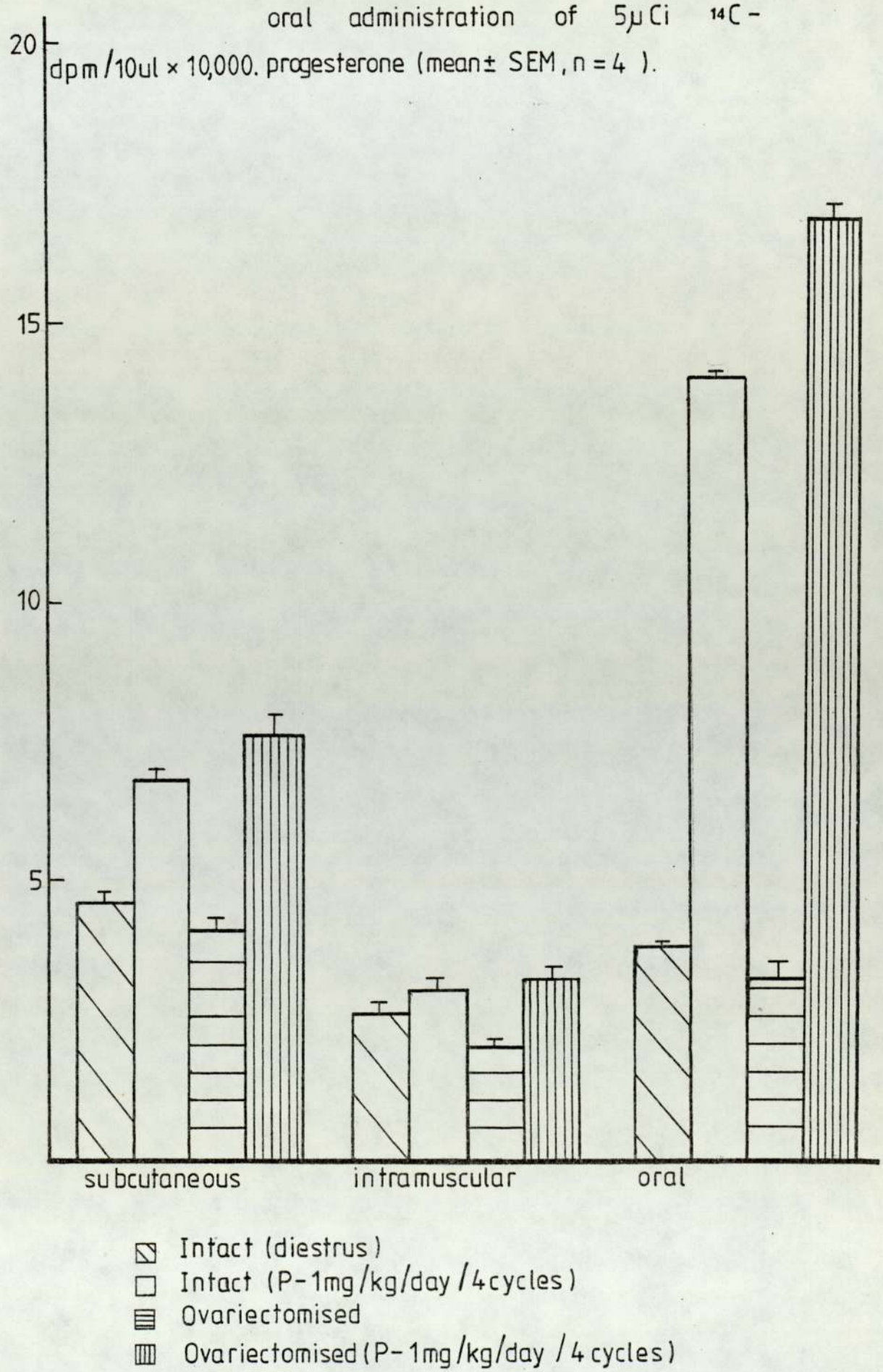


Table 7 IN VIVO DISTRIBUTION OF ESTRADIOL (n = 4) - CHAPTER 5

TISSUE (dpm/mg) & PLASMA (dpm/10µl) ^{14}C - ESTRADIOL LEVELS						
	subcutaneous		intramuscular		oral	
INTACT DIESTRUS	mean	SEM	mean	SEM	mean	SEM
plasma	119,378	6,168	112,215	2,800	45,090	3,597
liver	12,481	161	17,176	932	9,223	882
pancreas	3,101	171	2,518	35	2,076	35
AT	2,831	132	2,444	158	2,070	111
SM	3,338	61	2,488	186	2,279	11
CM	2,620	110	2,194	71	1,832	43
uterus	4,421	348	3,097	68	2,800	106
pituitary	2,633	110	2,215	11	1,957	108
INTACT + ESTRADIOL						
plasma	196,150	2,645	128,299	1,116	97,235	2,419
liver	19,738	1,339	18,025	446	15,898	1,105
pancreas	12,562	490	10,054	175	7,416	64
AT	9,227	331	6,076	294	4,940	180
SM	3,861	273	2,928	49	2,545	205
CM	4,524	179	3,233	94	2,432	21
uterus	5,384	224	4,004	226	3,329	11
pituitary	4,080	95	3,683	94	2,622	66
OVARIECTOMY						
plasma	92,810	1,985	78,714	615	36,302	2,116
liver	7,163	482	4,959	307	8,194	715
pancreas	2,075	50	1,931	54	2,136	65
AT	1,695	48	1,654	9	1,939	13
SM	1,361	73	965	20	1,331	49
CM	1,403	17	1,211	52	1,537	42
uterus	1,734	71	1,615	35	1,883	57
pituitary	1,499	60	1,450	68	1,643	12
OVARIECTOMY + ESTRADIOL						
plasma	145,865	1,342	121,021	3,125	45,596	398
liver	13,771	217	18,485	279	10,862	759
pancreas	7,233	28	4,812	235	4,040	91
AT	8,843	14	4,881	416	4,667	208
SM	3,663	114	2,771	172	2,456	113
CM	3,149	198	2,558	109	2,333	97
uterus	4,588	21	3,489	178	2,838	35
pituitary	5,358	184	3,457	15	2,965	18

Where AT - adipose tissue

SM - skeletal muscle

CM - cardiac muscle

Table 8 ^{14}C - ESTRADIOL DISTRIBUTION - STATISTICAL COMPARISONS

TISSUE (dpm/mg) & PLASMA (dpm/10µl) ^{14}C-ESTRADIOL LEVELS				
	Intact/Intact + Estradiol	OVX/OVX + Estradiol	Intact/OVX	Intact + E_2/ OVX + E_2
SUBCUTANEOUS				
plasma	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$
liver	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$
pancreas	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$
AT	$p < 0.001$	$p < 0.001$	$p < 0.001$	NS
SM	$p < 0.02$	$p < 0.001$	$p < 0.001$	NS
CM	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$
uterus	$p < 0.01$	$p < 0.001$	$p < 0.001$	$p < 0.001$
pituitary	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$
INTRAMUSCULAR				
plasma	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.01$
liver	NS	$p < 0.001$	$p < 0.001$	NS
pancreas	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$
AT	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.01$
SM	$p < 0.01$	$p < 0.001$	$p < 0.001$	NS
CM	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$
uterus	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.05$
pituitary	$p < 0.001$	$p < 0.001$	$p < 0.001$	NS
ORAL				
plasma	$p < 0.001$	$p < 0.001$	$p < 0.02$	$p < 0.001$
liver	$p < 0.001$	$p < 0.01$	NS	$p < 0.001$
pancreas	$p < 0.001$	$p < 0.001$	NS	$p < 0.001$
AT	$p < 0.001$	$p < 0.001$	NS	NS
SM	NS	$p < 0.001$	$p < 0.001$	NS
CM	$p < 0.001$	$p < 0.001$	$p < 0.001$	NS
uterus	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$
pituitary	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$

Where AT - adipose tissue

SM - skeletal muscle

CM - cardiac muscle

n = 4 for all groups

$p < 0.05$ is significant

$p < 0.02$ & $p < 0.01$ are highly significant

$p < 0.002$ & $p < 0.001$ are very highly significant

OVX - ovariectomised mice

Table 9 IN VIVO DISTRIBUTION OF ^{14}C-PROGESTERONE (n = 4) - CHAPTER 5

TISSUE (dpm/mg) & PLASMA (dpm/10µl) ^{14}C - PROGESTERONE LEVELS						
	subcutaneous		intramuscular		oral	
INTACT DIESTRUS	mean	SEM	mean	SEM	mean	SEM
plasma	46,730	1,265	26,389	1,965	37,735	935
liver	4,872	213	1,160	138	2,596	347
pancreas	706	74	464	37	639	39
AT	554	161	299	15	430	62
SM	576	46	351	39	470	58
CM	477	83	248	36	410	70
uterus	346	23	165	29	224	54
pituitary	230	37	124	11	133	33
INTACT + PROGESTERONE						
plasma	68,223	1,844	29,967	2,603	139,243	1,518
liver	5,221	340	1,306	112	5,679	524
pancreas	883	13	504	46	1,379	64
AT	658	22	402	44	656	35
SM	581	82	515	48	665	39
CM	544	74	365	100	600	63
uterus	403	57	331	56	475	48
pituitary	203	41	188	15	334	35
OVARIECTOMY						
plasma	41,827	1,177	20,119	1,019	32,853	1,945
liver	4,079	441	788	55	1,360	190
pancreas	607	125	382	34	444	52
AT	410	22	237	21	297	41
SM	441	57	247	11	320	64
CM	332	53	221	57	267	77
uterus	270	10	121	12	201	32
pituitary	150	31	47	15	87	12
OVARIECTOMY + PROGESTERONE						
plasma	76,422	2,750	31,968	1,753	168,102	2,372
liver	5,752	367	1,655	215	6,599	420
pancreas	1,141	25	700	17	2,428	179
AT	766	26	445	60	1,273	149
SM	1,296	135	556	133	1,350	154
CM	1,094	104	499	49	1,725	92
uterus	694	109	471	61	813	87
pituitary	660	56	406	9	723	108

Where AT - adipose tissue

SM - skeletal muscle

CM - cardiac muscle

Table 10 ^{14}C-PROGESTERONE DISTRIBUTION - STATISTICAL COMPARISONS

TISSUE (dpm/mg) & PLASMA (dpm/10µl) ^{14}C-PROGESTERONE LEVELS				
	Intact/Intact + Estradiol	OVX/OVX + Estradiol	Intact/OVX	Intact + E_2/ OVX + E_2
SUBCUTANEOUS				
plasma	$p < 0.001$	$p < 0.001$	$p < 0.01$	$p < 0.01$
liver	NS	$p < 0.002$	$p < 0.05$	NS
pancreas	NS	$p < 0.001$	NS	$p < 0.001$
AT	$p < 0.05$	$p < 0.001$	$p < 0.01$	$p < 0.002$
SM	NS	$p < 0.001$	$p < 0.02$	$p < 0.001$
CM	NS	$p < 0.001$	$p < 0.05$	$p < 0.001$
uterus	NS	$p < 0.001$	$p < 0.002$	$p < 0.01$
pituitary	NS	$p < 0.001$	$p < 0.05$	$p < 0.001$
INTRAMUSCULAR				
plasma	NS	$p < 0.001$	$p < 0.01$	NS
liver	NS	$p < 0.001$	$p < 0.01$	$p < 0.05$
pancreas	NS	$p < 0.001$	$p < 0.05$	$p < 0.001$
AT	$p < 0.02$	$p < 0.002$	$p < 0.01$	NS
SM	$p < 0.01$	$p < 0.01$	$p < 0.01$	NS
CM	NS	$p < 0.001$	NS	NS
uterus	$p < 0.01$	$p < 0.001$	$p < 0.05$	$p < 0.05$
pituitary	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$
ORAL				
plasma	$p < 0.001$	$p < 0.001$	$p < 0.01$	$p < 0.001$
liver	$p < 0.001$	$p < 0.001$	$p < 0.002$	NS
pancreas	$p < 0.001$	$p < 0.001$	$p < 0.01$	$p < 0.001$
AT	$p < 0.001$	$p < 0.001$	$p < 0.05$	$p < 0.001$
SM	$p < 0.01$	$p < 0.001$	$p < 0.02$	$p < 0.001$
CM	$p < 0.02$	$p < 0.001$	$p < 0.05$	$p < 0.001$
uterus	$p < 0.001$	$p < 0.001$	NS	$p < 0.002$
pituitary	$p < 0.001$	$p < 0.001$	NS	$p < 0.002$

Where AT - adipose tissue

SM - skeletal muscle

CM - cardiac muscle

n = 4 for all groups

$p < 0.05$ is significant

$p < 0.02$ & $p < 0.01$ are highly significant

$p < 0.002$ & $p < 0.001$ are very highly significant

OVX - ovariectomised mice

Chapter 6

EFFECT OF SEX STEROIDS AND OVARIECTOMY ON THE IN VITRO PERIPHERAL TISSUE SENSITIVITY TO THE HYPOGLYCAEMIC ACTION OF INSULIN

CHAPTER 6

IN VITRO PERIPHERAL TISSUE STUDIES

INTRODUCTION

The foregoing chapters have shown that both chemical structure and administration route of sex steroids markedly affect their action on glucose tolerance, insulin secretion and glycogen deposition. There is considerable evidence to suggest that endogenous sex hormones and contraceptive steroids influence many aspects of carbohydrate metabolism, as discussed in Chapter 1 (1 - 3, 5, 126).

The precise nature of the effects of sex steroids on carbohydrate homeostasis remains to be clearly determined, but they may be differentiated at the level of glucose uptake and metabolism by the peripheral tissues. Peripheral tissue insensitivity to insulin is characterised by the presence of hyperglycaemia and concomitant hyperinsulinaemia (480 & 481). This condition has been frequently observed in the present study. The causes of peripheral tissue insensitivity are uncertain, but a combination of effects is probably involved, including alterations in the number, structure and function of insulin receptors (98 & 103), and intracellular defects in glucose transport and/or phosphorylation (100, 107 & 393).

Peripheral tissue sensitivity to insulin has been examined in many tissues, but muscle and adipose tissue have been studied in particular depth (103, 324, 384 - 387). Quantitativly these tissues represent the most important sites of insulin-mediated glucose uptake, metabolism and storage (388 & 389). In addition, muscle accounts for approximately 50% and adipose tissue 15% of the total body weight in rodents (388), and similar figures have been presented for human subjects (482). Futhermore, it has been suggested that muscle and adipose tissue could be important sites at which steroids influence carbohydrate metabolism (3). Several studies have indicated that

natural estrogens might improve the sensitivity of these tissues to the action of insulin (169, 170 & 173). Circumstantial evidence has also been presented which indicates that progestogens might exert a small contra-insulin effect at these sites (143 & 165). However, these reports have yet to be confirmed by detailed in vitro studies, and studies with synthetic estrogens and progestogens have not been reported.

The present study was performed to make a more detailed analysis of peripheral tissue sensitivity to insulin during sex steroid treatment in skeletal muscle and adipose tissue. The effect of three administration routes have been compared and the influence of the prevailing estrogen and progestogen environment has been taken into account using both intact and ovariectomised mice. The results obtained will enable a more comprehensive interpretation of the effects of sex steroid administration on carbohydrate metabolism in relation to peripheral insulin action.

METHODS

Intact and bilaterally ovariectomised adult female mice were used in this study. Four weeks were allowed to elapse between ovariectomy and incorporation of the mice into the study. The mice were divided into ten groups and treated for four cycles as described below -

Groups 1 Intact - arachis oil

2 Ovariectomised - arachis oil

3 E_2 - 5µg/kg (body wt.) / day

4 EE - 5µg/kg (body wt.) / day

5 P - 1mg/kg (body wt.) / day

6 N - 1mg/kg (body wt.) / day

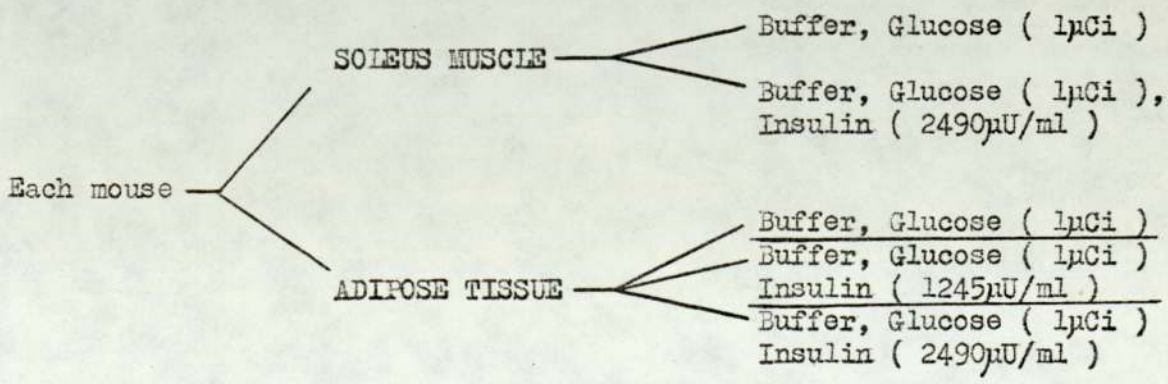
7 NA - 1mg/kg (body wt.) / day

8 E_2 & P - 5µg & 1mg/kg (body wt.) / day

9 EE & N - 5µg & 1mg/kg (body wt.) / day

10 EE & NA - 5µg & 1mg/kg (body wt.) / day

Within each group the Sc, Im and oral routes of administration were examined. After four cycles of treatment, each mouse was killed by cervical dislocation. Tissue samples were rapidly excised from the parametrial fat pad (lateral margin) and skeletal muscle (intact soleus muscle). The tissues were rinsed in warm oxygenated buffer, blotted and weighed. Incubations, as described in Chapter 2, were performed for 60 minutes. The following protocol was utilised -



Glucose uptake by adipose tissue and muscle was assessed by the concentration of radioactivity in the tissue. This was determined by LSC, as described in Chapter 2. Specific activity was expressed as dpm/mg wet weight.

