THE INFLUENCE OF OVARIAN SEX STEROIDS ON INSULIN RECEPTOR STATUS IN MICE

BY

GARY BRIAN WILLARS

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The University of Aston in Birmingham

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The hypoglycaemic action of exogenously administered insulin was significantly increased in mice at oestrus compared to mice at dioestrus, two stages of the oestrous cycle which represent high and low plasma concentrations of the ovarian sex steroids respectively. Unchanged insulin binding data suggested that this increase in insulin action was mediated at the post-receptor level in both skeletal muscle and liver. Neither 2 weeks ovariectomy nor the 2 week oral administration of oestradiol (5ug/kg/day) to ovariectomised mice had any significant effect on the insulin binding capacity of hepatocytes in the presence of insulin concentrations ranging from 0 to 1000ng/ml. The insulin binding capacity of hepatocytes was, however, significantly reduced by the 2 week oral administration of oestradiol at 500ug/kg/day and either progesterone alone (1mg/kg/day) or in combination with oestradiol (5ug/kg/day). This observation suggested a possible short-term antagonism to insulin action in the liver induced by these hormone replacement regimes. The maximum specific ^{125}I-insulin binding capacity of soleus muscle was not significantly altered either by 2 weeks ovariectomy or any of the above 2 week hormone replacement regimes. Ovariectomy for 10 weeks resulted in a reduced hypoglycaemic action of exogenously administered insulin, partially mediated via a post-receptor reduction in the ability of insulin to promote glucose oxidation in skeletal muscle. This effect might also have been due to a reduction in insulin action in the liver, a possibility that was suggested by the reduced insulin binding capacity of hepatocytes and a reduction in liver glycogen content. All the reductions in parameters observed after ovariectomy were either partially or totally redressed by 10 weeks oral administration of either oestradiol (5ug/kg/day), progesterone (1mg/kg/day), the two in combination or oestradiol at 500ug/kg/day. These observations support the hypothesis that natural oestrogens and progestogens, at least in the long-term, act on peripheral insulin target tissues to increase the action of insulin.

Key words : ovariectomy oestradiol progesterone
insulin receptor

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C O N T E N T S

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1. Introduction.

There are numerous accounts within the medical and scientific literature that have drawn attention to the fact that the female sex steroids play an important role in the regulation of glucose homeostasis. In Man, studies have centred around a consideration of glucose tolerance in the female both during different reproductive states and during the administration of contraceptive steroid preparations. Associations between glucose tolerance and circulating concentrations of ovarian steroids has often been implied in such studies. Generally, however, these reports highlighted the requirement for more controlled studies and a more thorough investigation of the underlying mechanisms. The following represents an overview of the literature relating to the impact of the ovarian sex steroids on carbohydrate metabolism in both Man and animals.

1.1.1. The effect of oral contraceptive agents on glucose tolerance.

The oral administration of female sex steroids as a fertility control, first introduced in the mid 1950's (1), has now become widely accepted as the most efficient form of contraception and the preferred choice of many women (2). It appears that oral contraceptives inhibit ovulation by the suppression of pituitary luteinizing hormone (LH) release but additional effects on the endometrium, cervical mucus and fallopian tubes cannot be excluded as a mechanism of contraception, particularly with the advent of the low dose progesterone only pills which have no inhibitory effect on gonadotrophins (3).

As oral contraceptives are hormonal they do, however, produce effects other than the inhibition of ovulation. To date the extensive use of the 'pill' has not been contra-indicated although consideration of the many reported side effects (3) may indicate clinical situations in which their use would be inadvisable.

Altered glucose tolerance represents only one aspect of the reported side effects of oral contraceptive therapy (3), and in this respect many attempts have been made since the early 1960's to define the effects of oral contra-ceptive agents on carbohydrate metabolism. Such work followed early reports (4) that women receiving ovulatory suppressants had reduced glucose tolerance. Subsequent work

has, however, been unable to precisely define, and thus allow an understanding of the effects of oral contraceptives on carbohydrate metabolism. Indeed there have been many conflicting reports comprehensively reviewed elsewhere (5,6,7, 8,9,10). To draw general conclusions from these reports has proved difficult due to differences in the chemical nature of the drugs, their mode of administration, the effect of interactions between drug components, doses, duration of treatment, pathophysiological condition of subjects, the parameters used for consideration of carbohydrate metabolism and the criteria used to assess the results (9). In general, the more recent literature supports the view that in some women the administration of oral contraceptives may induce glucose intolerance and possibly hyperinsulinaemia. The major detrimental effect of oral contraceptives on carbohydrate metabolism has been attributed to the progestogenic component with oestrogens providing a contributory effect (9). Other evidence, however, suggested that oestrogens produced the most marked decrease in glucose tolerance while progestogens had a lesser effect.(7).

A clear indication arising from the literature is that glucose tolerance is more likely to be aggravated by oral contraceptive therapy if the status of the pancreatic B cell is at all compromised. Hence subjects with potential or latent diabetes (and possibly obesity) may develop subclinical diabetes during oral contraceptive therapy, although the condition appears to be reversed after the withdrawal of therapy (9,11,12). On exposure to oral

contraceptives, women with already impaired glucose tolerance may develop overt diabetes (13), and overt diabetics show a marked deterioration in glucose tolerance (14,15). However, it is noteworthy that there has been a report of an improvement in glucose tolerance in type II diabetics undergoing oral contraceptive therapy (16), although in a similar study subjects with normal glucose tolerance subsequently developed abnormal glucose tolerance (17).

The synthetic steroids used in oral contraceptive preparations differ chemically from the naturally occuring endogenous ovarian sex steroids and as such may indeed differ in their biological properties and impact on carbohydrate metabolism. Synthetic steroids generally have a ethinyl or ester group attached at position 17 which renders the product less susceptable to hepatic degradation after intestinal absorption and thus makes them ideal for oral administration. It is not therefore entirely valid to equate the metabolic effects of natural and synthetic steroids.

Thus, while of relevance in assessing the suitability of oral contraceptive therapies for patients of differing pathophysiological conditions, in particular those with or susceptable to disturbances in carbohydrate metabolism, little information relating to the physiological effects of endogenous ovarian sex steroids on carbohydrate metabolism may be gained. Our attention must therefore turn to the impact of natural variations in plasma concentrations

of endogenous female sex steroids, namely the menstrual cycle and pregnacy.

1.1.2. The effect of the menstrual cycle on glucose tolerance.

Improvements in oral glucose tolerance at menses, a time of reduced sex steroid titres, have been reported on several occasions (18,19,20). Macdonald and Crossley (20) having earlier noted changes in the rate of gastric emptying during the menstrual cycle (21), suggested that the midcycle surge in the rate of gastric emptying could account for the reported deterioration in oral glucose tolerance at mid-cycle (20) and on entry into the luteal phase (19). Reports that intravenous glucose tolerance is unaltered during the menstrual cycle (22,23,24) lend weight to this argument. However, several reports indicate that fasting blood glucose levels are increased at the time of menstruation (25,26).

Seemingly contrary to the reports of an improvement in oral glucose tolerance in non-diabetics at the time of menstruation, diabetics may show a heightened hyperglycaemia and ketosis may be precipitated at this time (27,28,29). Thus there may be a marked deterioration in diabetic control at this time (29,30,31), but it is not a necessary consequence of menstruation (32),

Although alterations in glucose tolerance and diabetic control occur in some women during the menstrual cycle, it is difficult to equate the observations with specific changes in sex steroid titres. Moreover it would be

imprecise to single out altered sex steroid titres as a specific cause of altered glucose tolerance.

1.1.3. The effect of pregnancy on glucose tolerance and insulin action.

Both human and animal pregnancy represent conditions of naturally altered female sex steroid titres, and have long been associated with imbalances of carbohydrate metabolism. The following represents a brief review of the literature relating to carbohydrate metabolism and pregnancy in man and animals and considers the suitability of such reports for defining the effects of endogenous female sex steroids on carbohydrate metabolism.

Although the foetal drain of maternal fuel sources produces accelerated starvation during periods of fast in pregnancy, the maternal response to a nutritional load is characterised by hyperinsulinaemia, hyperglycaemia, hypertriglyceridaemia and a diminished sensitivity to insulin (33).

Hyperinsulinaemia after an appropriate challenge is most marked in late pregnancy. Hyperinsulinaemic responses in pregnancy in both Man and animals appear to be due to an enhanced sensitivity of the pancreatic islets to a glucose or amino-acid stimulus (34,35,36,37) rather than a more indirect effect such as an alteration in gastric-inhibitory polypeptide response (38).In the face of such hyperinsulinaemia and concomitantly reduced glucagon secretion, the observed elevated blood glucose responses

in gravid women during both intravenous and oral glucose challenges (39) are indicative of an insulin resistant state. The contention of pregnancy as a condition of peripheral target tissue resistance to insulin is further supported by the observation that increased plasma insulin levels are required by pregnant women to maintain a fixed degree of glycaemia during glucose infusion (40).

An increase in insulin resistance with advancing human pregnancy (33), the possible precipitation of diabetes and the increased insulin requirement of gravid diabetics fuel the argument for pregnancy as a condition of diabetogenic stress. Such development of insulin resistance in human and animal pregnancy (41,42,43) has been attributed to the production of certain hormones by the placenta, namely; placental lactogen (42), oestrogens and progestogens (45,46).

Human placental lactogen shares some of the biological actions of, and cross reacts immunogenically with human growth hormone (45,46), a substance of known diabetogenic action (47) and may therefore contribute to the insulin resistance during pregnancy. Indeed placental lactogen impairs glucose tolerance and promotes lipolysis in rats (48,49) and acute infusions in Man reduce glucose tolerance (50) particularly in cases of pre-existing diabetogenic stress (51,52). However, a consistent relationship between plasma placental lactogen concentrations and insulin requirement in gravid diabetics has not been observed (53) suggesting the involvement of other factors in the

development of insulin resistance during pregnancy. Thus, before a full understanding of this phenomenon can be achieved, a better knowledge of the individual and combined effects of the many hormonal alterations associated with pregnancy is required. Furthermore, determination of the time and dose dependency of the actions of the hormones is essential in view of the unusually long exposure to such high concentrations of the hormones.

In summary, the physiological significance of oestrogens and progestogens in controlling insulin action and carbohydrate metabolism during pregnancy remains unclear. Currently available data suggests that in Man and other animal species both sex steroids may contribute towards the hyperinsulinaemia of pregnancy (pages 12-15). It seems, however, unlikely that oestrogens per se cause insulin resistance although high levels of progestogens may well contribute toward the diabetogenic stress of the gravid condition (page 11).

Consideration of the impact of oral contraception and the menstrual cycle on glucoregulation, although often used to implicate sex steroid modulation of carbohydrate metabolism, does not permit the precise definition of these relationships in Man. The complex physiological changes exsisting in pregnancy in both Man and other animal species also render this area of little value in attempting to define precise associations between

sex steroid titres and carbohydrate metabolism. However, the enhanced ability to control experimental criteria in animals has led to the emergence of an understanding of the role of sex steroids in regulating carbohydrate metabolism and it is to these studies, predominantly in rodents, that our attention must focus.

1.1.4. The effect of oestrogens and progestogens on experimental diabetes.

The diabetes obtained in rats after subtotal (95%) pancreatectomy as described by Foglia (54), has provided an ideal animal model in which factors affecting both the incidence and severity of diabetes mellitus, and by inference insulin and glucose metabolism can be studied.

Early work investigating the influence of sex steroids revealed a sex difference in the production of diabetes by subtotal pancreatectomy. The frequency of induction was greater in males than females (55,56) and this was shown to be due to a protective action of the ovaries and provocative action of the testes (57).

The administration of sex steroids to castrated, subtotally pancreatectomised rats enabled the influence of individual sex hormones on experimental diabetes to be investigated. Oestradiol mediated protection was first demonstrated as a consequence of its administration to partially pancreatectomised rats (57). Subsequently

the protective action of many oestrogenic steroids was demonstrated after their injection for several months to subtotally pancreatectomised rats. These steroids included; oestrone, stilboestrol, dienoestrol, oestradiol benzoate, mono-benzyl-diethl-stilboestrols, phenocycline and to a lesser extent ethinyl oestradiol and ethinyl testosterone (58).

Several other reports using dogs (59), cats (60,61), rabbits (62) and monkeys (63) as experimental models have also shown attenuating effects of oestrogens on the severity of the diabetes induced by total pancreatectomy. However, it must be noted that in similar studies in dogs (64) and monkeys (65), oestrogens were shown to have no significant effect while their administration to ferrets (66), force fed rats (67) and castrated male rabbits (68) actually aggravated the diabetes induced by pancreatectomy.

Species differences should not be overlooked when interpreting these results. However, it is probable that inconsistencies within the various methodologies might produce such variations, in particular, the method of assessment of the diabetic condition and the type, dose and period of oestrogen administration. For example, in rats after subtotal pancreatectomy oestrogen treatment produced a biphasic effect, at first increasing the incidence and severity of diabetes but later (> 1 month) attenuating or even completely

suppressing diabetes (69). The diabetogenic effect of oestrogen administration shown by other workers, for example, in force fed normal rats (67), and in force fed rats made diabetic by partial pancreatectomy (67,56) or alloxan administration (70) may therefore represent the primary phase of oestrogen action. However, the work of Foglia, Schuster and Rodriguez (57) and Lewis, Foglia and Rodriguez (58) outlined earlier showing the protective nature of oestrogens against experimental diabetes may represent the secondary protective phase of oestrogen action.

There is very little evidence in the literature concerning the effects of progesterone on experimental diabetes. Progesterone has been shown to have no effect either on the incidence of diabetes after subtotal pancreatectomy in rats (58), or its severity in similarly treated rats (71) and ferrets (66). However, supra-physiological doses of progesterone have been observed to increase the incidence of diabetes in pancreatectomised rats (72).