RESULTS

(i) Group 1 - Intact diestrus mice

Intact diestrus mice (figure 65) received the vehicle only, arachis oil, by the Sc, Im and oral administration routes. The results show that labelled glucose was taken up by adipose tissue and soleus muscle in the absence of insulin. Addition of insulin increased the uptake of glucose in both tissues, a dose-related elevation was apparent. The absolute amount of glucose accumulated, expressed per mg wet weight of tissue, was greater in muscle than fat, and higher levels of radioactivity were consistently observed in muscle throughout the study. Intact soleus muscle in diestrus mice accumulated approximately three times the radioactivity of adipose tissue. The higher dose of insulin (2490μU/ml) produced an increase in glucose uptake that was of proportionally similar magnitude in both tissues, approximately 15 - 20%. There were no significant differences associated with the administration route.

To maintain consistency with previous studies, the intact mice were used during the diestrus stage of the estrous cycle. At this time carbohydrate tolerance is relatively impaired in comparison with the other stages during the cycle (120 & 123). Thus the radioactivity levels shown in figure 65 correspond to a time of impaired glucose tolerance in the adult female mouse. A comparison with the steroid treated mice will thus illustrate if any impairment of sensitivity to insulin in the treated mice is greater than the least sensitive period of the normal estrous cycle.

(ii) Group 2 - Ovariectomised mice

The effect of ovariectomy on tissue glucose accumulation is illustrated in figure 66. These animals were not pretreated with steroids, but were given arachis oil. Since no significant alterations

of glucose uptake attributable to the administration route were seen in intact diestrus mice (figure 65), it was decided to study only one route, the Sc administration route.

The results show that labelled glucose was taken up by adipose tissue and muscle in the absence of insulin. A dose-related accumulation of labelled glucose was observed with increasing concentrations of insulin in the incubation medium. Both basal and insulin-mediated glucose uptake were marginally higher in this group compared with intact diestrus mice. This is difficult to explain, since glucose tolerance is impaired to a greater extent following ovariectomy than during the diestrus stage of the estrous cycle in intact mice (119). These results suggest endogenous estrogens and progestogens do not significantly modulate glucose uptake by muscle or fat. In addition, it appears that alterations of the sensitivity and/or responsiveness of these tissues do not totally account for the presence of glucose intolerance in steroid treated mice described in Chapter 3. However, exogenous natural and synthetic estrogens and progestogens appear to exert a significant influence on glucose uptake by peripheral tissues, as described in the following sections of this chapter.

Group 3 - Estradiol 17β treatment

The influence of estradiol treatment on glucose uptake is shown in figure 67. The natural estrogen produced a marked elevation in basal glucose uptake (accumulation in the absence of insulin) in both adipose tissue and muscle in comparison with diestrus levels. However, whereas the increase in glucose accumulation in the presence of insulin was greater than seen in the intact diestrus mice after Sc administration, the Im and oral routes abolished the usual elevation produced by insulin. These data suggest the sensitivity of muscle and

adipose tissue was impaired in the Im and oral administration routes.

The large elevation of glucose uptake by the tissues of estrogen treated mice compares favourably with the lower plasma glucose titres observed in these animals compared with diestrus controls (see figure 18, chapter 3). Differences in plasma glucose titres and tissue glucose accumulation associated with the administration route were similar. Sc E_2 treatment produced the lowest plasma glucose levels whilst Im E_2 was associated with the higest levels (see figure 18, chapter 3).

(iv) Group 4 - Ethynl estradiol treatment

The influence of EE treatment in intact mice on peripheral tissue accumulation of glucose is illustrated in figure 68. EE, like E_2, noticeably raised basal glucose uptake in both adipose tissue and soleus muscle. However, the synthetic estrogen consistently produced a less marked effect on adipose tissue than E_2. Whereas both estrogens had similar effects on muscle (figures 75 & 76). EE inhibited insulin-mediated glucose uptake when administered by either the Im or oral routes, though when given by the Sc route a normal response was observed. Thus the influence of EE was very similar to that of the natural estrogen, E_2.

These observations correlate with plasma glucose titres at four cycles (see figure 19, chapter 3). The elevated glucose accumulation, compared with diestrus controls, corresponds with the lower plasma glucose titres produced by EE treatment in comparison with the control levels. Furthermore, the administration route associated with the highest tissue radioactivity uptake was also the one associated with the lowest plasma glucose titres.

(v) Group 5 - Progesterone treatment

Intact mice treated with P are shown in figure 69. Comparison with intact diestrus mice shows that P treatment elevated basal glucose uptake in both soleus muscle and adipose tissue after Im and oral administration, but not after Sc administration. Insulin-mediated glucose uptake was increased in both tissues, except following oral P administration. This may in part explain some of the reports in the literature that oral P produces a slight antagonism of insulin action (143 & 165), whereas this is not the case with Sc or Im P administration (120 & 126). A possible explanation for this effect following oral P treatment is that hepatic degradation produces a metabolite of P that is taken up by peripheral tissues, particularly adipose tissue, and produces insensitivity to insulin therein.

It is of particular interest that Im P produced the greatest uptake of glucose in adipose tissue and muscle since GTTs performed in similarly treated mice showed correspondingly low plasma glucose titres (see figure 25, chapter 3). This suggests adipose tissue and muscle are indeed important sites at which progestogens act to modify glucose homeostasis. This is further substantiated by the observation that the lower uptake of glucose produced by Sc P administration corresponds with high plasma glucose titres also produced by this regime (see figure 25, chapter 3). From the present data, it appears that insulin-mediated glucose uptake was similar in P treated and intact diestrus mice (figure 65).

(vi) Group 6 - Norethnodrel treatment

Tissue radioactivity levels of intact mice treated with N are illustrated in figure 70. In the absence of insulin, glucose uptake by soleus muscle and adipose tissue was not significantly different from the levels seen in intact diestrus mice (see figure 65 for comparison),

with the exception of orally administered N which marginally raised glucose accumulation. A similar response was seen during P treatment, but basal glucose uptake was often higher following synthetic progestogen treatment. Insulin-mediated glucose uptake by adipose tissue was inhibited by N at all times, whereas such inhibition was only observed during oral P administration. However, insulin-mediated glucose uptake in muscle was similar to both P treated and intact diestrus levels. These results suggest that N, or a metabolite of N, reduces the sensitivity of the adipose tissue to insulin, especially the higher insulin concentration.

As seen previously, the peripheral tissue studies and the GT studies yielded compatible results. Thus for each administration route, the greatest accumulation of glucose corresponded with the lowest plasma glucose titres.

(vii) Group 7 - Norethisterone acetate treatment

The effect of NA administration on peripheral tissue glucose uptake is shown in figure 71. Basal glucose uptake was significantly higher than diestrus levels in both soleus muscle and adipose tissue after Sc and Im administration, but not after oral administration. These results compare favourably with the previous evidence that progestogen treatment often elevates glucose uptake in the absence of insulin (figures 5 & 76). NA treatment in fact produced the highest basal glucose levels of the three progestogens examined. As seen during N administration, insulin-mediated glucose uptake by adipose tissue was consistently impaired in the NA treated mice (compare figures 70 & 71). This inhibition is a feature of the effect of synthetic progestogen treatment on adipose tissue, but it is not typical of natural progestogen treatment (see figure 69). All three progestogen regimes increased insulin-mediated glucose uptake

in muscle, but this was limited in NA treatment (figure 71). As seen previously, the results suggest that NA, or a metabolite of NA, reduces the sensitivity of both adipose tissue and muscle to insulin, particularly the response of these tissues to increased insulin concentrations in the incubation medium.

As noted earlier, the peripheral tissue studies and the GT studies produced compatible results. Higher plasma glucose titres were observed after oral NA treatment when glucose uptake by muscle and adipose tissue was reduced, whereas lower plasma glucose titres corresponded with greater glucose uptake following Sc and Im NA treatment (see figure 27, chapter 3).

(viii) Group 8 - Combined estradiol and progesterone treatment

Glucose uptake after E_2 and P treatment is illustrated in figure 72. Basal glucose uptake was significantly raised above the intact diestrus levels. However, insulin-mediated glucose uptake was similar to intact diestrus levels. The Im route of administration produced marginally, though consistently, higher levels of glucose uptake than the other administration routes. This was predominantly the case with E_2 and P treatments in the single preparations (see figures 67 & 69 for comparison). Comparison of the data shows that the combined preparation produced levels of glucose uptake that were generally lower than those produced by E_2, but higher than those produced by P. This suggests that P partially antagonised the uptake of glucose produced by E_2 in both adipose tissue and soleus muscle in the combined E_2 and P treated mice. These data support studies which have suggested that P exerts a slight contra-insulin effect in the presence of estrogens (143 & 165).

From the results presented in this section it is deduced that combined E_2 and P treatment enhances glucose uptake, and this may

explain at least in part, the lower plasma glucose levels seen in these mice compared with the intact diestrus mice (see figure 31, chapter 3). Accordingly, plasma glucose titres were lowest at four cycles during Im combined E_2 and P treatment, and tissue glucose uptake was greatest at this time.

(ix) Group 9 - Combined ethynyl estradiol and norethnodrel treatment

Glucose uptake in intact animals after treatment with the synthetic combination, EE and N, is shown in figure 73. Glucose uptake in the absence of insulin was considerably elevated above diestrus levels in adipose tissue and soleus muscle. The levels were generally intermediate between those produced by the two steroid components alone. Thus the presence of the progestogen in the combined preparation reduced the marked elevation of basal glucose uptake produced by the estrogen component alone. Insulin-mediated glucose uptake was essentially similar to the intact diestrus mice. Thus the combined preparation produced greater insulin-mediated increments in glucose uptake by adipose tissue than either EE or N given alone (see figures 68 & 70 for comparison). However in muscle, insulin-mediated uptake was similar to that produced by the individual steroids.

The effect of the administration route on glucose accumulation was slight, with marginally but consistently higher levels occurring after Im combined EE and N administration. A similar pattern was seen during EE treatment (figure 68), but N therapy produced the greatest accumulation after oral administration.

The high levels of radioactivity in comparison with the intact diestrus mice, correspond with the low plasma glucose titres produced by EE and N treatment (see figure 32, chapter 3). At four cycles, both the lowest plasma glucose titres and the highest glucose uptake were observed following Im EE and N administration.

(x) Group 10 - Combined ethynyl estradiol and norethisterone acetate

The influence of EE and NA treatment on glucose accumulation in adipose tissue and soleus muscle is illustrated in figure 74. Basal glucose uptake was significantly higher than diestrus levels in both tissues. Labelled glucose uptake was intermediate between those produced by EE and NA individually. Thus in the combined preparation, NA appears to partially antagonise the effect of EE on tissue glucose uptake. Insulin-mediated glucose uptake was severely limited after Im and oral combined EE and NA treatment in fat and muscle (figure 74). However, in the Sc route the response was similar to, or greater than that seen in the intact diestrus mice (see figure 65 for comparison). A similar pattern was observed in the EE treated mice (figure 68), whereas the NA regime inhibited insulin-mediated glucose uptake in all three administration routes (figure 71).

The differences in uptake attributable to the administration route were not consistent. The highest levels in the adipose tissue were produced by Sc administration, whilst the highest levels in muscle were produced by oral administration (figure 74). Nevertheless, the high glucose accumulation produced by combined EE and NA treatment corresponded with low plasma glucose titres observed in similarly treated mice (see figure 33, chapter 3 for comparison).

(xi) Comparison and discussion of the different treatment regimes

Figures 75 and 76 illustrate the glucose uptake data of all the groups treated by the Sc route of administration. Similar relationships were obtained with the Im and oral administration routes.

All steroid treatments significantly elevated muscle and adipose tissue accumulation of glucose in comparison with the intact diestrus mice. The greatest uptake of glucose was produced by the estrogens, and the smallest by progestogen administration. The combined

preparations produced intermediate levels. It is evident from these data that the marked increase in basal glucose uptake produced by estrogen treatment was considerably reduced by the use of combined regimes. Progestogens, given alone, may produce small elevations in basal glucose uptake, however in the combinations they antagonise the effects of the estrogen component on glucose uptake. This is compatible with conclusions obtained from insulin hypoglycaemia tests after fifteen weeks of E_2 and P, both singularly and in combination, treatment in mice (126). However, this antagonism between the steroids did not abolish glucose uptake, it only reduced the effect of the estrogen.

In both the progestogen and combined treatment regimes, the natural steroids had the smallest influence on glucose uptake (figures 75 & 76). After steroid administration, the uptake of glucose by soleus muscle was consistently greater than adipose tissue at the same insulin concentration. These data implicate muscle as a quantitatively more important tissue than adipose tissue for the uptake and metabolism of glucose, as already suggested by other studies (388 & 389). No consistent alterations associated with administration route were apparent. However, comparison of the glucose data described in this chapter with the plasma glucose titres observed in similarly treated mice, described in chapter 3, shows that a close correlation exists: such that higher glucose uptake generally corresponded with lower plasma glucose concentrations. This stresses the quantitative importance of muscle and adipose tissue as regulators of plasma glucose homeostasis, and highlights the need for additional studies to investigate these interactions in more detail.

The present data indicate that improved glucose tolerance in estrogen treated mice (see figure 18, chapter 3) may be attributed, in part to increased glucose uptake by muscle and adipose tissue in the

presence and absence of insulin. This effect is co-existent with increased circulating insulin titres (see chapter 3). Progestogens also elevate circulating insulin concentrations, but they do not consistently improve GT (see chapter 3). This may be attributed to the impairment of insulin-mediated glucose uptake by muscle and adipose tissue, and antagonism of the influence of estrogens on glucose uptake by these tissues. Thus although most sex steroids increase plasma insulin, additional factors, possibly synergism between estrogens and insulin and antagonism between progestogens and insulin, also appear to be important in the regulation of glucose homeostasis. The present study also raises the possibility that E_2 may have an insulin-like action in soleus muscle and adipose tissue. The high glucose uptake occurring in the absence of insulin supports this hypothesis. However, the present experimental technique does not exclude the possibility of of small quantities of residual insulin in the interstitial fluid. This insulin might conceivably act as a permissive agent to facilitate E_2-stimulated glucose uptake. The concept that estrogens may increase glucose uptake and metabolism independently of insulin has been suggested previously (162 & 418). In support of this view, natural estrogens have been shown to elevate glucose and amino acid penetration into uterine muscle via an inducible carrier-mediated transport system (171 & 483). However, the present data are the first to indicate that estrogens may be able to induce similar systems in adipose tissue and skeletal muscle.

Figure 65 Intact diestrus mice - ^{14}C glucose uptake by adipose tissue & soleus muscle in vitro (mean ± SEM, n = 5).

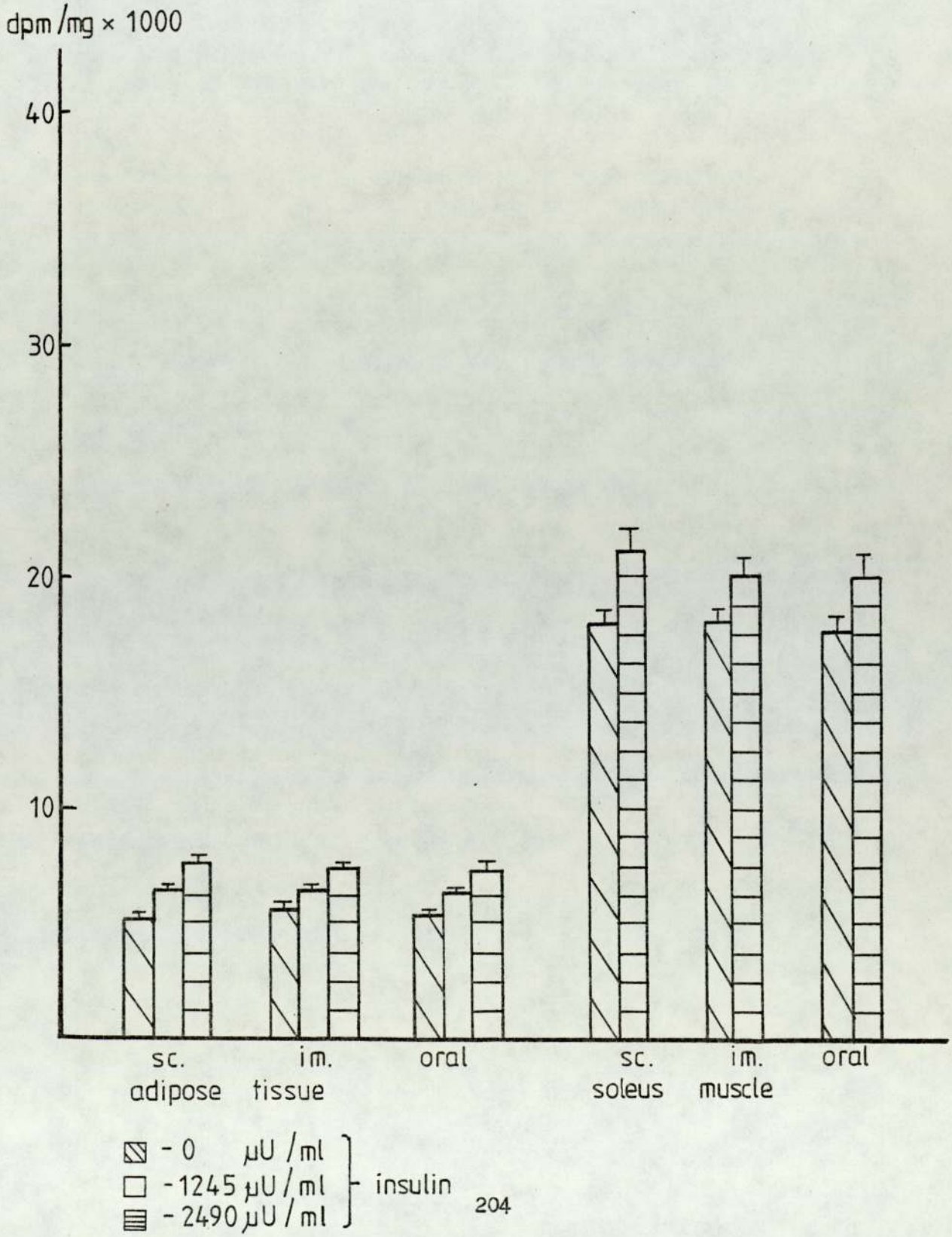


Figure 66 Ovariectomised mice - ^{14}C glucose uptake by adipose tissue & soleus muscle in vitro (mean± SEM , n = 5).