Thus oestrogens, particulary after long term administration, provide a protective action against experimental diabetes. Progesterone, at least at physiological concentrations, appears to have little or no effect while higher concentrations may indeed provoke the experimental production of diabetes.

The mechanism of the initial diabetogenic action of the oestrogens remains obscure. Although normal levels of adreno-cortical hormones have been reported as being conditioning factors (73), this has not been substantiated (56). The subsequent protective action of oestrogens could not be attributed to alterations in food intake (74) and required neither hypophyseal nor adrenal interaction (69,71). However, protection coincided with pancreatic islet hypertropy and hyperplasia (69) and the effect of oestrogens on experimental diabetes have thus generally been attributed to specific effects on the pancreas (69,75).

1.2. The effects of oestrogens and progestogens on the endocrine pancreas.

In view of the proposed mechanism of oestrogen mediated protection against experimental diabetes, that is through the production of pancreatic islet hypertrophy and hyperplasia (see above), the following represents a brief consideration of the reported effects of sex steroids on pancreatic morphology, constitution and function.

Although no sex differences in islet mass have been reported (75,76), reduced female sex steroid titres produced by ovariectomy have been shown to be associated with a reduction in B cell numbers (77) and a reduction in total pancreatic and islet insulin content (78). Hormone replacement regimes (oestradiol, progesterone or the two in combination), however, reversed the effects of

ovariectomy (78). Similarly, oestrogen administration appears to be associated with an increase in islet weight (76), new islet formation (79), an increase in the number of B cells (80) and increased pancreatic insulin content (81,82,83,84). The stimulatory effects of progestogens on the pancreas (78) have also been noted and are represented by an increase in islet weight (76) and size (75) although not in total pancreatic insulin content (82,83).

In general, the presence of ovarian sex steroids appears therefore to be associated with an enhanced pancreatic insulin secretory capacity. Furthermore, the increased amount of B cell granulation (69,71,75,85) and enlarged B cell Golgi apparatus (86) have provided circumstantial evidence of enhanced insulin secretion during oestrogen administration. It was not, however, until the developement of a suitable procedure for the use of isolated islets (87), that the direct in vitro quantification of insulin secretion from islets exposed in vivo and in vitro to various sex steroid titres could be performed.

As would be expected from the presently accepted mechanism of action of steroids, that is at the level of gene transcription (88) ,the incubation of isolated islets with sex steroids for 1-2 hours, had no effect on insulin secretion (35,89). However, ovariectomy reduced glucose stimulated (but not basal) insulin release from both

statically incubated and perifused islets (78). It is also well established that the administration of progesterone, oestrogen, or the two in combination at concentrations within the physiological range, increase glucose stimulated insulin release from subsequently isolated islets of both rats and mice (35,78,89,90,91, 92). Studies with perfused rat pancreas, particulary after exposure to progesterone have shown similar effects (93,94) of the ovarian hormones.

An increased insulin secretory capacity and an increased insulin secretion, specifically in response to glucose, are therefore associated with the long term exposure of the pancreas to female sex steroids. These effects require time and are long lasting thus excluding direct effects of sex steroids on any of the components believed to be involved in insulin release (for review see 95) including alterations in ionic fluxes or intracellular concentrations of cyclic AMP, Ca^{++}, intermediary metabolites or pyridine nucleotides. It appears therefore, that along with effects on islet hypertrophy, hyperplasia and insulin content, sex steroids mediate their effects on insulin secretion at the level of gene transcription, consistent with the known specific interaction of steroids with nuclear chromatin (88).

It is noteworthy that although both oestrogens and progestogens have stimulatory effects on the pancreas, only oestrogens alleviate experimental diabetes (69)

suggestive of extrapancreatic effects of one or both steroids. It is thus pertinent to consider the role of the ovarian sex steroids in modulating carbohydrate metabolism and insulin action within peripheral insulin target tissues.

1.3. The effects of oestrogens and progestogens on carbohydrate metabolism and insulin action in peripheral target tissues.

Glucose tolerance, hepatic glycogen levels and the total rate of gluconeogenesis from L-alanine, have been shown to fluctuate throughout the oestrous cycle in rats (96,97). Improved glucose tolerance, heightened insulin response to a glucose challenge and depressed gluconeogenesis were associated with high plasma levels of sex steroids present at proestrus and oestrus. However, it must be noted that alterations in hepatic glycogen concentrations and hepatic glycogen production from L-alanine were more closely associated with changes in plasma corticosteroid levels known to occur during the oestrouscycle (97). Thus, although hepatic glycogen production and concentration were greatest at proestrus they were significantly reduced at oestrus followed by slight increases during metoestrus and dioestrus. These data suggest therefore that the effects of gonadal steroids on carbohydrate metabolism during the oestrous cycle may at least in part, be mediated by their influence on adrenocortical secretions.

However, physiological variations in both oestrogen and progestogen concentrations throughout the oestrous

cycle complicate the interpretation of the effects of individual sex steroids on carbohydrate metabolism and insulin action. Thus depletion of sex steroids by ovariectomy and the administration of hormone replacement regimes have been widely used to further investigate the role of the ovarian steroids in modulating both carbohydrate metabolism and insulin action.

Ovariectomy of mice on a chronic basis (15 weeks) increased fasted but not fed plasma glucose levels, impaired glucose tolerance and reduced the insulin response to glucose loading compared to intact (dioestrus) mice (78). Ovariectomy of mice also increased total *in vivo* gluconeogenesis from L-alanine (98) reduced the hypoglycaemic action of insulin, enhanced the hyperglycaemic action of glucagon and adrenaline (99) and reduced the glycogen contents of liver, heart and skeletal muscle (98). In general, the long term administration (15 weeks) of either oestradiol or progesterone, or the two in combination in physiological doses was associated with the partial or total rectification of these ovariectomy induced alterations in carbohydrate metabolism (78,98, 99).

Similarly, administration of oestrogen in physiological doses for 2-3 weeks to female rats reduced blood glucose levels, increased plasma insulin concentrations (35, 96,100) and was associated with increased glycogen deposition in liver and uterine myometrium (101-106). This

increased rate of glycogen deposition, at least in the uterus, was probably due to the increased activation or induction of glycogen synthase (107,108,109) and/or oestrogen induced activation of specific glucose transport systems (110,111).

Thus it is apparent that natural oestrogens promote glucose metabolism. The increased pancreatic islet function associated with oestrogen administration (pages 12 -15), may, in part account for reduced blood glucose concentrations (35,78,96,100) and increased glycogen contents of peripheral insulin target tissues (101-106). However, oestrogen induced increases in insulin sensitivity of skeletal muscle and adipocytes particularly in respect of glucose uptake (105,112-115), suggest specific alterations in carbohydrate metabolism at the cellular level in peripheral insulin target tissues. Such alterations are exemplified by the oestrogen mediated increase in key glucoregulatory enzymes, namely the increased activation or induction of glycogen synthase in uterine myometrium (107-109) and skeletal muscle (116) and the increased insulin stimulated hexokinase and oxoglutarate dehydrogenase activity in skeletal muscle from oestradiol treated ovariectomised mice (116).

The effects of progesterone on the same parameters of carbohydrate metabolism are less clear. Specific effects of progesterone suggesting a role for this sex steroid in the enhancement of carbohydrate metabolism have been observed. These

include decreased blood glucose and elevated plasma insulin levels in rats and mice (35,96,100). More specifically, the administration of progesterone produced alterations in glycogen metabolism, and this was associated with either an increased activation or induction of uterine glycogen synthase (108,109) and increased liver glycogen contents in rats (101) and ferrets (117). These alterations in the hepatic handling of glucose were also indicated by the increased incorporation of L-alanine and pyruvate into hepatic glycogen, while the decreased incorporation of both substrates into blood glucose (101) revealed a reduced rate of total *in vivo* gluconeogenesis by progesterone. Taken together, these data suggest an insulin enhancing effect of progesterone on carbohydrate metabolism although it must be noted that there have been several reports indicating an insulin antagonistic effect of progesterone, specifically on skeletal muscle and adipocyte carbohydrate metabolism. Thus, the observed reduction in the *in vivo* hypoglycaemic action of insulin after progesterone administration was reflected in adipocytes by a reduced insulin stimulated glucose uptake and oxidation (120) a reduced glucose phosphorylation and lipogenesis and an increased rate of lipolysis (121). The primary cause of these progesterone induced reductions in carbohydrate metabolism is suggested to be the result of indirect inhibition of hexokinase which has been observed in rat adipocytes (122). Such inhibition has not, however, been observed in skeletal muscle of progesterone treated ovariectomised mice (116).

It is also significant that progesterone antagonism of oestrogen elevations of *in vivo* gluconeogenesis (98), the hypoglycaemic action of insulin (99) and glycogen deposition in both myometrial tissue (109,123) and liver (102) have been observed. Thus the effects of individual sex steroids on carbohydrate metabolism and insulin action appear to be dependent upon the tissue studied and the molar concentrations of other gonadal steroids.

It is apparent, however, that progesterone does not show the characteristics of an insulin antagonist in the liver and indeed appears to facilitate insulin action in this organ, as exemplified by the promotion of hepatic glycogen deposition (101,108,109,117). These effects coincide, however, with hyperinsulinaemia (124) thus preventing the distinction between either a direct effect of progesterone on insulin action in the liver or an effect mediated via an increased plasma insulin concentration.

Progesterone administration to rodents has been reported to both increase (99) and decrease (118) the hypoglycaemic action of exogenous insulin and it's effect on carbohydrate metabolism and insulin action in adipose tissue and skeletal muscle is equally equivocal (98,116,118-120).These reported alterations in the hypoglycaemic action of insulin and insulin action in isolated adipocytes and skeletal muscle, suggest, however, that progesterone is capable of modulating insulin action at the level of peripheral insulin target tissues.

There is therefore evidence within the literature to suggest that both natural oestrogens and natural progestogens influence insulin action at the level of peripheral insulin target tissues. The following literature survey therefore considers the presently proposed mechanisms of insulin action in an effort to distinguish the level(s) at which gonadal steroids may regulate insulin action.

1.4. The mechanism of insulin action.

In mammals, the biological actions of insulin are concerned primarily with the control of rapid adjustments of intermediary metabolism, to accommodate fluctuations in nutrient supply and energy demand (125,126).

The immediate effects of insulin involve alterations in the cellular metabolism of glucose and lipids through changes in the activities of pre-existing enzymes and membrane transporters. Insulin rapidly promotes glucose uptake into muscle and fat cells, where in the former, glycogen production is greatly enhanced and in the latter, glucose is converted mainly to glycerol and fatty acid moieties of triacylglycerol. Lipolysis in fat cells, particularly that enhanced by other hormones, is depressed, while in the liver insulin initiates a reduction in gluconeogenesis and causes an increase in glycogen storage and triacylglycerol synthesis.

The hypoglycaemic action of insulin is reflected at the cellular level either by an increased glucose uptake mediated by an enhanced capacity of the specific carrier

system through covalent modification (127) or recruitment of carrier molecules from intracellular sites (128-130). Many other alterations in enzyme activities have been found in extracts of cells previously exposed to insulin (131) and are consistent with the known metabolic actions of insulin (Figure 1).

Although it has been known since the early 1970's that the metabolic actions of insulin are initiated by its interaction with specific receptors located within the plasma membrane (132) there is as yet no satisfactory explanation of the molecular events which then occur and preceed the recognizable metabolic effects of insulin.

The biological actions of insulin brought about by the altered activity of enzymes controlling key metabolic pathways, and the regulation of many if not all of the intracellular activities are accompanied by changes in the phosphorylation of the affected enzymes, usually but not always, dephosphorylation (131), (Figure 1).

Since 1968, cAMP (133), cGMP (134), Ca^{++} (135), Mg^{++} (136), H_2O_2 (137), membrane hyperpolarisation (138) and intracellular pH (139) have been suggested as mediators of insulin action. It is unlikely, however, particularly because of the coincident phosphorylation and dephosphorylation of separate enzymes, that any one of the above could individually mediate the entire range of the effects of insulin (131).

Figure 1. Insulin exerts its metabolic effects by altering the activities of enzymes responsible for the regulation of key metabolic pathways. This figure represents the well established alterations in enzyme activities and glucose transport that persist into extracts of various cell types previously exposed to insulin, and indicates the roles of the enzymes in controlling cellular glucose metabolism. The arrows, ↑ and ↓ indicate an increase or decrease in activity respectively after exposure of intact cells to insulin, where the cell types in which the changes have been observed are represented by;

B=brown fat, H=heart muscle, L=liver

MG=mammary gland, SM=skeletal muscle, W=white fat.

Ⓟ and Ⓓ indicate whether the alteration in activity was induced by phosphorylation or dephosphorylation of the enzyme respectively.