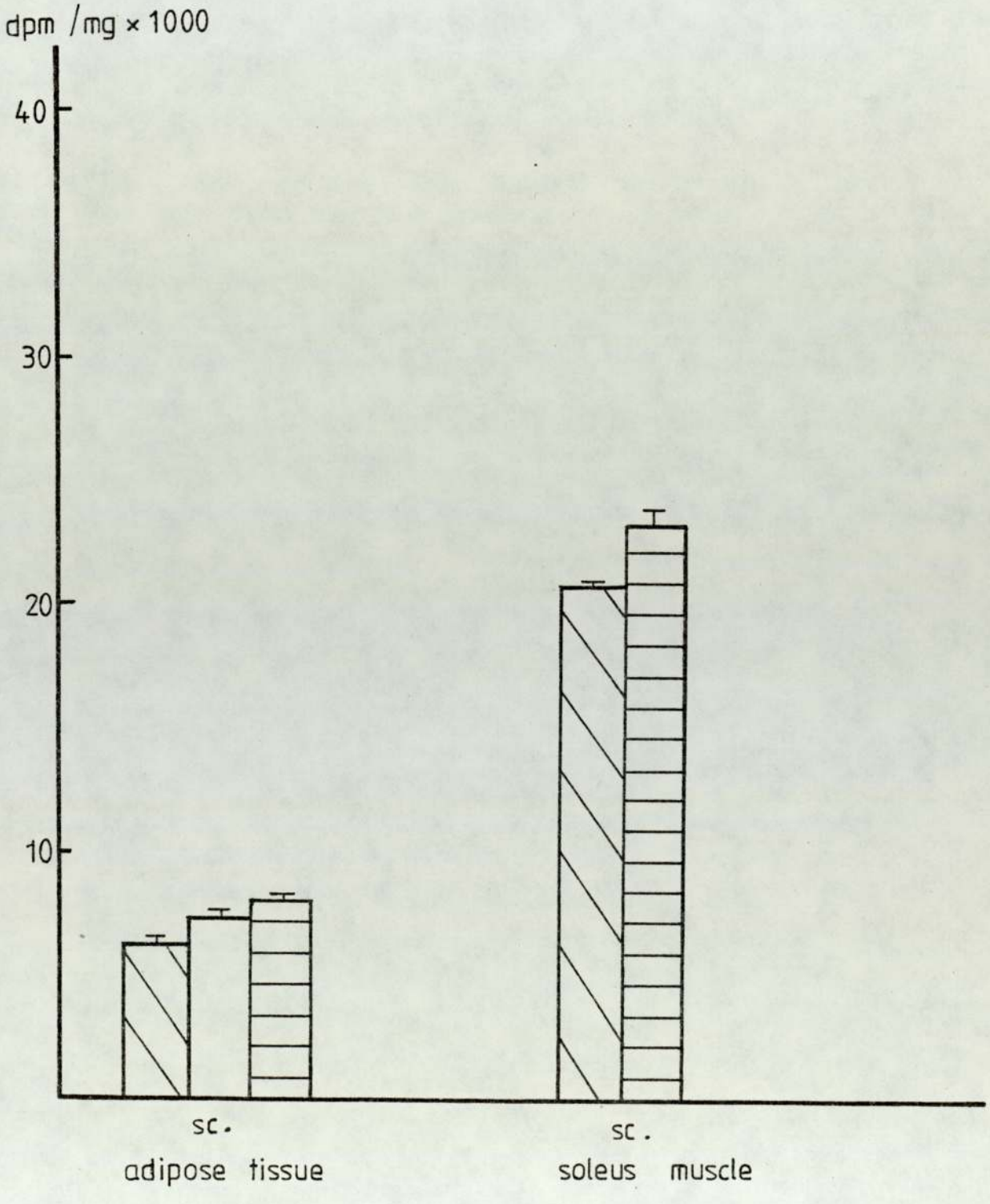


Figure 67 Subcutaneous, intramuscular & oral estradiol treated mice (5ug /kg / d / 4 cycles) - ^{14}C glucose uptake by adipose tissue & soleus muscle in vitro (mean ± SEM , n = 5).

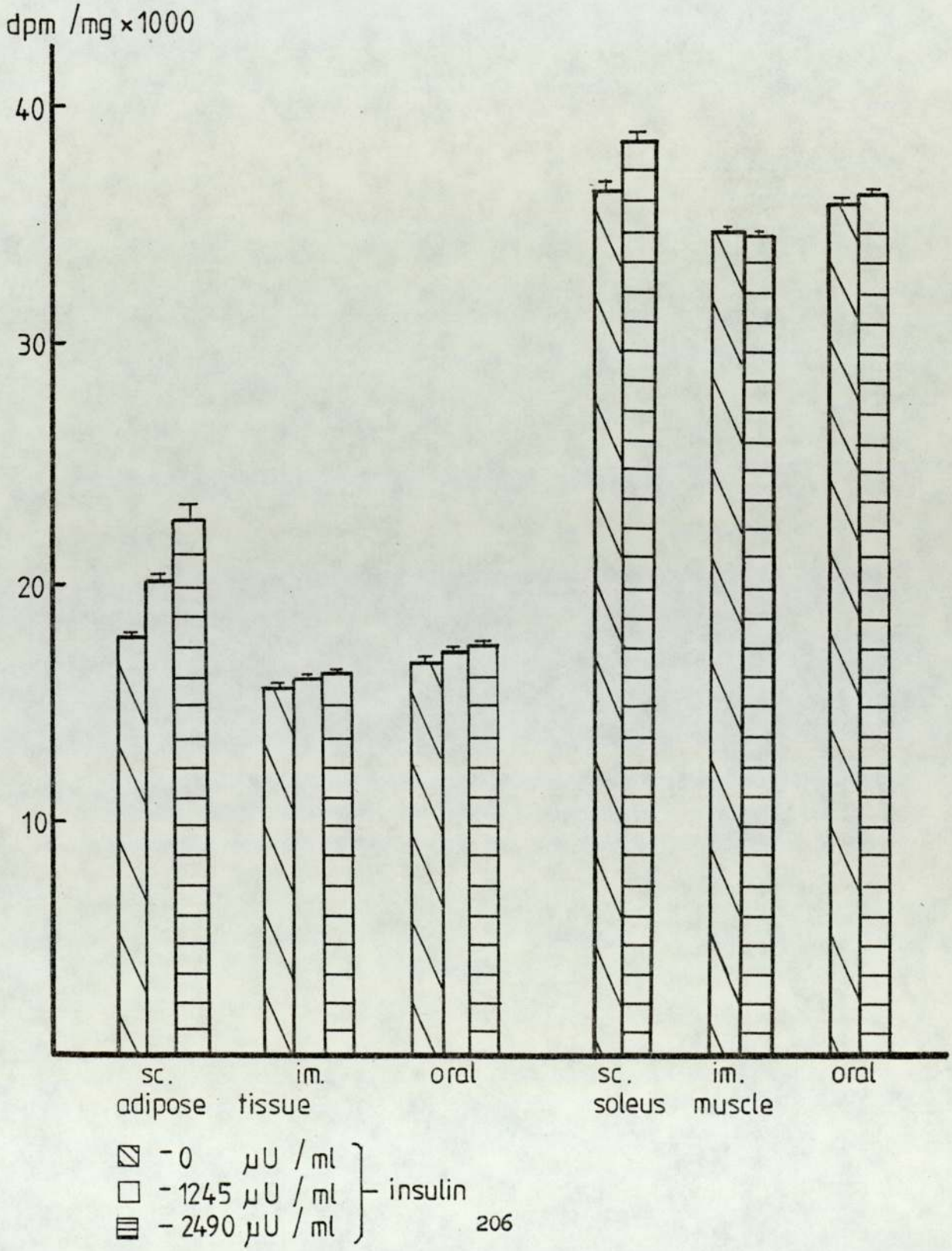


Figure 68 Subcutaneous, intramuscular & oral ethynyl estradiol treated mice (5ug/kg/d /4 cycles) - ^{14}C glucose uptake by adipose tissue & soleus muscle in vitro (mean ± SEM , n = 5).

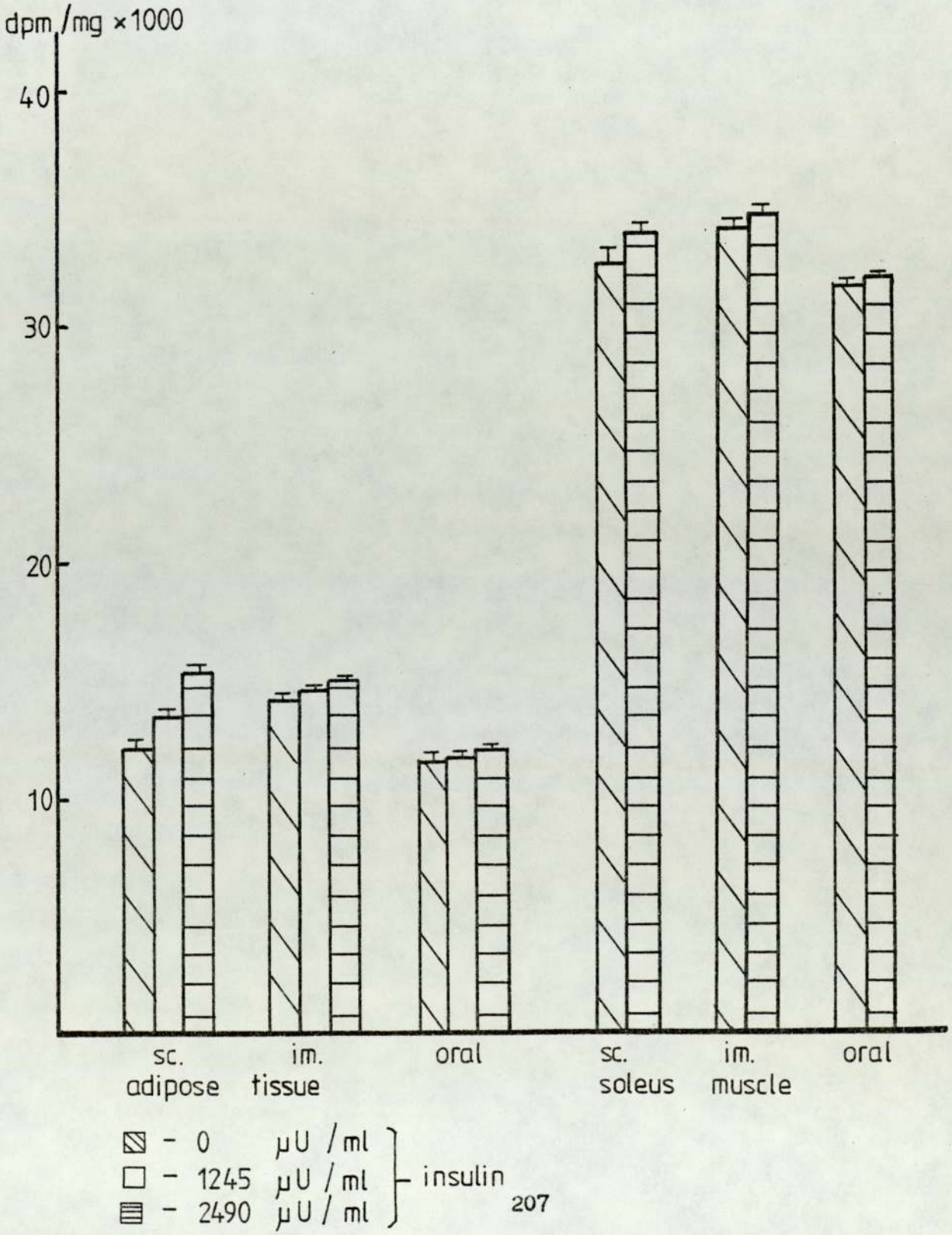


Figure 69 Subcutaneous, intramuscular & oral progesterone treated mice (1mg / kg / d / 4 cycles) – ^{14}C glucose uptake by adipose tissue & soleus muscle in vitro (mean ± SEM, n = 5).

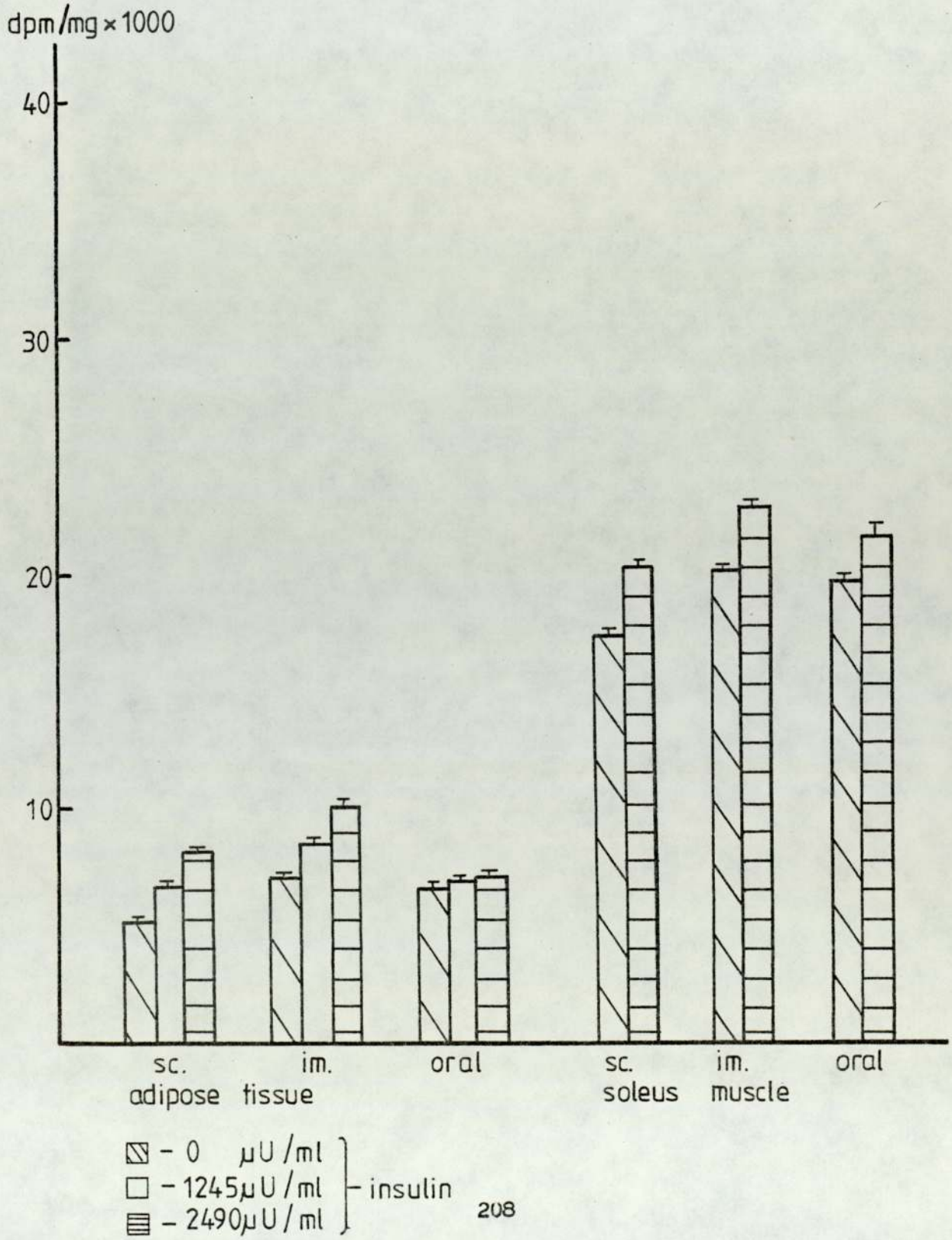


Figure 70 Subcutaneous, intramuscular & oral norethnodrel treated mice (1mg / kg /d /4cycles) - ^{14}C glucose uptake by adipose tissue & soleus muscle in vitro (mean ± SEM , n = 5).

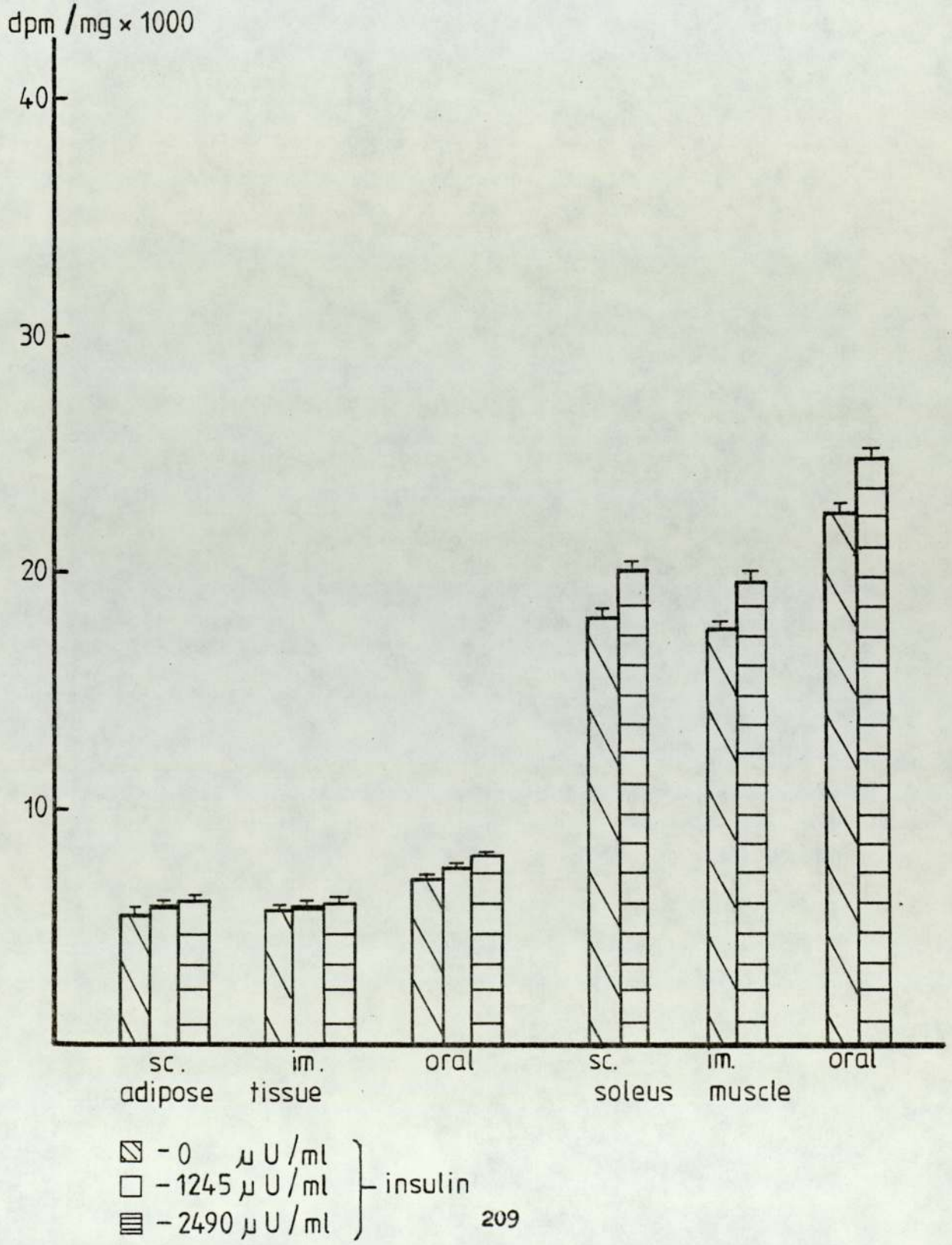


Figure 71 Subcutaneous, intramuscular & oral norethisterone acetate treated mice (1mg/kg/d/4 cycles) -^{14}C glucose uptake by adipose tissue & soleus muscle <u>in vitro</u> (mean ± SEM, n = 5).

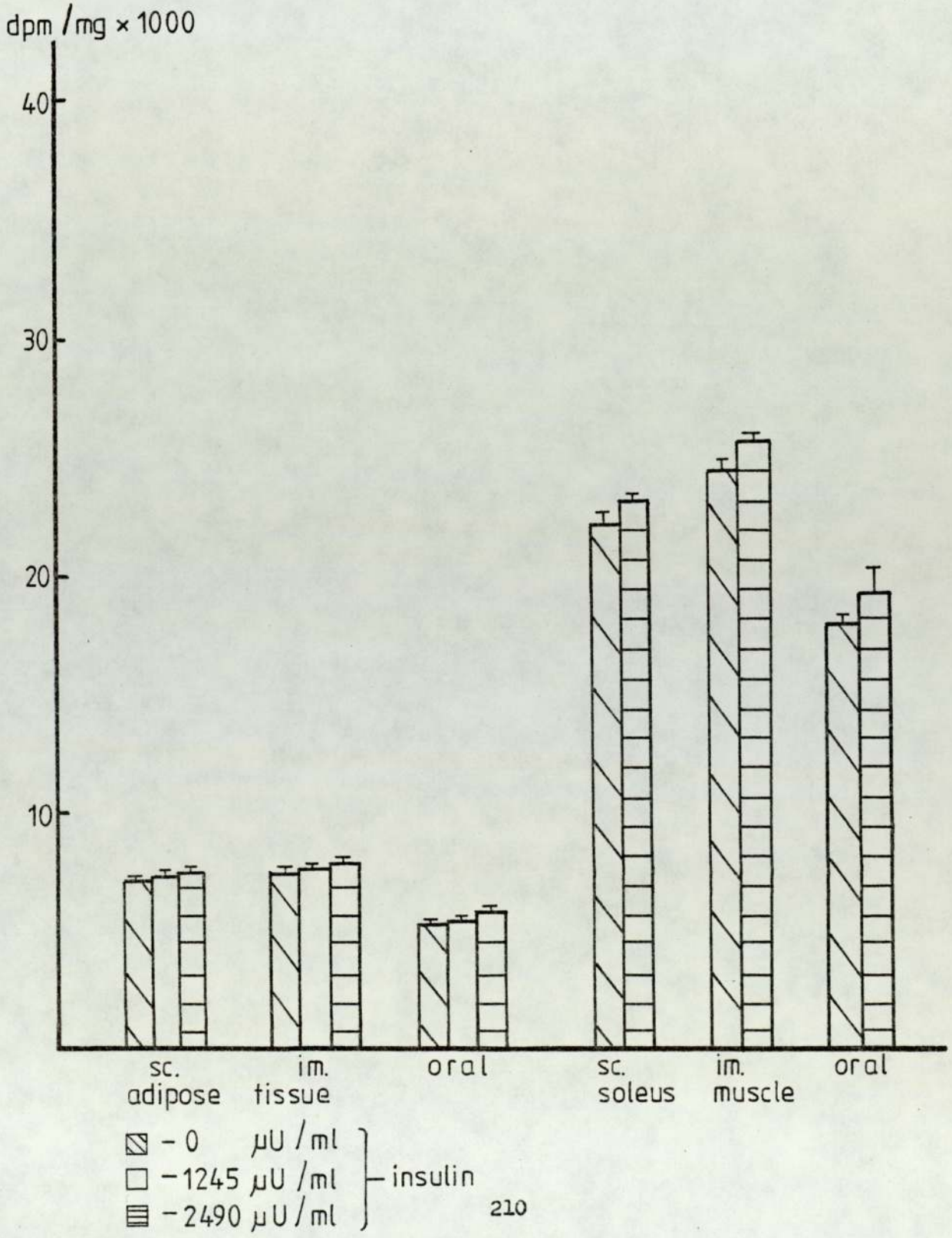


Figure 72 Subcutaneous, intramuscular & oral estradiol + progesterone treated mice (5ug+1mg /kg /d /4 cycles) - ^{14}C glucose uptake by adipose tissue & soleus muscle <u>in vitro</u> (mean ± SEM , n = 5).

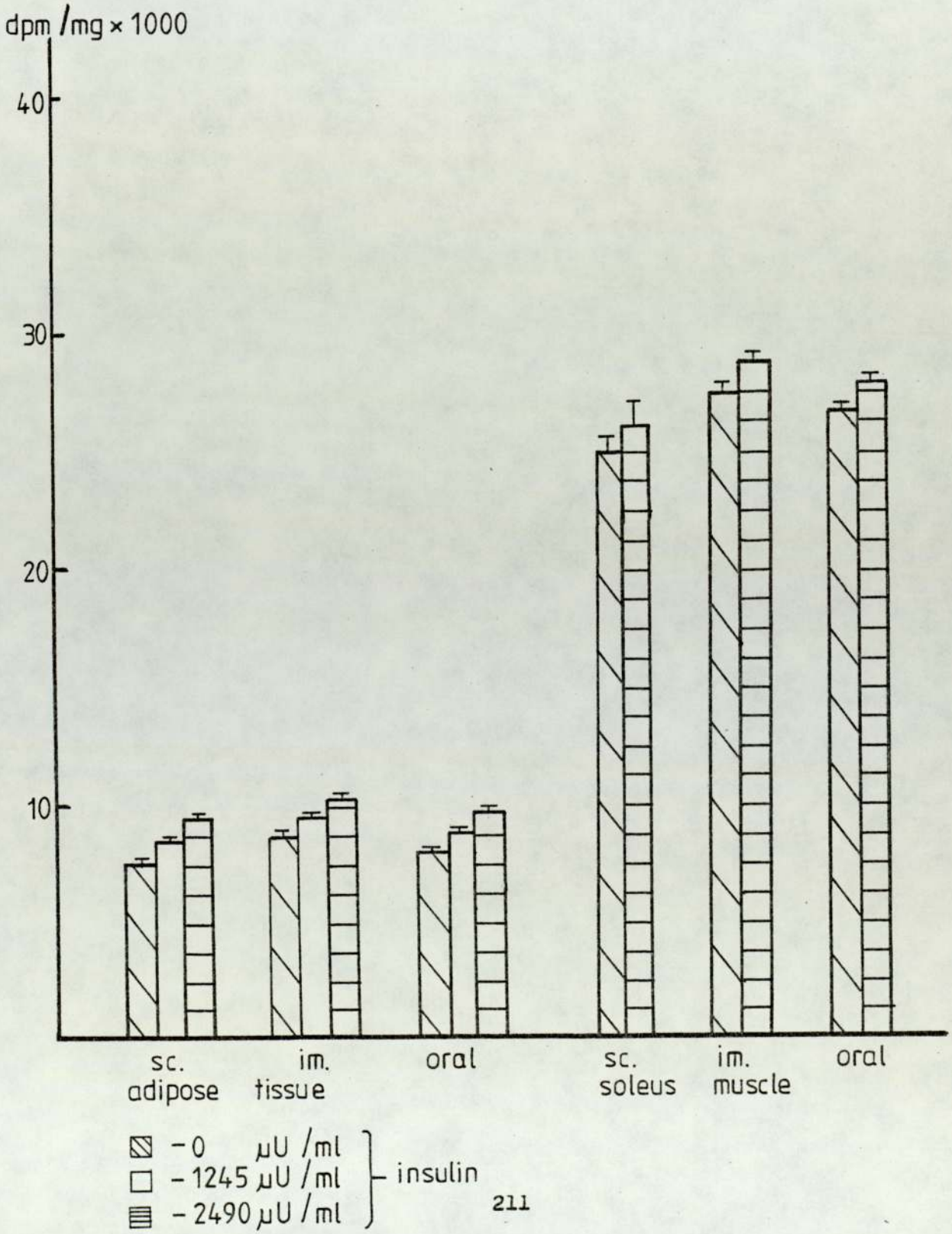


Figure 73 Subcutaneous, intramuscular & oral ethynyl estradiol + norethnodrel treated mice (5ug+1mg/kg/d/4cycles)- ^{14}Cglucose uptake by adipose tissue & soleus muscle in vitro (mean ± SEM, n= 5).

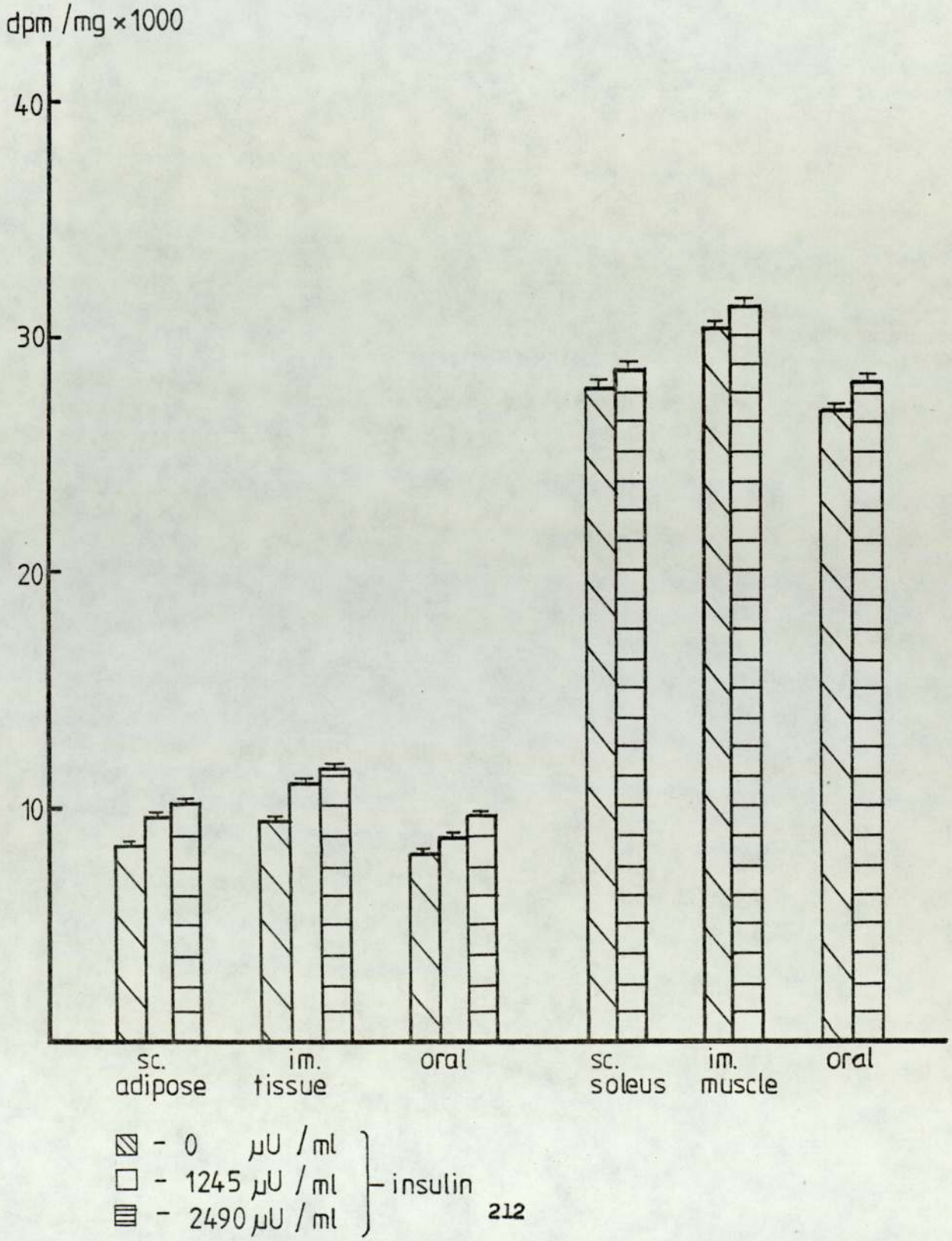


Figure 74 Subcutaneous, intramuscular & oral ethynyl estradiol + norethisterone acetate treated mice (5ug+1mg /kg / d /4cycles) - ^{14}C glucose uptake by adipose tissue & soleus muscle in vitro (mean ± SEM, n = 5).

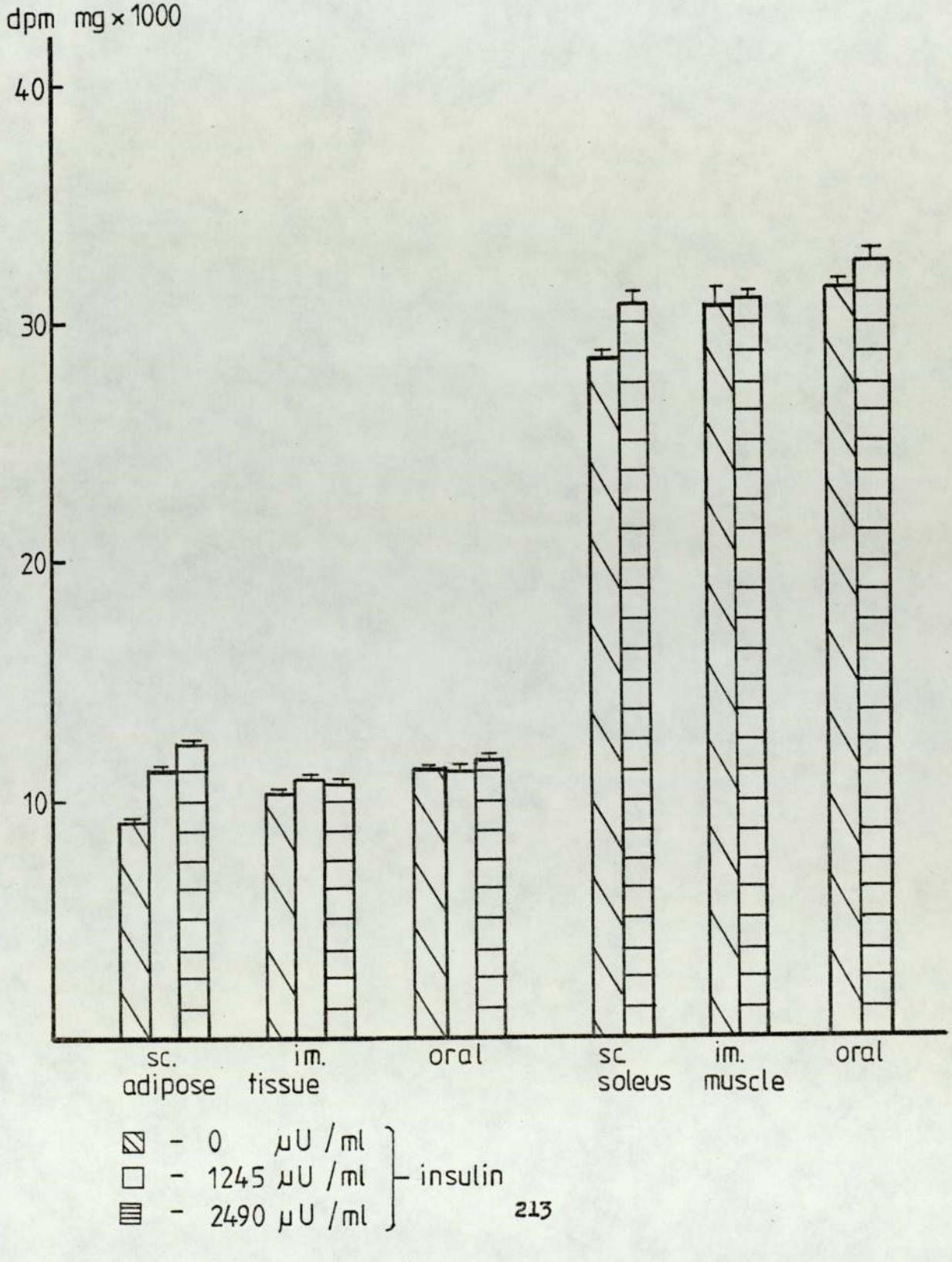


Figure 75 Comparison of ^{14}Cglucose uptake by adipose tissue <u>in vitro</u> during subcutaneous steroid treatment in mice (mean ± SEM, n = 5).