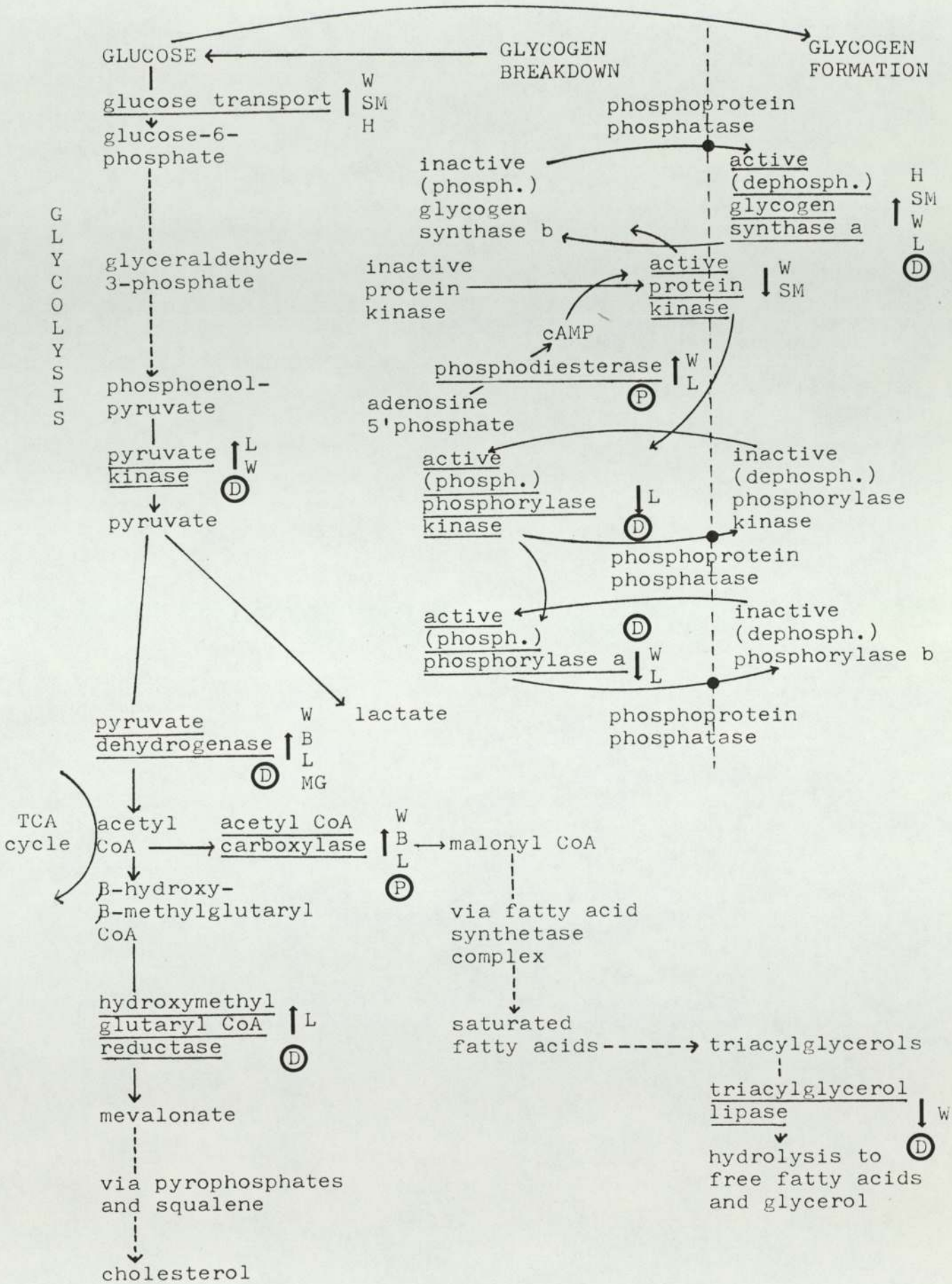
GLUCOSE
GLYCOGEN BREAKDOWN
GLYCOGEN FORMATION
glucose transport
W SM H
glucose-6-phosphate
GLYCOLYSIS
glyceraldehyde-3-phosphate
phosphoenol-pyruvate
pyruvate kinase
L W
D
pyruvate
phosphoprotein phosphatase
inactive (phosph.) glycogen synthase b
active (dephosph.) glycogen synthase a
H SM W L
D
inactive protein kinase
active protein kinase
W SM
cAMP
phosphodiesterase
W L
P
adenosine 5'phosphate
active (phosph.) phosphorylase kinase
L
D
inactive (dephosph.) phosphorylase kinase
phosphoprotein phosphatase
active (phosph.) phosphorylase a
W L
D
inactive (dephosph.) phosphorylase b
phosphoprotein phosphatase
lactate
pyruvate dehydrogenase
W B L MG
D
TCA cycle
acetyl CoA
acetyl CoA carboxylase
W B L
P
malonyl CoA
β-hydroxy-β-methylglutaryl CoA
via fatty acid synthetase complex
hydroxymethyl glutaryl CoA reductase
L
D
saturated fatty acids
triacylglycerols
triacylglycerol lipase
W
D
hydrolysis to free fatty acids and glycerol
mevalonate
via pyrophosphates and squalene
cholesterol

Figure 1.

A chemical mediator of insulin action, released into the cytoplasm after exposure of target cells to insulin was first proposed in 1972 (140). By 1979 an insulin generated substance had been identified that specifically inhibited cAMP dependent protein kinase, stimulated glycogen synthase phosphoprotein phosphatase (141) and activated mitochondrial pyruvate dehydrogenase (142) by stimulation of pyruvate dehydrogenase phosphatase (143). This insulin mediator was subsequently identified as an oligoglycopeptide with a molecular weight of 1000-1500 daltons (141). By 1980, two peptides had been separated from the mediator cell extract which had opposing effects on protein phosphorylation (144) although it was not known if one represented a metabolic product of the other or a separate entity (145). It was, however, suggested that the presence of two such mediators would provide a mechanism by which insulin could induce both the phosphorylation and dephosphorylation of intracellular proteins (146).

Mediators of insulin action have also been isolated from extracts of adipocytes exposed to very mild trypsin action, and this observation along with the blocking of insulin action by proteolytic inhibitors, has lead to the suggestion that insulin acts via a proteolytic mechanism (145). A general hypothesis has therefore been forwarded (145-147) suggesting that insulin binding facilitates a proteolytic reaction at the plasma membrane involving an arginine-specific serine protease and an endogenous membrane substrate, followed by the internalisation of

free rather than vesicular bound mediator(s) with the subsequent alterations in phosphorylation of the key enzymes (Figure 2). Present estimates indicate that the mediator(s) is/are present at 100-1000 times in excess of the insulin receptor and it appears therefore unlikely that proteolysis of the receptor itself generates the mediator(s) (145). Thus in essence insulin is a cofactor for the membrane-bound protease which it might activate by initially cross linking the receptor (145).

The validity of the experimental procedures used to demonstrate the presence of mediators of insulin action has, however, been questioned and indeed there have been claims that similar work has failed to substantiate the findings outlined above (131). Alternatively, at least in fat cells, insulin action is proposed to be mediated through changes in the states of phosphorylation of certain proteins (notably ATP-citrate lyase and uncharacterised proteins of subunit molecular weights of 61,000, 35,000 and 22,000 daltons) catalysed by a mediator (possibly a protein kinase) released from the plasma membrane after insulin-receptor interaction (131), (Figure 3).

It has been observed that the insulin receptor is itself phosphorylated in intact cells (148) and in solubilized rat liver membranes (149) after exposure to insulin, due to the phosphorylation of a tyrosine residue (149). Indeed it has recently been reported that the insulin receptor itself is a tyrosine-specific kinase which phosphorylates the B subunit

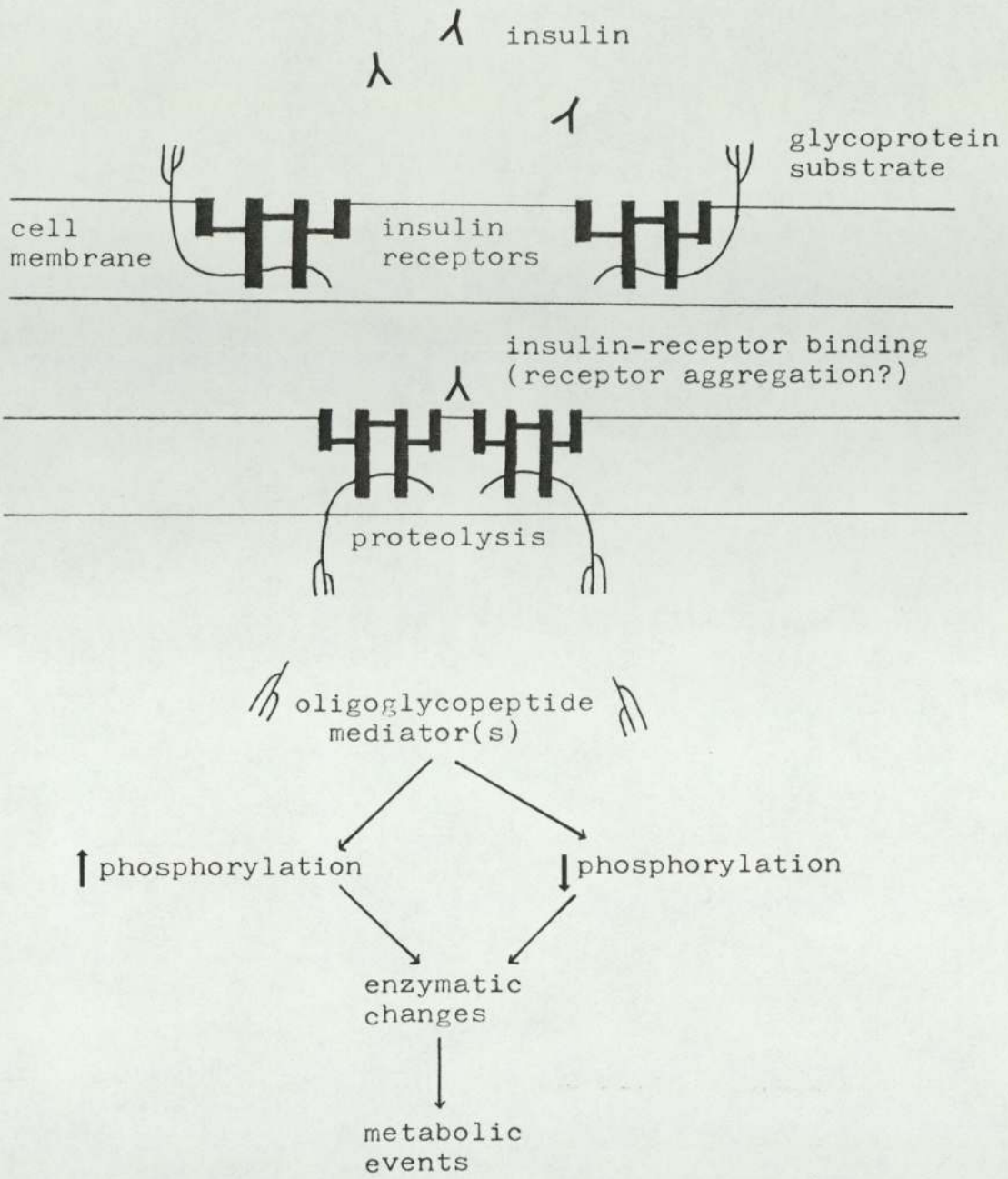

Figure 2. One proposed mechanism of insulin action, involving insulin mediated proteolysis of membrane located glycoprotein substrate, with the subsequent production of an oligopeptide mediator(s) capable of altering the states of phosphorylation and thus activity of key glucoregulatory enzymes. After Larner et al(1982),(145) and Jarett et al(1982) (146).