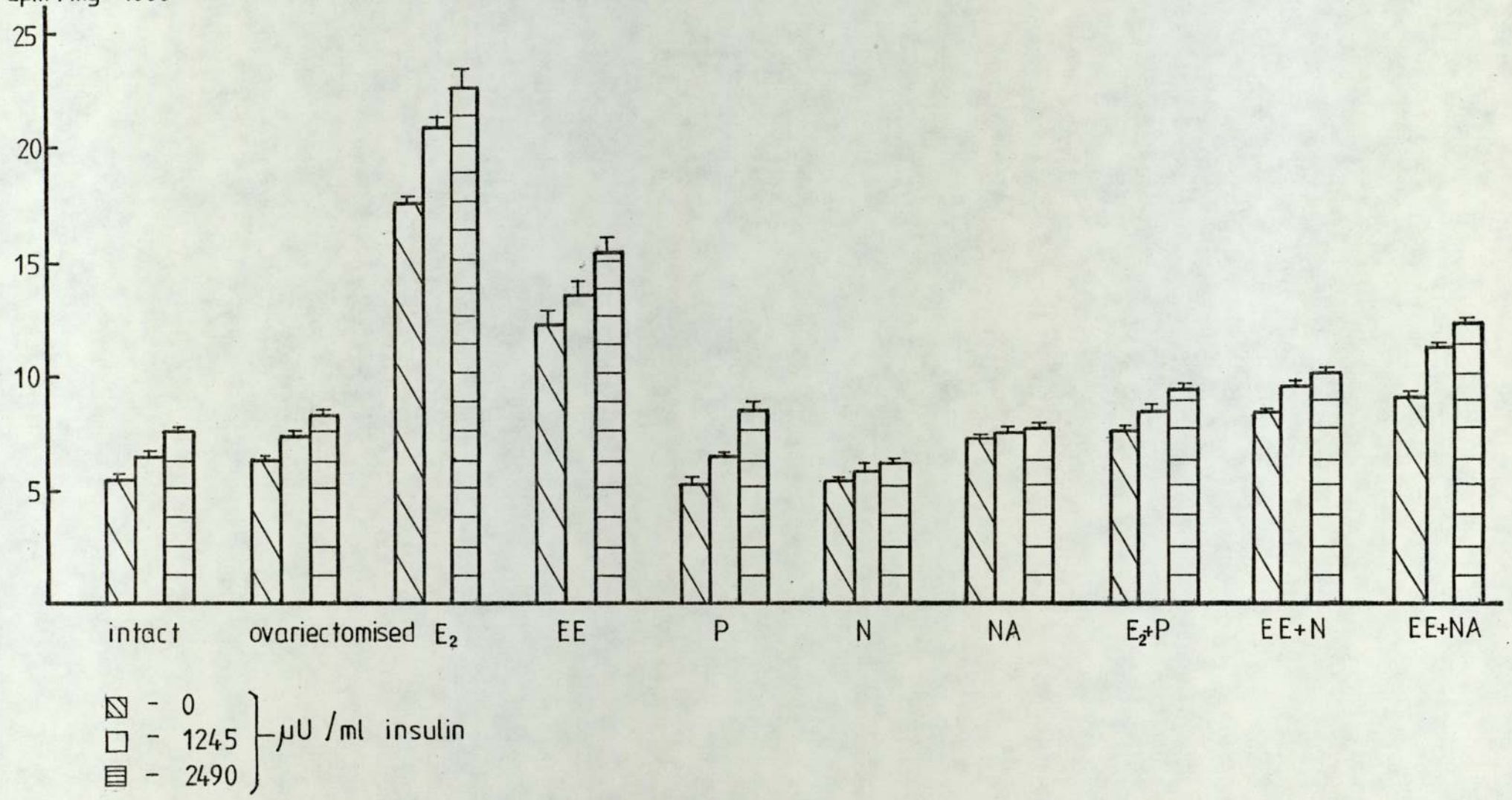


Figure 76 Comparison of ^{14}C glucose uptake by soleus muscle in vitro during subcutaneous steroid treatment in mice (mean ± SEM, n = 5).

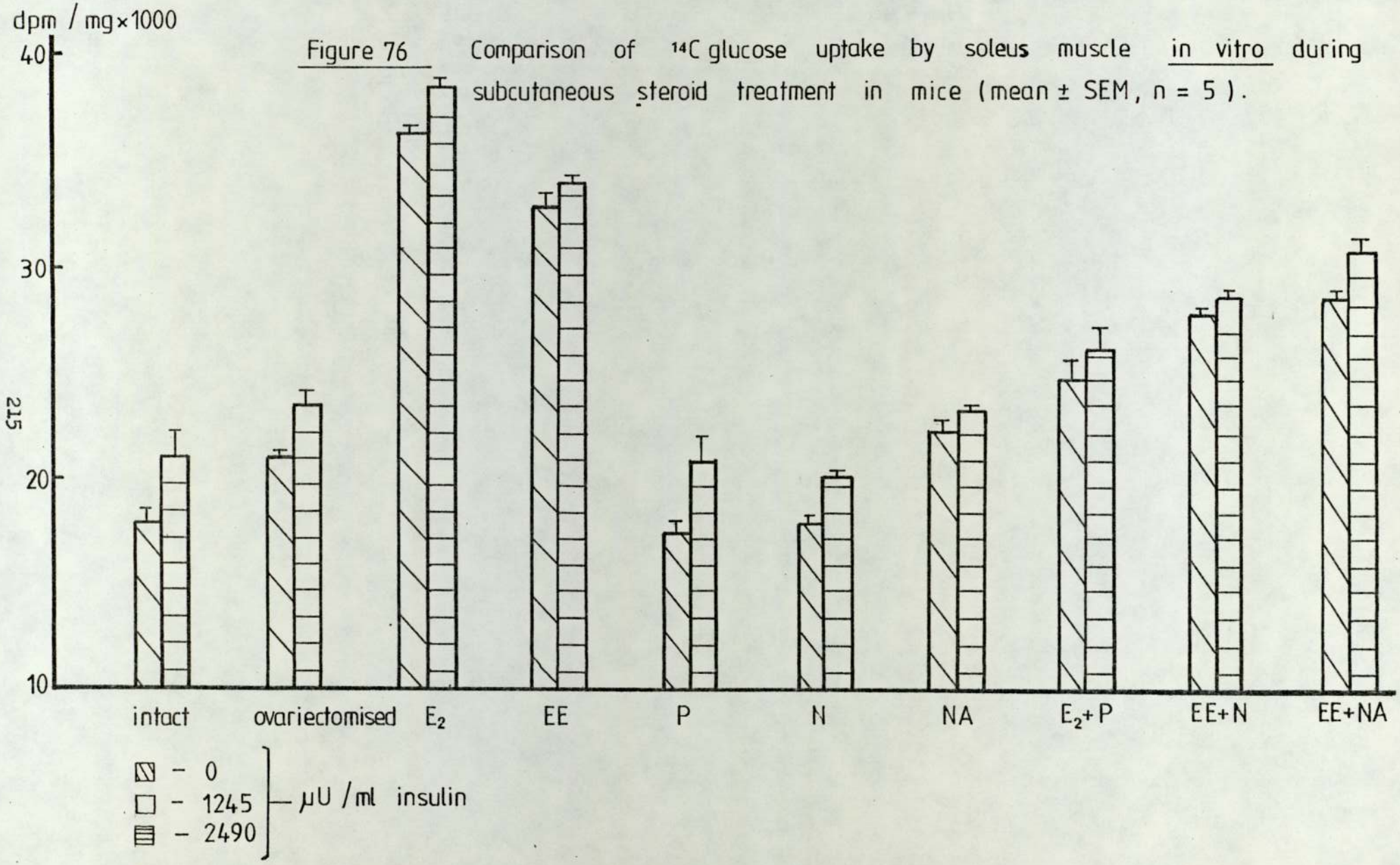


Table 11 PERIPHERAL TISSUE UPTAKE OF ^{14}C-GLUCOSE - CHAPTER 6 (n = 5)

TISSUE ^{14}C-GLUCOSE LEVELS (dpm/mg)						
	Subcutaneous mean	SEM	Intramuscular mean	SEM	Oral mean	SEM
DIESTRUS CONTROLS						
AT						
0μUI/ml	5,345	268	5,415	247	5,384	128
1245μUI/ml	6,307	153	6,368	142	6,291	155
2490μUI/ml	7,491	306	7,237	237	7,342	340
SM						
0μUI/ml	17,922	563	17,971	531	17,617	609
2490μUI/ml	20,915	1,143	19,976	756	20,082	856
OVX						
AT						
0μUI/ml	6,252	167				
1245μUI/ml	7,255	98				
2490μUI/ml	8,085	159				
SM						
0μUI/ml	20,859	225				
2490μUI/ml	23,433	712				
ESTRADIOL						
AT						
0μUI/ml	17,594	184	15,576	86	16,671	248
1245μUI/ml	20,055	268	15,932	71	16,970	166
2490μUI/ml	22,564	741	16,158	78	17,258	232
SM						
0μUI/ml	36,452	410	34,690	200	35,854	355
2490μUI/ml	38,546	327	34,570	212	36,173	171
ETHYNYL ESTRADIOL						
AT						
0μUI/ml	12,169	475	14,317	230	11,686	336
1245μUI/ml	13,525	284	14,754	81	11,718	360
2490μUI/ml	15,401	310	15,057	177	11,942	204
SM						
0μUI/ml	32,846	738	34,235	286	31,783	209
2490μUI/ml	33,934	412	34,739	336	31,950	293
PROGESTERONE						
AT						
0μUI/ml	5,235	158	7,069	111	6,557	74
1245μUI/ml	6,653	178	8,436	66	6,795	206
2490μUI/ml	8,230	231	10,028	168	6,810	239
SM						
0μUI/ml	17,382	276	20,031	186	19,489	274
2490μUI/ml	20,220	324	22,703	440	21,591	695
NORETHNODREL						
AT						
0μUI/ml	5,467	91	5,709	84	7,047	54
1245μUI/ml	5,860	340	5,801	64	7,445	125
2490μUI/ml	6,085	49	5,929	150	7,951	109
SM						
0μUI/ml	17,854	451	17,587	202	22,576	456
2490μUI/ml	19,979	260	19,564	353	24,803	172

Table 11 contn.

TISSUE ^{14}C-GLUCOSE LEVELS (dpm/mg)						
	Subcutaneous mean	SEM	Intramuscular mean	SEM	Oral mean	SEM
NORETHISTERONE ACETATE						
AT						
0μUI/ml	7,261	93	7,474	154	5,481	201
1245μUI/ml	7,318	144	7,557	182	5,548	148
2490μUI/ml	7,445	172	7,655	118	5,704	115
SM						
0μUI/ml	22,194	569	24,555	504	18,045	220
2490μUI/ml	23,162	357	25,774	426	19,303	1,161
ESTRADIOL + PROGESTERONE						
AT						
0μUI/ml	7,490	48	8,664	38	7,906	69
1245μUI/ml	8,379	169	9,437	198	8,590	114
2490μUI/ml	9.327	126	10,353	158	9,601	114
SM						
0μUI/ml	24,871	709	27,437	411	26,508	280
2490μUI/ml	26,030	1,003	28,778	454	27,637	373
ETHYNYL ESTRADIOL + NORETHNODREL						
AT						
0μUI/ml	8,465	97	9,394	147	7,932	89
1245μUI/ml	9,532	174	11,030	189	8,787	133
2490μUI/ml	10,152	71	11,794	167	9,540	138
SM						
0μUI/ml	27,760	433	30,402	281	26,849	358
2490μUI/ml	28,586	275	31,244	304	27,941	326
ETHYNYL ESTRADIOL + NORETHISTERONE ACETATE						
AT						
0μUI/ml	9,147	118	10,374	106	11,414	66
1245μUI/ml	11,137	60	10,820	166	11,396	319
2490μUI/ml	12,102	151	10,785	222	11,501	394
SM						
0μUI/ml	28,562	411	30,820	881	31,488	291
2490μUI/ml	30,895	566	31,041	377	32,629	658

Where AT - adipose tissue

SM - soleus muscle

I - insulin

Table 12 PERIPHERAL TISSUE UPTAKE OF ^{14}C-GLUCOSE - STATISTICAL COMPARISON WITH DIESTRUS CONTROLS - CHAPTER 6

TISSUE ^{14}C-GLUCOSE LEVELS (dpm/mg)			
	Subcutaneous	Intramuscular	Oral
OVX			
AT			
0μUI/ml	$p < 0.001$		
1245μUI/ml	$p < 0.001$		
2490μUI/ml	$p < 0.01$		
SM			
0μUI/ml	$p < 0.001$		
2490μUI/ml	$p < 0.01$		
ESTRADIOL			
AT			
0μUI/ml	$p < 0.001$	$p < 0.001$	$p < 0.001$
1245μUI/ml	$p < 0.001$	$p < 0.001$	$p < 0.001$
2490μUI/ml	$p < 0.001$	$p < 0.001$	$p < 0.001$
SM			
0μUI/ml	$p < 0.001$	$p < 0.001$	$p < 0.001$
2490μUI/ml	$p < 0.001$	$p < 0.001$	$p < 0.001$
ETHYNYL ESTRADIOL			
AT			
0μUI/ml	$p < 0.001$	$p < 0.001$	$p < 0.001$
1245μUI/ml	$p < 0.001$	$p < 0.001$	$p < 0.001$
2490μUI/ml	$p < 0.001$	$p < 0.001$	$p < 0.001$
SM			
0μUI/ml	$p < 0.001$	$p < 0.001$	$p < 0.001$
2490μUI/ml	$p < 0.001$	$p < 0.001$	$p < 0.001$
PROGESTERONE			
AT			
0μUI/ml	NS	$p < 0.001$	$p < 0.001$
1245μUI/ml	$p < 0.02$	$p < 0.001$	$p < 0.01$
2490μUI/ml	$p < 0.01$	$p < 0.001$	$p < 0.05$
SM			
0μUI/ml	NS	$p < 0.001$	$p < 0.001$
2490μUI/ml	NS	$p < 0.001$	$p < 0.05$
NORETHNODREL			
AT			
0μUI/ml	NS	NS	$p < 0.001$
1245μUI/ml	NS	$p < 0.001$	$p < 0.001$
2490μUI/ml	$p < 0.001$	$p < 0.001$	$p < 0.02$
SM			
0μUI/ml	NS	NS	$p < 0.001$
2490μUI/ml	NS	NS	$p < 0.001$
NORETHISTERONE ACETATE			
AT			
0μUI/ml	$p < 0.001$	$p < 0.001$	NS
1245μUI/ml	$p < 0.001$	$p < 0.001$	$p < 0.001$
2490μUI/ml	NS	$p < 0.02$	$p < 0.001$

Table 12 contn.

TISSUE ^{14}C-GLUCOSE LEVELS (dpm/mg)			
	Subcutaneous	Intramuscular	Oral
NORETHISTERONE ACETATE			
SM			
0μUI/ml	$p < 0.001$	$p < 0.001$	NS
2490μUI/ml	$p < 0.01$	$p < 0.001$	NS
ESTRADIOL + PROGESTERONE			
AT			
0μUI/ml	$p < 0.001$	$p < 0.001$	$p < 0.001$
1245μUI/ml	$p < 0.001$	$p < 0.001$	$p < 0.001$
2490μUI/ml	$p < 0.001$	$p < 0.001$	$p < 0.001$
SM			
0μUI/ml	$p < 0.001$	$p < 0.001$	$p < 0.001$
2490μUI/ml	$p < 0.001$	$p < 0.001$	$p < 0.001$
ETHYNYL ESTRADIOL + NORETHNODREL			
AT			
0μUI/ml	$p < 0.001$	$p < 0.001$	$p < 0.001$
1245μUI/ml	$p < 0.001$	$p < 0.001$	$p < 0.001$
2490μUI/ml	$p < 0.001$	$p < 0.001$	$p < 0.001$
SM			
0μUI/ml	$p < 0.001$	$p < 0.001$	$p < 0.001$
2490μUI/ml	$p < 0.001$	$p < 0.001$	$p < 0.001$
ETHYNYL ESTRADIOL + NORETHISTERONE ACETATE			
AT			
0μUI/ml	$p < 0.001$	$p < 0.001$	$p < 0.001$
1245μUI/ml	$p < 0.001$	$p < 0.001$	$p < 0.001$
2490μUI/ml	$p < 0.001$	$p < 0.001$	$p < 0.001$
SM			
0μUI/ml	$p < 0.001$	$p < 0.001$	$p < 0.001$
1245μUI/ml	$p < 0.001$	$p < 0.001$	$p < 0.001$

Where AT - adipose tissue

SM - soleus muscle

I - insulin

$p < 0.05$ is significant

$p < 0.02$ & $p < 0.01$ are highly significant

$p < 0.002$ & $p < 0.001$ are very highly significant

Chapter 7

DISCUSSION

CHAPTER 7

DISCUSSION

This study has shown that both natural and synthetic female sex steroids are intimately involved in the regulation of glucose homeostasis in the adult female mouse.

The effect of various sex steroid preparations on glucose and insulin metabolism has been examined using ipGTTs. The results show that not only the chemical composition of the steroid preparation, but also the route of administration and the duration of treatment play an important role in determining the alterations of carbohydrate metabolism.

The tolerance tests demonstrated that circulating plasma insulin was characteristically elevated above the diestrus levels in sex steroid treated mice. The natural hormones, E_2 and P, had a greater stimulatory effect on insulin than their synthetic counterparts. Indeed, N and NA often produced a lower plasma insulin response to glucose than the diestrus animals. This was unexpected since these progestogens are partly converted to EE *in vivo* (484 & 485) which has a marked insulinogenic influence. Thus synthetic progestogens appear to antagonise the insulinogenic influence of EE produced during their metabolism. Whether this antagonism is due to the intact progestogen molecule or due to other metabolites is unclear, but it is not a transient effect which subsides as metabolism of the progestogen proceeds.

In the estrogen treated mice, elevations of plasma insulin corresponded with significant reductions of plasma glucose, but the effect of progestogen treatment was more variable. Some progestogen regimes produced elevations and others reductions in the glucose titre. Nevertheless, E_2 and P treatment lowered circulating glucose to a greater extent than synthetic steroids. A relationship was observed

between the plasma insulin and glucose titres. The highest insulin and glucose levels occurred during oral steroid treatment, and the lowest insulin and glucose levels were seen during Sc treatment. Thus in contraceptive steroid treated mice, the rise in circulating insulin was not always accompanied by a corresponding reduction in circulating glucose. Hence the hypoglycaemic action of insulin was altered by contraceptive steroids. Generally, Im and oral administration of these agents was associated with a reduction of the hypoglycaemic action of insulin, whilst Sc administration was associated with a potentiation of the hypoglycaemic action of insulin compared with the diestrus mice. These data suggest that differences in the distribution and metabolism of steroids due to the administration route were responsible to a large extent for differences in glucose homeostasis.

GTTs were performed in mice receiving a combined preparation containing an estrogen and a progestogen. The combined preparations rarely elevated the plasma insulin titre above diestrus levels, but circulating glucose was significantly reduced in these mice. In contrast to the single steroid regimes, the combined synthetic preparations were more effective than the natural combinations in raising the insulin titre. In addition, combined preparations given by the oral route generally produced a high insulin response to glucose, and those given by the Sc route produced a lower response. These data are compatible with those of the single steroid regimes, and support the hypothesis that differences in the distribution and metabolism of steroids play an important role in determining the alterations of glucose homeostasis.

Comparison of glucose homeostasis in single and combined regimes shows that with the latter, the two components interact to produce plasma insulin and glucose titres that differ to those produced by either component alone. For example, single steroid treatment was

associated with a characteristically large elevation of plasma insulin, but when the same agent is given in a combined preparation, insulin levels were rarely elevated above the diestrus levels. Thus the effect of combined preparations on insulin metabolism is not cumulative, antagonism of the estrogen component by the progestogen appears to be responsible for a reduction in the insulinogenic influence of combined preparations. The alterations of plasma glucose produced by combined preparations showed an effect that was intermediate between that produced by the estrogen and progestogen alone. Thus it appears that the estrogen and progestogen interact to mutually oppose their individual effects on glucose homeostasis, ie. the hypoglycaemic influence of estrogens (at a dose of 5ug/kg/d) is approximately opposed by the hyperglycaemic influence of progestogens (at a dose of 1mg/kg/d).

A consideration of both the insulin and glucose status demonstrates that combined steroid treatment did not affect carbohydrate metabolism adversely and often improved it, particularly when given by the Sc route. The beneficial influence of the combined preparations may be due partly to the interactions between the two components, and partly to the closer approximation to the normal circulating proportions of estrogen and progestogen than produced during single steroid administration. However, absolute circulating sex steroid titres would be higher than normal following combined treatment. The more advantageous effects of combined contraceptive preparations may be a particularly important factor to consider when prescribing contraceptive preparations to women with either a compromised carbohydrate status or a potential disposition towards developing impaired carbohydrate tolerance. In the following sections, the GT data are discussed in terms of the effects of contraceptive sex steroids on (i) in vivo distribution of sex steroids, (ii) tissue

glycogen deposition and (iii) peripheral tissue glucose utilisation.

(i) Distribution studies

These studies examined the hypothesis that alterations of carbohydrate homeostasis produced by different administration routes of the various contraceptive steroids are due to differences in the subsequent distribution of the steroid, which in turn affects the metabolism and action of these drugs in various tissues.

The results show that alterations of the prevailing sex hormone environment, whether by ovariectomy or sex steroid administration, affects both plasma and tissue uptake of labelled E_2 and P. These hormones are bound in considerable amounts to tissues that are quantitatively important in glucose homeostasis, yet are not normally regarded as target tissues for these hormones, namely skeletal muscle, adipose, pancreatic and hepatic tissues. The physiological significance of this binding has received virtually no attention in the literature compared with the extensive research effort directed towards the reproductive target tissues. The present study suggests that sex steroids may exert some direct effects on carbohydrate metabolism in the afore mentioned tissues. Detailed studies are required to identify specific steroid receptors in these 'non-classical' target tissues, and to determine their exact role in sex steroid-induced alterations of carbohydrate homeostasis.

The plasma and tissue binding capacity of P was lower than E_2, suggesting that the number and/or affinity of specific receptors was correspondingly lower for P. It is feasible that P is selectively taken up by tissues not examined in the present study, for example other adipose depots. Indeed it has been suggested that the fat depots respond differently to sex hormones (487). However, P may be more rapidly excreted from the body than E_2 .This hypothesis is supported

by the higher metabolic clearance rate reported for P (475 & 476).

From a consideration of the GT data in relation to the distribution characteristics of E_2 and P, it is possible to equate alterations of glucose homeostasis with the binding of these steroids. For example, mice receiving oral P exhibited the highest plasma insulin and glucose titres, and this corresponded with a high plasma and tissue ^{14}C-progesterone content. In the same way Sc and Im P administration produced low plasma insulin and glucose titres, and low plasma and tissue radioactivity levels.

Pancreatic P accumulation was particularly elevated by P pretreatment and hence this hormone may have a direct insulinogenic effect in this tissue. Such a direct effect has been proposed on several occasions (119 & 177). In addition to the insulinogenic effect of P in the present study, this steroid also reduced the hypoglycaemic action of insulin. The latter effect could involve a direct antagonistic effect on the action of insulin on tissues such as liver, muscle and adipose depots. The high uptake of P by these tissues is consonant with this view. Since P pretreatment increased labelled P accumulation, particularly after oral administration, it may facilitate absorption of this hormone in addition to contributing to a reduction in the metabolic clearance rate (475 & 476).

Glucose homeostasis in E_2 treated mice was related to the distribution of this hormone in a different manner to that noted for P. Oral E_2 produced the smallest accumulation of label in plasma and tissues, whilst also producing high plasma insulin and glucose titres. Furthermore, Sc E_2 produced the highest accumulation of label in conjunction with low plasma insulin and glucose titres.

The marked elevation of pancreatic E_2 uptake in pretreated mice suggests this hormone, like P, may play a direct role in regulating insulin secretion. However, this was not observed in short-term

studies (119 & 186). In contrast to P, E_2 treatment appears to accelerate peripheral glucose utilisation, and the distribution studies suggest that it may be associated with the accumulation of this hormone by peripheral tissues, namely muscle and adipose tissue. E_2 appears to potentiate the hypoglycaemic action of insulin and these two hormones are known to act synergistically in certain diabetic women (146) and in muscle and adipose tissue preparations in vitro (173, 175 & 418).

The distribution studies are consistent with the view that orally administered steroids are more rapidly absorbed into the circulation and more rapidly removed than Sc or Im administered steroids. The latter two thus produce a more stable circulating steroid titre over an extended period. The higher plasma and tissue accumulation of labelled hormone following Sc administration suggests that release occurs more rapidly than from the Im depot over the two hour time interval studied.

The distribution data confirm that a close positive relationship exists between the sex steroid titre and the sex steroid accumulation by plasma and tissues. It must be noted that the absolute number and/or affinity of sex steroid receptors may vary in different tissues. This awaits confirmation, but it may, in part explain why some tissues are more responsive to pretreatment with steroids than others. Thus the capacity of a tissue to respond may depend on the inherent receptor capacity of that tissue.

(ii) Tissue glycogen deposition

The alterations of glucose homeostasis produced by contraceptive steroids are complementary with many of the alterations of glycogen metabolism observed in these animals. For example, the elevated plasma insulin and reduced plasma glucose titres are consistent with increased tissue glycogen levels. The more pronounced elevations of glycogen were produced by estrogen treatment, E_2 had a stronger glycogenic effect than

its synthetic counterpart. The important glycogenic role of insulin has been discussed in Chapter 1, and presumably insulin mediates this effect to a considerable extent. E_2 produced a more rapid, greater and more sustained glycogenic effect than EE. This was unexpected since E_2 is less resistant to hepatic metabolism. Obviously a high specificity exists for E_2-stimulated glycogenesis which is not fully shared by EE. The high accumulation of ^{14}C-estradiol in the glycogen storing tissues favour an effect produced by the intact molecule as opposed to a metabolite.

The responsiveness of the different tissues to sex steroids varied. Liver responded rapidly and this is consistent with the role of this organ in adjusting to short-term changes in the availability of metabolic fuels to maintain normal glycaemia. Thus in the short-term, contraceptive steroids may affect glucose homeostasis via their influence on liver (162). The distribution studies highlighted liver as an important site for uptake of both E_2 and P. However, over the long-term, muscle glycogen was elevated to a greater extent than liver glycogen, indicating that changes in glucose metabolism in muscle exert an increasingly important role in glucose homeostasis.

Alterations of glucose and glycogen status associated with the various administration routes were complementary. For example, mice receiving Sc E_2 had the highest tissue glycogen levels and correspondingly low plasma glucose levels. Oral EE treatment produced low glycogen levels and correspondingly high plasma glucose titres.

Progestogen administration significantly elevated tissue glycogen above the diestrus levels. The natural progestogen had a greater insulinogenic influence than the synthetic progestogens. However, complementary alterations of glycogen deposition were not evident. Indeed, the synthetic progestogens raised glycogen deposition to a greater extent than P. Thus it appears that factors in addition to

the plasma insulin titre also contribute to the regulation of glycogen metabolism in progestogen treated mice. Possibly the conversion of synthetic progestogens to EE (485 & 486) and subsequent synergism with insulin could account, in part for the elevated glycogen deposition. Indeed, the results suggest the glycogenic effect of a sex steroid is a function of its estrogenicity. Synthetic progestogens may also directly influence glycogen metabolism, or may have a further indirect influence that does not involve insulin metabolism, but these hypotheses remain to be confirmed.

There were similarities between glucose and glycogen homeostasis with respect to the administration route. Sc administration, compared with other routes, of both P and NA produced the highest glycogen and the lowest plasma glucose concentrations. Likewise mice receiving oral P showed low tissue glycogen and high plasma glucose levels. In contrast, during N treatment the relationship was reversed. During oral N administration, elevated glycogen deposition was not associated with reductions in plasma glucose. This suggests that hepatic glucose production is also elevated in these animals. During Sc N administration, low glycogen and low glucose levels were seen. These data suggest that glucose was not stored as glycogen in these animals, but was preferentially disposed via other pathways, possibly triglyceride pathways. In addition, the intact N molecule may partially antagonise glycogenesis. Thus when present in high concentrations (after Sc and Im administration) glycogen deposition is limited, but when present in lower concentrations (after oral administration) the antagonism is reduced and glycogen deposition is increased.

Combined steroid treatment rarely elevated the insulin titre significantly, but plasma glucose levels were reduced. The increased tissue glycogen deposition seen in these mice was compatible with the

reduced plasma glucose titres. Glycogen deposition was generally highest following combined E_2 and P treatment and this corresponded with the low plasma glucose titres in these mice. Indeed, reduced hepatic gluconeogenesis (123, 162 & 488) has also been reported and this would contribute towards a reduction of circulating glucose.

The relationship between glycogen and insulin metabolism in the combined groups was not as straight-forward as that noted during single sex steroid administration. Interactions between the two components in the combined preparations, and their synergistic or antagonistic influences on glycogen metabolism may play a more dominant role than the plasma insulin titre. The combined preparations produced glycogen concentrations that were intermediate in comparison with the high levels seen during estrogen and the low levels seen during progestogen treatment. Interactions, such as partial antagonism between the two steroids may result in addition to the glycogenic effects of the estrogen component.

(iv) Peripheral glucose utilisation

The effect of sex steroids on carbohydrate metabolism may be differentiated at the level of peripheral glucose utilisation. Peripheral tissue insensitivity to insulin, as evidenced by concomitant hyperinsulinaemia and hyperglycaemia (2, 202, 480 & 481), has been frequently observed during sex steroid administration. Indeed, this was seen on several occasions during the present study. Muscle and adipose tissue were examined in the present study as quantitatively these tissues are important sites for insulin-mediated glucose uptake and metabolism (388 & 389). In addition, these tissues were identified in the present study as sites of sex steroid uptake, and they may be important tissues at which these agents influence carbohydrate metabolism.

The present study demonstrated that muscle ^{14}C-glucose uptake was greater than that of adipose tissue at all times during the investigation. This suggests that muscle is more important than fat for peripheral glucose metabolism, as indicated by other studies (388 & 389). The influence of contraceptive steroids on muscle glycogen deposition confirms the importance of this tissue in the glucose economy of the body. The effects of steroids on plasma glucose titres were closely related to their effects on glucose uptake by muscle and fat in vitro. All steroid treatments elevated glucose uptake by muscle and adipose tissue above the diestrus levels. Low plasma glucose titres were accompanied by high peripheral tissue glucose accumulation in steroid treated mice, and vice versa. For example, estrogen treatment produced the highest tissue glucose uptake and the lowest plasma glucose titres, whilst progestogen treatment produced the lowest tissue glucose uptake and the highest plasma glucose titres in comparison with the diestrus mice. Combined steroid treatment produced intermediate glucose uptake and plasma glucose titres due to a partially antagonistic effect of the progestogen component. Partial antagonism by P of the estrogen- facilitated hypoglycaemic action of insulin has been reported in similarly treated mice (126).

Ovariectomised mice were also examined in this study. In these animals, endogenous sex hormone titres are low (124 & 489) and glucose tolerance is impaired (126 & 127). The degree of impairment is greater than that seen in diestrus mice (119), hence peripheral glucose utilisation might be expected to be lower in the ovariectomised mice. However, muscle and adipose tissue glucose uptake were higher in the ovariectomised than in the diestrus mice. In the muscle of ovariectomised mice this may be consequent to the low levels of glycogen within the tissues(126), and the 'spare capacity' for glycogen synthesis that exists (170). The increased adiposity in

ovariectomised mice appears to be associated with a more avid uptake of glucose eventhough the adipocytes of these mice frequently show insulin resistance (62). An alternative explanation for these observations is not apparent. Thus it appears that alterations of perpheral tissue sensitivity to the hypoglycaemic action of insulin do not totally account for alterations of glucose homeostasis in ovariectomised mice. Nevertheless, this study demonstrated that exogenous contraceptive steroids are important modulators of glucose metabolism in muscle and adipose tissue.

The high uptake of ^{14}C-glucose in the absence of insulin in the tissues of steroid treated mice raises the possibility of an insulin-like action of sex steroids, particularly estrogens, in muscle and adipose tissue. Estrogens generally improve GT by increasing plasma insulin and peripheral glucose utilisation. Progestogens had similar effects without significantly improving GT, partly due to the contra-insulin effect of these steroids *in vivo* (143 & 179). The present study supports the possibility of synergism between estrogens and insulin with respect to peripheral glucose utilisation (418 & 490). The present data also lend credence to the view that a non-insulin dependent glucose carrier may be induced by estrogens in muscle and adipose tissue, as already noted in uterus (171 & 483).

In contrast to estrogens, synthetic progestogens exerted a greater effect on glucose uptake than P. This was also evident during combined steroid treatment. This effect may be attributed to the more rapid degradation of P in comparison with the synthetic agents.
In addition, the conversion of synthetic progestogens to EE (485 & 486), and the stimulation of peripheral glucose utilisation by this conversion product may also be an important factor in the mode of action of progestogens.

The proportion to which glucose uptake was increased by insulin

in muscle and fat was often limited by some of the contraceptive steroid regimes. Tissue resistance to the hypoglycaemic action of insulin was mostly evident following only the Im and oral administration of steroids, but it was present in all administration routes during N and NA treatment. These results correspond with high circulating glucose titres, particularly following oral steroid treatment. The occurrence of concomitant hyperinsulinaemia and hyperglycaemia, frequently observed during contraceptive steroid treatment, implies a reduction of peripheral tissue sensitivity to the hypoglycaemic action of insulin, this was confirmed in the present study.

Insulin-mediated glucose uptake was elevated during Sc EE and P treatment, whilst the plasma glucose titre was reduced thus suggesting the hypoglycaemic action of insulin was increased in these animals. As discussed previously, the administration route affects the circulating steroid titre, the distribution and the subsequent metabolism of steroid hormones.

Mechanism of action of sex steroids on carbohydrate metabolism

Several theories, with varying amounts of supporting evidence, have been proposed for the mechanism of action of contraceptive steroids on carbohydrate metabolism. However, no clear mechanism is apparent and it seems likely that a number of mechanisms are involved to a greater or lesser extent, depending on the particular steroid in question. From the data presented in this thesis, it appears that some of the mechanisms contributing to the alterations of carbohydrate metabolism during sex steroid treatment include alterations of insulin metabolism, peripheral tissue sensitivity to insulin, liver metabolism, gastrointestinal function and CNS function. These mechanisms are discussed below and they are followed by a brief discussion of other mechanisms not implicated in this thesis, but which may also play an important role in the steroid-induced alterations of carbohydrate metabolism.

(1.1) Alterations of insulin metabolism

Insulin is the most important regulator of glucose homeostasis, as discussed in Chapter 1. Elevations of plasma insulin were frequently observed in the sex steroid treated mice used in this study, and it is thought that alterations of insulin metabolism are one of the main mechanisms through which these agents exert their effects on carbohydrate metabolism (162). There are numerous ways in which contraceptive steroids could influence insulin homeostasis, including direct and indirect actions via antagonism or synergism with other hormones. Indeed, different steroids may have a similar net effect on plasma insulin even though they might alter insulin metabolism in different ways. However, more detailed experimentation is required to support or refute this speculation. A direct action of sex steroids on insulin homeostasis may involve alterations of any of the following

parameters.

Elevation of intracellular cAMP levels stimulates insulin secretion by the pancreatic β cells (see reviews by 491 & 492). cAMP levels may be raised by an action of contraceptive steroids on adenylate cyclase, and/or an action on phosphodiesterase activity. However, contraceptive steroids have not been shown to have a significant effect on cAMP levels in other tissues. Furthermore, the importance of cAMP in the prolonged stimulation of insulin has been questioned since this nucleotide is known to produce only a quick and rapid response by the β cells (81 & 493). Moreover, in vitro experiments suggest that there is a considerable delay before steroid-induced insulin secretion is observed (119).