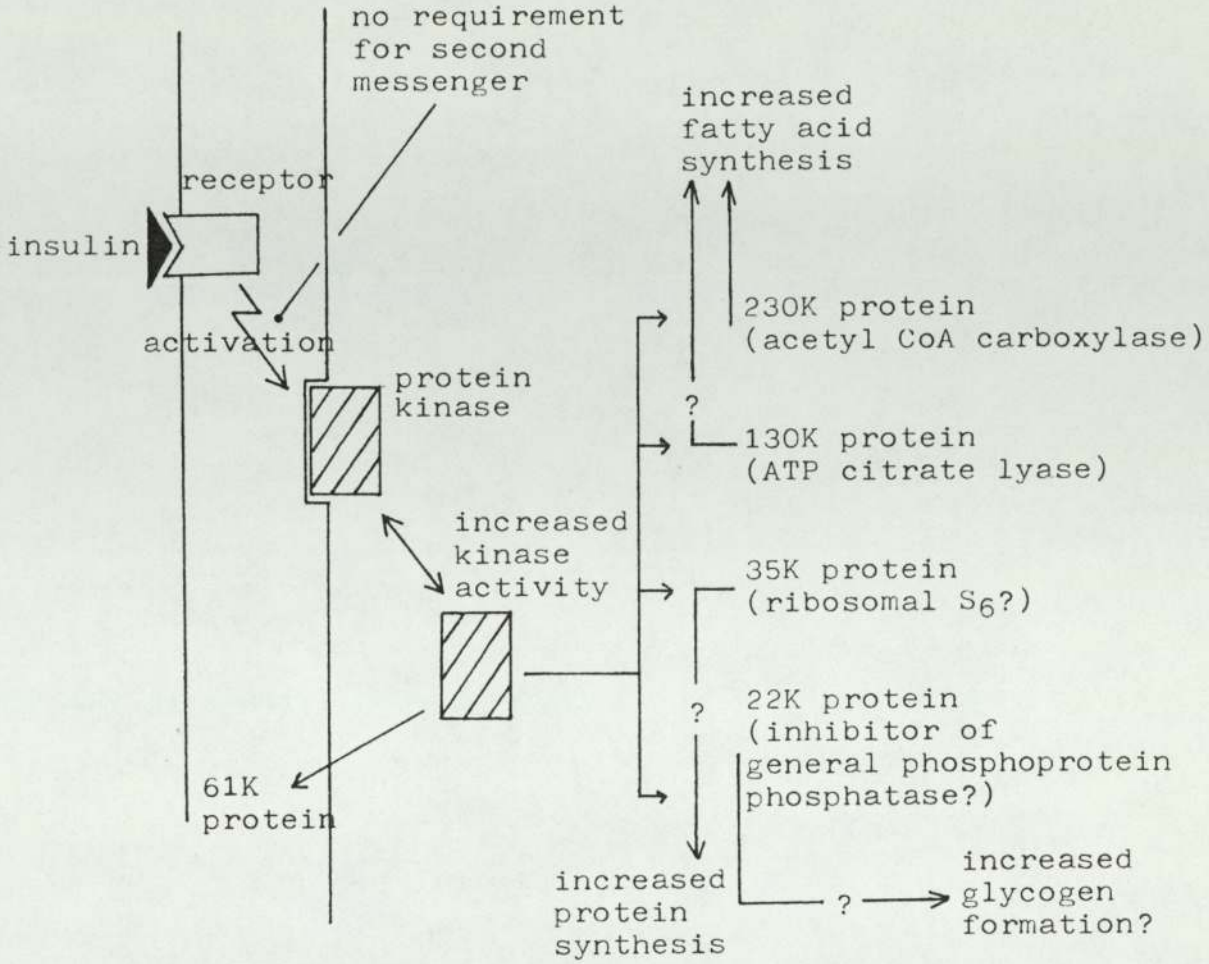

Figure 3. Proposed mechanism for insulin in fat cells, after Denton et al(1981),(131). Although the increased phosphorylation of several proteins has been observed after exposure of fat cells to insulin, the full implications of these changes has yet to be identified.

of the insulin receptor after insulin binding to the α-subunit of the receptor (150). However, it is not clear whether or not the increased kinase activity of the receptor would be sufficient to induce the entire range of the effects of insulin suggesting that it may be wise to consider multiple mechanisms for the mediation of the action of insulin.

The interaction of insulin with a specific plasma membrane receptor marks the first link in a chain of cellular events which culminate in the biological actions of insulin (151). It is apparent, however, that at present the explanation for the post-receptor events of insulin action is far from complete and an understanding of these events will provide a challenge for some time to come.

1.4.1. The biological actions of insulin.

Along with the rapid and potent effects on carbohydrate, fat and amino-acid metabolism considered above, insulin exerts a more mild long-term stimulatory effect on cell growth, division and differentiation (152). The effects of insulin on intermediary metabolism, such as glucose transport and glucose oxidation, occur within minutes of the cell's exposure to insulin concentrations of less than 10^{-9}M (153-155). Growth promoting effects, however, show a lag time of hours to days before onset, require protein biosynthesis and require insulin concentrations of 10^{-8} to 10^{-7}M (156). These growth promoting effects appear to be mediated by a class of plasma membrane receptors distinctly independent from receptors mediating the effect of insulin

on intermediary metabolism. In addition it has, however, been recently reported that insulin may act directly on the nucleus, inducing enhanced mRNA synthesis (157), thus questioning the assertion that all the actions of insulin are initiated by insulin interaction with its specific membrane receptors (151). The plasma membrane receptors that mediate the growth effects of insulin show a high degree of specificity for a group of polypeptides known as the insulin-like growth factors (IGF's). Structural similarities exist between IGF's and insulin, allowing weak interactions with each other's receptors (158,160,161). For the purpose of the present work, "insulin receptor" will refer solely to that receptor which shows a high degree of specificity for insulin and which mediates its effect upon intermediary metabolism.

1.5. The insulin receptor and insulin action.

That insulin is able to mediate its biological effects without entering the cell has been known since the middle to late 1960's (162-164). Thus a role for a specific receptor located within the plasma membrane has been confirmed. Although insulin binding sites have been identified on subcellular organelles (165-167) it is now well established that they are located predominantly within the plasma membrane (168) and that the interaction of insulin with this specific receptor initiates a chain of cellular events which culminate in it's biological action.

Clearly, alterations in either post-receptor events responsible for the mediation of insulin action or in

insulin binding to its receptor, are possible mechanisms by which alterations in insulin action could be initiated. Indeed an evaluation of biological dose/response curves in conditions of altered insulin action provide information about the nature of the defect.

1.5.1. Target cell sensitivity and responsiveness to insulin.

A typical dose/response curve for insulin is shown in Figure 4, in which biological activity is plotted against the $\log_{10}$ of the hormone concentration required to bring about that effect. A displacement to the right of this curve is termed insensitivity and is classically considered to be a receptor defect, while a reduction in the maximum biological response attainable is termed unresponsiveness and considered to be a post-receptor defect. It is also possible that both insulin insensitivity and unresponsiveness may occur simultaneously.

1.5.2. The 'spare-receptor' theory.

It is well established that for insulin to initiate its maximum biological effect upon cells of normal sensitivity, only a small percentage of the receptors need to be occupied. Depending on the effect and tissue under study this fraction may vary from as little as 2% to 35% (169-173). The unoccupied receptors, generally called 'spare' receptors may have the function of regulating the cellular sensitivity to insulin. Provided insulin-receptor binding follows the law of mass action, alterations in the total number of receptors responsible for a biological effect will alter the

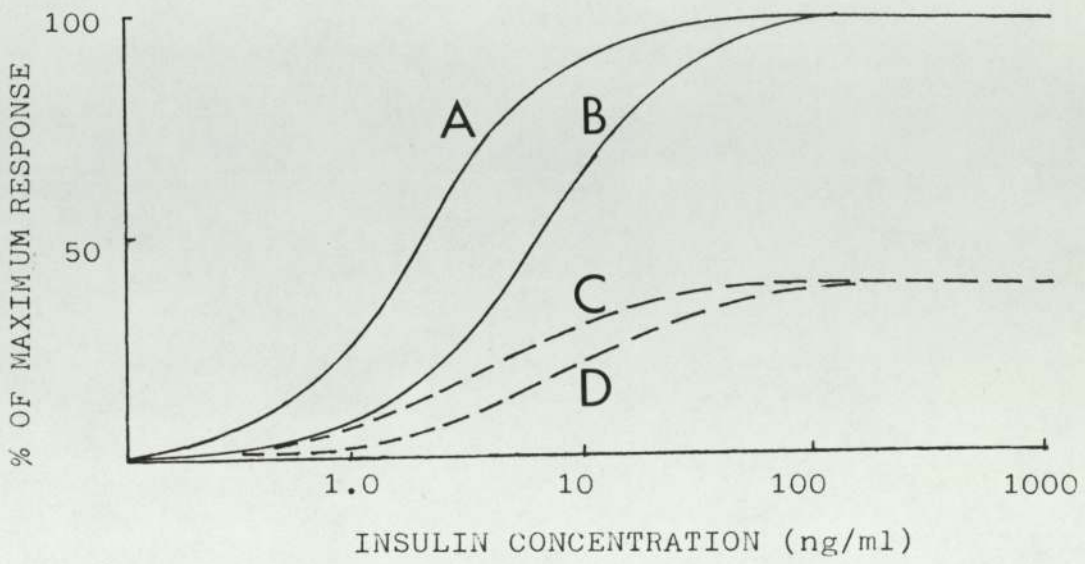

Figure 4. Theoretical dose response curves for a biological action of insulin indicating alterations in insulin action. A= normal curve. B= decreased sensitivity (receptor defect). C= decreased responsiveness (post-receptor defect). D= decreased sensitivity and responsiveness (combined receptor and post receptor defect).

insulin concentration required to bring about such an effect. Thus, providing receptor numbers do not fall below the number required to initiate a maximum biological effect only the sensitivity will be altered by changes in receptor number and post-receptor events will be rate-limiting.