Sex steroids may stimulate insulin secretion by raising intracellular calcium levels. Elevations of the β cell calcium concentration are known to stimulate insulin secretion (75 & 494) and these increases may be produced by the action of steroids on the mobilisation of calcium from pools within the organelles of the β cell, and by a reduction of cellular calcium efflux. Experiments to examine this association have not been reported.

Liberation of lysosomally bound acid amylo-glucosidase facilitates the conversion of glycogen to glucose within the β cell and hence stimulates insulin secretion. Contraceptive steroids may regulate insulin homeostasis through this mechanism but it remains to be examined. Indeed, since contraceptive steroids are generally glycogenic in other tissues, this seems unlikely.

In vivo and in vitro studies show that sex steroids have a cytotrophic effect on the β cells, including hyperplasia and hypertrophy (3, 129, 166, 175, 177 & 178). The present study supports a direct action of steroids on the β cell, as evidenced by the large accumulation of both E_2 and P in the pancreas.

Studies are required to clarify what proportion of the accumulated steroid is associated with the β cells, but it is reasonable to speculate that specific steroid receptors are involved since P has recently been shown to accumulate in the β cell (186).

The data presented in this thesis agree with an enhanced β cell response to glucose in contraceptive steroid treated mice. The steroids may affect the membranal gluco-receptor system or intracellular glucose metabolism, both of which are believed to be intimately involved in the control of glucose-induced insulin secretion (492, 495 & 496). This action of steroids may be direct, as suggested by many investigators (120, 177 & 178). Recently, the time-course of such a direct action has been described. Steroids commonly act in cells via enzyme induction (497). Hence their effects are not manifest immediately, but appear after four to six hours, as observed with P stimulated insulin release (120 & 177). An immediate stimulatory effect of E_2 has been reported (151), but several other laboratories have not been able to reproduce this effect (see 498). Detailed in vitro investigations into the precise effects of contraceptive steroids on the β cell are required to identify the mechanisms involved.

Insulin homeostasis may be altered by the action of sex steroids on insulin binding. Contraceptive steroids have been reported to elevate adrenocorticoid and thyroid binding globulin (46 & 499) and synalbumin (218, 219 & 500). Insulin binds to these substances and as a result of increased binding the half-life of insulin is raised, but the effectiveness of the circulating insulin may be reduced producing a state of relative insulin insufficiency. This is believed to create hyperinsulinaemia if adequate β cell capacity exists. However if β cell status is compromised, a deterioration of glucose homeostasis can eventually result (37). The concomitant

hyperinsulinaemia and hyperglycaemia observed at times in the present study are consistent with a reduction of the biological effectiveness of the circulating insulin. Indirect actions of contraceptive steroids on insulin metabolism which involve other hormones, including adrenocorticoids, growth hormone and glucagon, are discussed later in this chapter.

It is not possible from the information available at the present time to deduce whether insulin secretion is affected directly or indirectly to produce the characteristic hyperinsulinaemia observed in sex steroid treated mice.

(1.2) Alterations of peripheral tissue sensitivity to insulin

Alterations of peripheral tissue sensitivity to the hypoglycaemic action of insulin appear to be a frequent occurrence during sex steroid therapy. Reduced sensitivity is characterised by the presence of concomitant hyperinsulinaemia and hyperglycaemia (480 & 481), which was seen on numerous occasions during the present study. This suggests insulin insensitivity may play an important role in the alterations of glucose homeostasis produced by contraceptive sex steroids.

Progestogens reduce peripheral tissue sensitivity to the hypoglycaemic action of insulin (143, 161, 480, 501 & 502) but the effect of estrogen treatment is still uncertain. Estrogens have been reported to enhance peripheral glucose utilisation (173) and it has been suggested that this may be due, in part to synergism with insulin (175). However, estrogens have also been reported to inhibit the hypoglycaemic action of insulin on peripheral tissues (408) possibly via an indirect mechanism. The present study supports these findings when Im and oral estrogens are administered, and may be used to support the view that the development of peripheral insensitivity is an

adaptive mechanism to counteract the hyperinsulinaemia produced by these steroids. It is well documented that hyperinsulinaemia causes down-regulation of insulin receptors and thus creates a condition of reduced insulin sensitivity (99 & 103). Nevertheless, contraceptive steroids may also influence peripheral glucose utilisation directly, as indicated by the distribution studies. Indeed, it is feasible that both mechanisms operate in steroid treated animals, further experiments are necessary to determine their relative contribution to changes in glucose homeostasis.

Peripheral tissue insensitivity to the hypoglycaemic action of insulin may be at the point of glucose entry into the cell. However, interference with one or more stages in the intermediary metabolism of glucose might also occur (107 & 389). It has also been suggested that peripheral tissue sensitivity may be related to increased daily food intake since this may increase insulin output (40 & 503). Increased food intake, however, during steroid treatment was not demonstrated in a recent long-term study (126). Food intake was not measured in the present study.

Estrogens and progestogens may produce their effects on peripheral glucose utilisation indirectly via other hormones, including adrenocorticoids, growth hormone and glucagon, these possibilities are discussed later in this chapter.

(1.3) Alterations of liver metabolism

The liver plays an important role in the maintenance of normal glycaemia, circulating glucose levels, the hypoglycaemic action of insulin and the hyperglycaemic action of other hormones are integrated and translated by this organ. It is conceivable that alterations of hepatic function are involved in the alterations of glucose homeostasis observed during contraceptive sex steroid treatment.

Alterations in several types of liver function tests have been reported in contraceptive steroid users and in pregnant women (500, 504 & 505), and sex steroids appear to influence the majority of physiological and biochemical systems so far examined in the liver (506 - 508). E_2 has been shown to be a potent inducer of various hepatic enzymes concerned with carbohydrate metabolism, including hexokinase, phosphofructokinase & glucose-6-phosphatase dehydrogenase (509 & 510).

Sex steroids elevate glycogen deposition (126 & 162), as confirmed in the present study. It is well established that the glycogen content of the liver, and indeed of other tissues, is the result of the balance between glycogenesis and glycogenolysis. It is possible that sex steroids alter the net increase in glycogen by favouring the glycogen synthetase to glycogen phosphorylase ratio. Such a mode of action for E_2 has been identified in uterine myometrium (478). In view of the very high uptake of sex steroids by hepatic tissue, it is possible that a direct action of steroids on glycogen synthetase and phosphorylase might occur in this tissue. However the hyperinsulinaemic action of most of the steroids represents another pathway through which these effects may be mediated. Reports by Sladek (123 & 488) and by Matute and Kalkhoff (162) indicate estrogens and possibly progestogens suppress gluconeogenesis in hepatic tissue. Thus reduced hepatic glucose output, by increased glycogenesis and suppressed gluconeogenesis, represent a general process through which plasma glucose titres are controlled by contraceptive steroids.

The oral administration route was frequently associated with the highest plasma insulin and glucose titres in comparison with the other administration routes. Orally administered steroids are immediately exposed to the liver and are bound and metabolised more rapidly than those administered by the other routes. These results indicate that metabolites of contraceptive steroids, as opposed to the intact

molecule may be responsible for the concomitant hyperinsulinaemia and hyperglycaemia observed in this study.

(1.4) Alterations of gastrointestinal function

Contraceptive steroids have been shown to alter glucose absorption from the gastrointestinal tract, and it has been suggested that the alterations of carbohydrate metabolism may result from alterations in the rate at which glucose enters the body. Sex steroids reduce gastric emptying (216), reduce glucose absorption (511) and enhance intestinal metabolic activity (513). The slower rate of glucose absorption from the gastrointestinal tract, thus produced by sex steroids has been proposed as too small to have an appreciable influence on plasma glucose levels (2 & 3). However, differences in the incidence of impaired glucose tolerance following oral and intraveneous GTTs do not support this view (206). Mechanisms involved in the alterations of glucose absorption are unknown, but alterations of the electrical potential of the laminal epithelial membranes may be involved (514). Sex steroids may also influence glucose absorption indirectly, via changes in the levels of other hormones, such as adrenocorticoids, glucagon and growth hormone, which are known to modulate glucose absorption (515). The association between sex steroids and these hormones is discussed later in this chapter.

It has been suggested that the hyperinsulinaemic effect of contraceptive steroids might be produced in part via various gut hormones (152). Numerous insulinogenic hormones have been identified (see 516 & 517), but GIP is the only one which has been shown to stimulate insulin secretion at physiological levels (516, 518 - 521). It is thought that this hormone lowers the glucose threshold necessary for insulin release, thus elevating the insulin response to glucose (70). An elevated secretion of GIP might help to explain how orally

administered steroids provoke a greater insulin response to glucose than those given by the Sc or Im administration routes. However, this remains to be investigated.

(1.5) Alterations of Central Nervous System function

Recent studies have shown that sex steroids play an important part in the normal physiological regulation of food intake and body weight (126, 522 & 523). E_2 (522 & 524) and preparations containing E_2 and P (126 & 523) reduce food intake and body weight in intact and ovariectomised animals. E_2 increases locomotor activity and decreases appetite, P antagonises this effect, but has little effect in the absence of circulating E_2 (524). The underlying mechanisms are uncertain, but probably involve alterations of gastrointestinal function, as discussed in the previous section, and alterations of neural function (525 & 526). Within the hypothalamus there are distinct areas concerned with satiety and hunger. The satiety centre, situated within the ventro-medial hypothalamus (VMH) exerts an inhibitory influence on the hunger centre situated in the ventro-lateral hypothalamus (VLH) (526). It has been proposed that sex steroids influence the regulation of body weight via an effect on the VMH satiety centre (525 - 527). The mechanism remains to be delineated.

The influence of contraceptive steroids on the pituitary may be involved in the subsequent alterations of glucose homeostasis (35). A diabetogenic effect of pituitary gonadotrophins has been reported (528). Thus sex steroids may improve glucose homeostasis, in part by inhibiting pituitary gonadotrophin release. However this proposal was not confirmed in hypophysectomised (529) or in diabetic (166) rats. The relatively large accumulation of steroids by the pituitary, particularly in the intact mice in the present study, strongly suggests that the pituitary might contribute to steroid-induced alterations of

carbohydrate metabolism, but this remains to be adequately investigated.

In the following sections, mechanisms through which contraceptive steroids may alter carbohydrate metabolism via their actions on other hormones are discussed. These mechanisms were not examined in the present study, but they may represent physiologically important modes of action for these agents.

(2.1) Adrenocorticoids

The influence of the secretions of the adrenal gland on carbohydrate metabolism is well established. Adrenal steroids elevate plasma glucose (293), plasma insulin (530), free fatty acids (175) and ketones (531). In addition they reduce peripheral glucose utilisation (532 & 533). The net effect depends on the adequacy of the compensatory islet response (49 & 175), but long-term exposure to adrenocorticoids may precipitate diabetes due to β cell exhaustion (49).

The influence of contraceptive steroids on adrenocorticoids has been extensively investigated. Many steroids, including E_2, EE, N, mestranol and megestrol acetate (534 - 537), exert a hypertrophic action on the adrenal cortex, thus elevating plasma glucocorticoids (538 & 539), particularly cortisol (540 & 541). Elevations of circulating adrenocorticoids are compounded by a reduced negative feedback effect (542) and an elevated response to adrenocorticotrophic hormone (ACTH) in sex steroid treated animals (538). Contraceptive steroid administration reduces the rate of disappearance of adrenocorticoids from the circulation (253 & 543). Sex steroids have been reported to inhibit hepatic adrenocorticoid metabolism (544 & 545), and to elevate adrenocorticosteroid-binding globulin (546 & 547). Thus circulating adrenocorticoid titres are increased

and the half-life of these hormones in the peripheral circulation is prolonged (548). However, the elevation of free adrenocorticoids is thought to be small, and its physiological importance in impairing glucose homeostasis is doubtful (548 & 549).

Contraceptive steroids may exert their effects on carbohydrate metabolism by altering the plasma adrenocorticoid titre and also by a synergistic action with these hormones. For example, glucocorticoid-induced alterations of carbohydrate metabolism are potentiated when estrogens are present (512, 548 & 550). Furthermore, E_2 has been reported to synergise the insulin-antagonistic effect of cortisone (126). Some sex steroids may possess an inherent corticoid-like activity (551) and their influence on carbohydrate metabolism may include this inherent activity.

Thus the diabetogenic action of certain contraceptive steroids may be mediated via adrenocorticoids. However, it should be noted that since the diabetogenic effect can still exist, albeit reduced, in the absence of the adrenal glands (175), it is clear that this cannot be the only mode of action of such contraceptives.

(2.2) Growth hormone

The possibility has been raised that alterations of carbohydrate metabolism produced by sex steroids are mediated via their effect on growth hormone (552 - 555). The diabetogenic effects of growth hormone are well documented (49 & 556) and this hormone is a potent antagonist of insulin (557 & 558). Recently it has been shown that growth hormone promotes somatomedin generation which subsequently affects the target tissues (559). Interactions between somatomedin and contraceptive steroids await confirmation. Other investigators have proposed that growth hormone preparations are heterogenetic with some moieties producing an anti-insulin effect and others an

insulin-like effect (560). The production of different growth hormone fractions, with different effects may account for the contradictory data concerning growth hormone participation in glucose homeostasis during contraceptive steroid treatment (3, 228, 265, 281 & 561).

(2.3) Thyroid hormones

Alterations of thyroid function during pregnancy and hormonal contraceptive therapy have been tentatively associated with changes of carbohydrate metabolism (563 & 564). The diabetogenic potency of thyroid hormones has been known for many years but the precise influence of these hormones on glucose homeostasis is unclear (565). Thyroid hormones do not inhibit insulin secretion *in vitro* (566), but *in vivo* they impair glucose tolerance (181), elevate plasma glucose (567 & 568) and reduce glucose-induced insulin secretion (565 - 568). However, these alterations do not correspond with data presented in this thesis, which show that glucose-induced insulin secretion is generally elevated by contraceptive steroid treatment. In addition, other laboratories have failed to demonstrate alterations of thyroid hormone titres during acute or chronic sex steroid treatment (2 & 3). Thus it appears that alterations of thyroid status do not play an important role in modulating the influence of contraceptive steroids on carbohydrate homeostasis.

(2.4) Glucagon

Recent experiments have shown that E_2 affects both α and β cell activities, so that the ratio of insulin to glucagon is elevated (254). As a result, liver enzyme activity is altered, lipogenesis is elevated and gluconeogenesis is reduced. The influence of sex steroids on glucagon secretion has not been extensively examined.

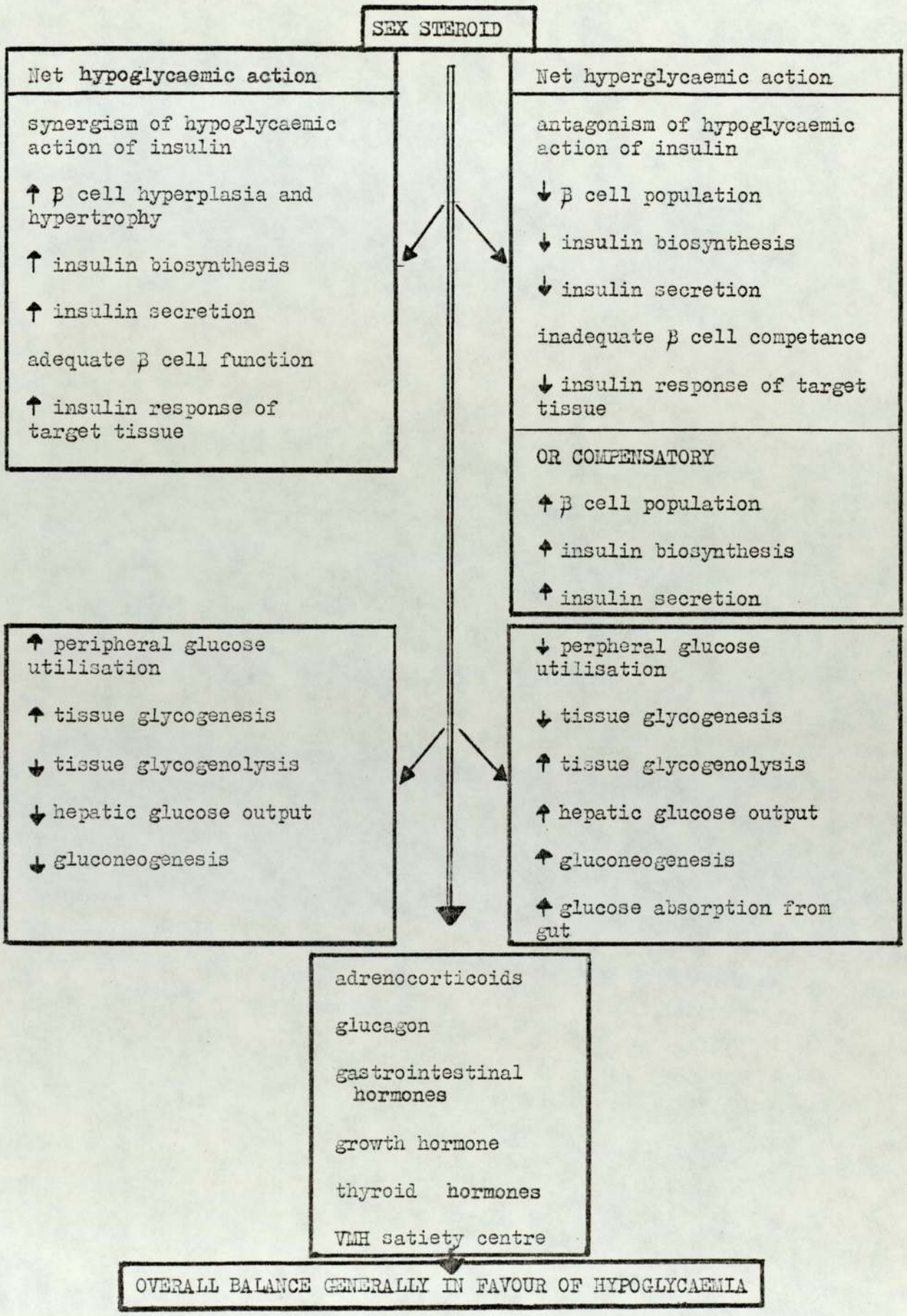
EE, mestranol and norethisterone have been shown to suppress the plasma glucagon response to arginine (264). Various sex steroids suppress gluconeogenesis, and this action may be a result, in part, to reductions of glucagon secretion (123, 126, 162 & 488). Thus it is possible that sex steroid-induced alterations of glucose homeostasis may be mediated to some extent by alterations of the plasma insulin:glucagon ratio, but further experiments are required to identify the effect of contraceptive steroids on this ratio.

(3.1) Conclusions

From the data presented in this study and from the available literature, it is proposed that the influence of contraceptive steroids on carbohydrate homeostasis is likely to involve several concurrent mechanisms of action. The relative importance of each of these mechanisms probably varies with the steroid, and this study has emphasised that the administration route and the treatment duration are also important. Evidence from the literature indicates that dose and patho-physiological status of the recipient are also likely to affect the relative importance of the different mechanisms within an individual.

The most important mode of action of steroids probably involves interaction with insulin metabolism, peripheral tissue sensitivity to insulin and adrenocorticoids. The response of the liver appears to be critical due to its central role in translating the influence of insulin, glucose, sex steroids and adrenocorticoids on glucose metabolism.

Figure 77 Mechanism of action of sex steroids



Structure-activity relationships

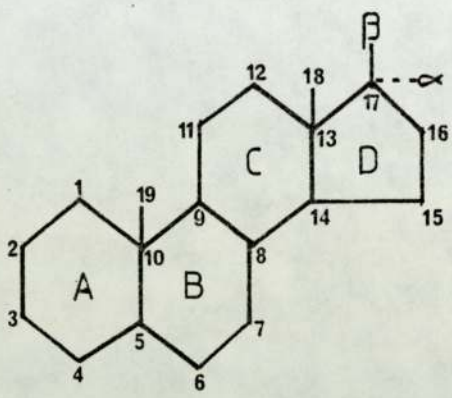
Clinical reports of the effect of contraceptive steroids on carbohydrate metabolism are often confusing, due largely to methodical differences (different preparations, doses, routes of administration, treatment duration, variations in the patho-physiological condition of the recipient and the criteria for assessment of data). However, when changes are correlated with chemical structure, general structure-activity relationships are apparent (165).

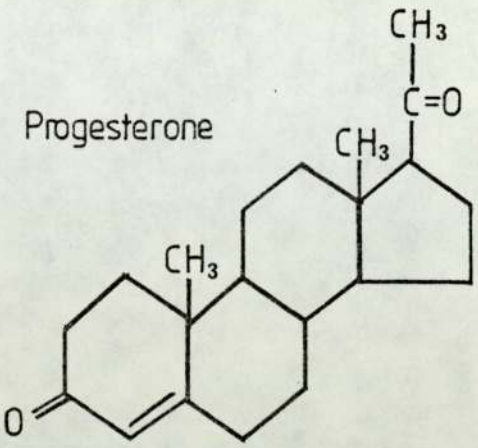
This section analyses the effect of contraceptive steroids on glucose homeostasis in terms of the side groups on the steroid molecule. Such analyses should eventually enable predictions to be made of which side groups will produce which effect on metabolism when a particular steroid is administered under defined circumstances to an individual of defined physio-pathological state. Eventually it should prove possible to formulate steroid preparations that are effective contraceptives, but have negligible or predictable metabolic side effects, and that can be modified in structure and administration regime to suit the metabolic circumstance of the individual.

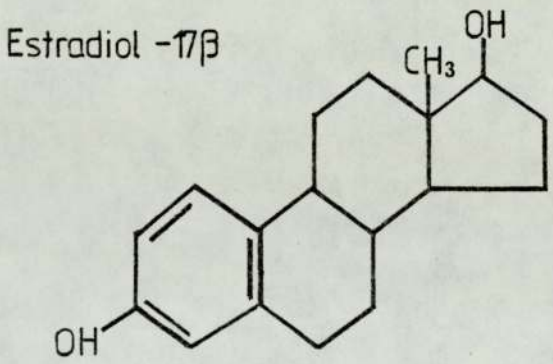
The basic estrogen molecule, illustrated in figure 78 (consisting of C3 and C17 hydroxyl groups, C18 methyl group and an unsaturated A ring), elevates fasting insulin and the insulin response to glucose. In addition, plasma glucose is reduced, tissue glycogen levels increased and peripheral glucose utilisation elevated.

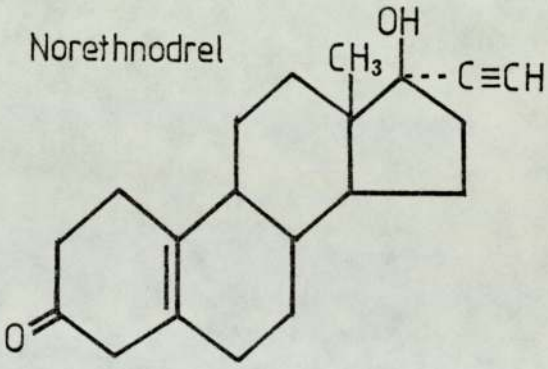
The basic progestogen molecule, shown in figure 78 (consisting of C3 carbonyl group, C18 methyl group, various C17 β groups and a positive charge at C5 (165)), also elevates plasma insulin titres, but this effect was weaker than that produced by estrogens at the doses used in this study. Furthermore, plasma glucose is often not significantly reduced as a result of the reduced peripheral glucose

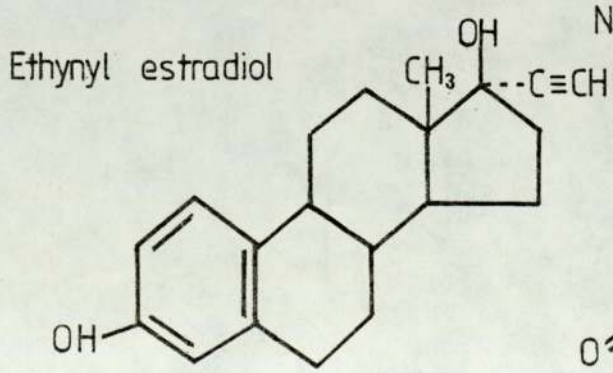
Figure 78 Estrogen & progestogen structures.

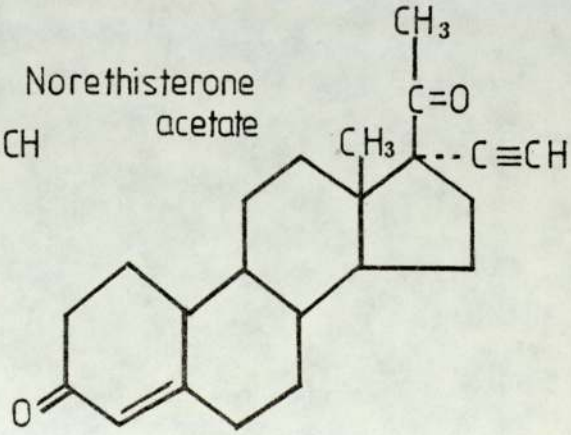












utilisation in these mice. Thus progestogens are without certain of the benefits conferred on glucose homeostasis by estrogens.

Comparison of E_2 and EE (figure 78) which differ only by the presence of a C17α-ethynyl group in the latter, shows that this side group lowers the insulinogenic influence, reduces the glycogenic effect and reduces peripheral glucose utilisation compared with the native E_2 molecule. As these alterations indicate, plasma glucose is higher during EE treatment than it is during E_2 treatment. Thus the C17α-ethynyl group in estrogens can be regarded as beneficial with respect to insulin secretion as these estrogens represent a smaller insulinogenic influence which may be exploited in those individuals with inadequate β cell capacity to respond to insulinogenic stimuli. However, glucose homeostasis generally does not benefit from the presence of the C17α-ethynyl group in estrogens.

Similar criteria apply to progestogens. Comparison of P and NA, which differ by the presence of a C17α-ethynyl group in the latter, shows that this side group lowers the insulinogenic influence shown by the native P molecule. However in contrast to estrogens, the C17α-ethynyl group elevates the glycogenic effect, increases peripheral glucose utilisation and lowers plasma glucose compared with P. P also differs from NA by the presence of a C19 methyl group in the latter. However it has been shown that this particular side group does not affect glucose or insulin homeostasis (165), and thus was not considered to be an important factor when comparing these and other molecules in this section. Thus the C17α-ethynyl group in certain progestogens (ie. those also possessing a C17 β-acetyl group) can be regarded as beneficial with respect to both insulin and glucose homeostasis compared with the native P molecule.

These progestogens represent a smaller hyperinsulinogenic stimulus yet exert a greater hypoglycaemic effect than the native molecule.

When EE and N (figure 78) are compared, the effect of changes in the A ring on glucose homeostasis is exposed. These steroids differ by the replacement of a C3 hydroxyl group by a carbonyl group, and by the presence of a positive charge at C5 in the N molecule. These alterations reduce the insulinogenic influence, reduce glycogen deposition and impair peripheral glucose utilisation, thus resulting in a significant elevation of plasma glucose compared with EE treatment. Thus such alterations in the A ring of the steroid molecule has a deleterious influence on glucose homeostasis.

The structural differences between E_2 and N allow estimation of the effects of alterations in the A ring (replacement of a C3 hydroxyl group by a carbonyl group and a positive charge at C5 in N) in the presence of a C17α-ethynyl group. The structural alterations reduce the extent of enhancement of peripheral glucose utilisation compared with the E_2 molecule. In consequence, plasma glucose is significantly raised. The magnitude of these effects is greater than observed when comparing EE and N. Thus the deleterious effect of alterations in the A ring (C3 carbonyl group and C5 positive charge) is potentiated by the simultaneous presence of a C17α-ethynyl group in the progestogen molecule.

Comparison of N and NA identifies the influence of different C17 β side groups on glucose homeostasis in the progestogen molecule, namely the C17 β-hydroxyl and C17 β-acetyl groups respectively. Both progestogens retain the C5 positive charge even though there is a different distribution of electrons within the A ring of the molecule

(165). The acetyl group produces a slight reduction in the insulinogenic influence, elevates glycogen deposition, increases peripheral glucose utilisation and thus exerts an overall hypoglycaemic effect in comparison with the hydroxyl group present in N. Thus progestogens possessing an acetyl group as opposed to a hydroxyl group at C17, other structural features of the molecule being equal, have a relatively beneficial influence on glucose homeostasis. This conclusion is supported when P and N are compared. The P molecule has a C17 β-acetyl group whereas N has a C17 β-hydroxyl group together with a C17α-ethynyl group. Plasma glucose titres are slightly lower during P treatment, and this appears to be due to decreased hepatic glucose output since both glycogen deposition and peripheral glucose utilisation are higher in N than P treated mice.

The structural differences between EE and NA (figure 78) result in a reduction of the insulinogenic influence, small elevations of plasma glucose and reduced enhancement of peripheral glucose utilisation in NA compared to EE. Over the period of the present study, NA did not influence glucose homeostasis adversely in comparison with EE. It is possible, however, that over a prolonged period, the reduced peripheral glucose utilisation might precipitate diabetes.

It is evident that side groups in the steroid molecule influence glucose homeostasis and that general structure-activity relationships can be identified. Substitutions at C17 alter the insulinogenic influence of the steroid. A C17 β-hydroxyl group produces a greater stimulation of plasma insulin than an acetyl group in this position. The effect is modified by the presence of other side groups in the steroid molecule. For example, the insulinogenic effect of the C17 β group is reduced in both estrogens and progestogens by the simultaneous

presence of a C17α-ethynyl group. In progestogens, a C17 β-acetyl group has a greater insulinogenic influence than a C17 β-hydroxyl group when the C17α-ethynyl group is absent eg. P. The inhibitory influence of the C17α-ethynyl group is greater in progestogens possessing a C17 β-acetyl group than it is in those possessing a C17 β-hydroxyl group. This may be associated with the C3 carbonyl group in these molecules.

Similarly, substitutions at C17 influence glucose metabolism. The plasma glucose titre is lower in estrogens, which have a C17 β-hydroxyl group, than in progestogens. In estrogen molecules, a C17α-ethynyl group reduces the hypoglycaemic influence of the steroid. This also occurs in progestogens which possess a C17 β-hydroxyl and a C17α-ethynyl group, such as N. However, in progestogens which have a C17 β-acetyl group the simultaneous occurrence of a C17α-ethynyl group has the reverse effect, as seen in NA. In these steroids the hypoglycaemic influence is potentiated, and this may be associated with the presence of the C3 carbonyl group.

Conclusions and Speculations

In this thesis I have attempted to define more clearly the effect of various contraceptive regimes on carbohydrate metabolism in the adult female mouse. It is hoped that this animal model will serve as a useful basis for similar studies in women. Indeed, there is a long awaited need for this type of systematic investigation to be performed in women.

Particular emphasis was placed on estimating the importance of the adminstration route, the type of steroid used and the time-course of the observed alterations of carbohydrate homeostasis. The relative importance of these factors is critical both with regard to future developments in the hormonal contraceptive field and also to enable investigators to compare different studies more effectively. The most popular administration route used today is the oral route, but future developments are likely to increasingly exploit other methods of administration, including subcutaneous and intramuscular methods. In the UK at present, intramuscular medroxyprogesterone acetate is given for short periods to many women, and there is increasing interest in the more widespread use of this type of regime. There have been few studies in either animal models or women comparing the influence of the various administration routes with respect to the incidence of metabolic side effects, and no conclusions of practical assistance to clinicions have been proposed. This study has demonstrated that both the intramuscular and oral administration routes are frequently associated with a reduction in the hypoglycaemic action of insulin. However, the subcutaneous administration route of sex steroids is often associated with an increase in the hypoglycaemic action of insulin. Exactly how this situation develops remains to be clarified, but differences in the distribution, metabolism and hence subsequent activity of these agents due to the administration route utilised are

undoubtedly important factors to be considered. The present study suggests that more frequent use of the subcutaneous administration route could reduce the incidence of impaired tolerance in contraceptive users, and might prove useful in future hormonal contraceptive developments. In addition, it is expected that dosage may be reduced if the subcutaneous rather than the oral route is used, since the disappearance rate of the steroids will be reduced. Dosage reductions are also likely to contribute towards a lowering of the incidence of metabolic side effects. The present study also suggests that increasing use of the intramuscular route of administration should be thoroughly examined since this route exaggerated the diabetogenic influence of certain steroid preparations. The balance between the convenience aspect of using oral contraceptive preparations against the relative inconvenience of using subcutaneous or intramuscular preparations will have to be considered, but it is proposed that in those individuals particularly susceptible to diabetogenic stresses, routes other than oral should be seriously evaluated.

One of the characteristic features of sex steroid treatment was the elevation of the plasma insulin titre, especially during the administration of single steroid preparations, and in particularly during estrogen treatment. It appears that glucose tolerance is maintained within normal limits by the development of hyperinsulinaemia in these treatment regimes. This may be an important factor to consider, especially in the light of an increasing trend towards the use of single progestogen preparations. The present study has demonstrated that progestogen regimes have a deleterious influence on glucose homeostasis, both plasma insulin and glucose titres being elevated. Indeed, glucose titres were frequently higher than the diestrus levels, which represent the normal maximum impairment of glucose homeostasis in the female mouse. The physiological

significance of prolonged hyperglycaemia remains to be clarified but obviously it cannot represent a beneficial environment. Thus the administration of progestogens as single preparations should be contra-indicated in those individuals with compromised β cell status or the high risk of developing such a condition.

An interesting result to appear in this study was the relatively beneficial influence of both natural and synthetic combined preparations on glucose homeostasis. The plasma insulin titre was often significantly lower than the diestrus titre, yet plasma glucose levels were reduced. This improvement of glucose tolerance was apparently due largely to the increased peripheral glucose utilisation by muscle and adipose tissue in these mice. Thus combined steroid preparations, which remain the most popular contraceptive regime presently used, do not appear to represent a diabetogenic influence in healthy normal individuals. Indeed an anti-diabetogenic influence may be exerted. This may reflect the sex steroid environment in which the proportion of circulating estrogen and progestogen are close to normal, even though the absolute levels may be raised. In addition, the frequent antagonistic effect of the progestogen on the estrogen component in these preparations may also help to maintain the status quo with respect to glucose homeostasis.

If hormonal contraceptive therapy is to be used to the maximum benefit by reducing the incidence of metabolic side effects, then the most suitable regime for each individual must be determined. The use of different administration routes, different types of steroids and the proportion of estrogen : progestogen in combined preparations are critical in this respect.

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Time-dependent effects of orally and intramuscularly administered oestradiol and ethynyl oestradiol on glucose tolerance and insulin secretion in mice. By L. J. Carrington and C. J. Bailey. *Department of Biological Sciences, The University of Aston, Birmingham, B4 7ET*

Natural and synthetic oestrogens are reported to alter carbohydrate metabolism, but the precise nature of their effects has been obscured by variations in dose, route of administration and duration of treatment used in the different studies. To evaluate these factors, female mice were treated with oestradiol (E_2) or ethynyl oestradiol (EE_2) both at 5 μg kg body weight^{-1} day^{-1} either orally or intramuscularly (i.m.). Carbohydrate metabolism was assessed by fasting intraperitoneal glucose tolerance tests with simultaneous determination of plasma insulin after one, four and six 4-day cycles of treatment. The 4-day cycles corresponded to the oestrous cycles of control mice.

Both oral and i.m. E_2 and EE_2 raised fasting and glucose-stimulated plasma insulin levels. The degree of increase produced by E_2 increased with the duration of treatment, whereas EE_2 induced maximal levels after four cycles. Both oral and i.m. E_2 lowered fasting plasma glucose levels. An improvement of glucose tolerance was observed at each time interval during i.m. administration of E_2, and at four and six cycles during oral E_2 administration. Ethynyl oestradiol did not alter fasting plasma glucose levels, and a consistent improvement of glucose tolerance was only observed at four cycles during both oral and i.m. administration.

These observations demonstrate that E_2 and EE_2 induce different alterations in carbohydrate metabolism which are dependent on both the route of administration and the duration of treatment. A more beneficial effect on glucose tolerance is exerted by E_2 than by EE_2 and the i.m. route of administration is associated with more consistent changes of glucose tolerance than is the oral route.

J. Endocrinol. 80, 67P, 1979