1.5.3. Conditions of altered insulin-receptor binding.

Situations in which alterations in insulin-receptor binding have been implicated in the induction of insulin resistant states covers a broad spectrum of both physiological and pathological states (174). Although the role of any changes in receptor binding in the aetiology of these many states has not been firmly established, the close correlations between these changes and alterations in insulin sensitivity and carbohydrate metabolism strongly support a patho-physiological role for alterations in insulin receptor binding. The diverse number of conditions under which receptor binding is altered also indicate why insulin binding studies are an important facet of many areas of research, particularly when the aetiology of alterations in insulin action is unknown.

1.6. The study of insulin-receptor interactions.

Much information on the nature of the specific interaction of insulin with its receptor has been obtained from kinetic and equilibrium binding studies. Initial work in this field was, however, hampered by the lack of a suitable high specific activity tracer hormone preparation. Indeed, it was not until the developement of a reliable insulin

iodination technique in the early 1970's (175-177) coupled with the preparation of suitable receptor populations, that useful and meaningful insulin receptor research became a reality.

1.6.1. Preparations used to study insulin-receptor binding.

Studies of both kinetic and equilibrium insulin binding have normally been performed on either isolated intact cells or plasma membrane preparations although binding to subcellular organelles, tissue pieces and intact organs have also been determined. The last two preparations provide cells in a more physiological environment, but the interpretation of results is complicated by the need for many theoretical considerations, including the high degree of non-specific binding (ie. that binding not mediated by specific insulin receptors) and the presence of barriers to the diffusion of hormone. Also the limited number of tissue pieces and intact organs available from individual animals often restrict analysis to the determination of a limited number of binding parameters.

Cells of insulin target tissues isolated by enzymatic digestion, and cell membrane fractions prepared by cell homogenisation and differential centrifugation therefore provide ideal tools for studies of insulin receptor status. The large number of aliquots of equivalent receptor populations that can be prepared by these methods also allow the determination of many more parameters of receptor status, including various affinity constants and the number of

receptors. Isolated cells are also metabolically active and thus have the distinct advantage of allowing both the biological effects and binding of insulin to be determined simultaneously.

Although assessment of insulin binding to the major insulin target tissues provides the most useful information when determining the role of changes of insulin binding in the mediation of alterations in insulin action, this is not always practical. This is particularly the case in clinical studies where muscle, liver and fat biopsies are difficult to obtain, and in which case more easily obtained tissues are used although not considered classically as sites of insulin action. In man, assessments of insulin receptor activity have relied mostly on the use of circulating monocytes (178-180) and erythrocytes (181-183).

The insulin binding properties of monocytes have been generally considered to mirror the properties of adipocyte insulin receptors in a number of conditions in man (179,180, 184,185), but doubt has now been raised as to the validity of such assumptions (186). Compared to monocytes, erthrocytes have the advantage that only a small volume of blood is required (181,182). However, changes in the binding characteristics of erythrocytes may occur more slowly (182,187,188) which may in part reflect the incapacity of erythrocytes to undertake de novo protein synthesis. The anucleate state of erythrocytes, their unresponsiveness to insulin, the higher specific insulin binding of reticulocytes within

erythrocyte preparations and the exponential decrease in the number of erythrocyte insulin receptors with cell age (189-191) also cast doubt on the suitability of such assays for reflecting insulin binding to insulin target tissues.

The criticisms of both monocyte and erythrocyte insulin receptor binding assays outlined above merely serve to illustrate the fact that data should only be extrapolated beyond the cell type studied with great caution.

1.6.2. Assessment of insulin-receptor binding.

Irrespective of the insulin receptor preparation used the basic theoretical considerations employed to study equilibrium binding are based upon the competitive binding assay originally described by Yalow and Berson (192) and now widely employed for the radioimmunoassay of many ligands. Modification of these assays for the quantitative determination of various parameters of hormone/receptor interaction involves the optimally controlled incubation of replicate aliquots of the receptor population (eg. cells) with a fixed amount of high specific activity ^{125}I-monoiodoinsulin (within the physiological range) and a range of cold standard insulin concentrations (typically 0-1000ng /ml). After a predetermined time, under optimal conditions, equilibrium binding is reached at which point the tracer and cold insulin are bound in proportion to their molar ratios. At this point the receptor bound and free insulin (labelled and unlabelled) are rapidly separated by filtration or centrifugation (through oil or buffer). After any required

washing steps, the radioactivity associated with the bound fraction of hormone is determined.

However, not all tracer binding represents the specific binding of insulin to specific receptors. This non-saturable component is determined in the presence of excess native insulin and designated the non-specific binding. After correction for the non-specific element of tracer binding at all insulin concentrations employed, binding data may be subjected to a variety of graphical analyses (for reviews see: 193-195).

This generalized scheme for a receptor binding assay may be applied to many receptor preparations although the conditions under which equilibrium binding is achieved may differ substantially. In most cases, those conditions by no means parallel the in vivo situation and it must therefore be appreciated that changes in receptor status may occur during the preparation and incubation of the tissue. Thus it must be remembered that the observed in vitro insulin receptor status may not be a precise reflection of their status in vivo prior to assay.

1.6.3. Analysis of equilibrium insulin-receptor binding data.

1.6.3.i. The competition curve.

A simple competitive (displacement) binding curve may be used for the analysis of steady state binding data and is the only plot of those to be considered to display raw, untransformed data. The plot considers as the ordinate, the

amount of ^{125}I-insulin bound as a function of the $\log_{10}$ of the total insulin concentration. The plot is typically sigmoidal (Figure 5) and may be used for affinity assessments (195)providing the concentrations of tracer hormone and binding sites are at least 10-fold below the dissociation constant (196).

Distortion of data due to the use of one log scale, the limited amount of information gained from such plots and the fact that tracer hormone and binding site concentrations and the dissociation constant are often unknown, render the plot of limited use for the quantitative interpretation of steady state binding data and are thus often used in conjunction with other methods of analysis.

1.6.3.ii. The law of Mass Action. The saturation plot.

The insulin receptor shows a high degree of affinity for insulin and reacts in a specific, rapid, saturable and reversible manner (197,198). The interaction of insulin with its receptor is generally considered as a simple, bimolecular reversible reaction described by the law of mass action (199);

$$[H] + [R] \underset{Kd}{\overset{Ka}{\rightleftharpoons}} [HR]$$

where;- $[H]$ = free hormone concentration

$[R]$ = concentration of unoccupied receptors

$[HR]$ = concentration of hormone-receptor complexes

Ka and Kd = association and dissociation rate constants respectively.

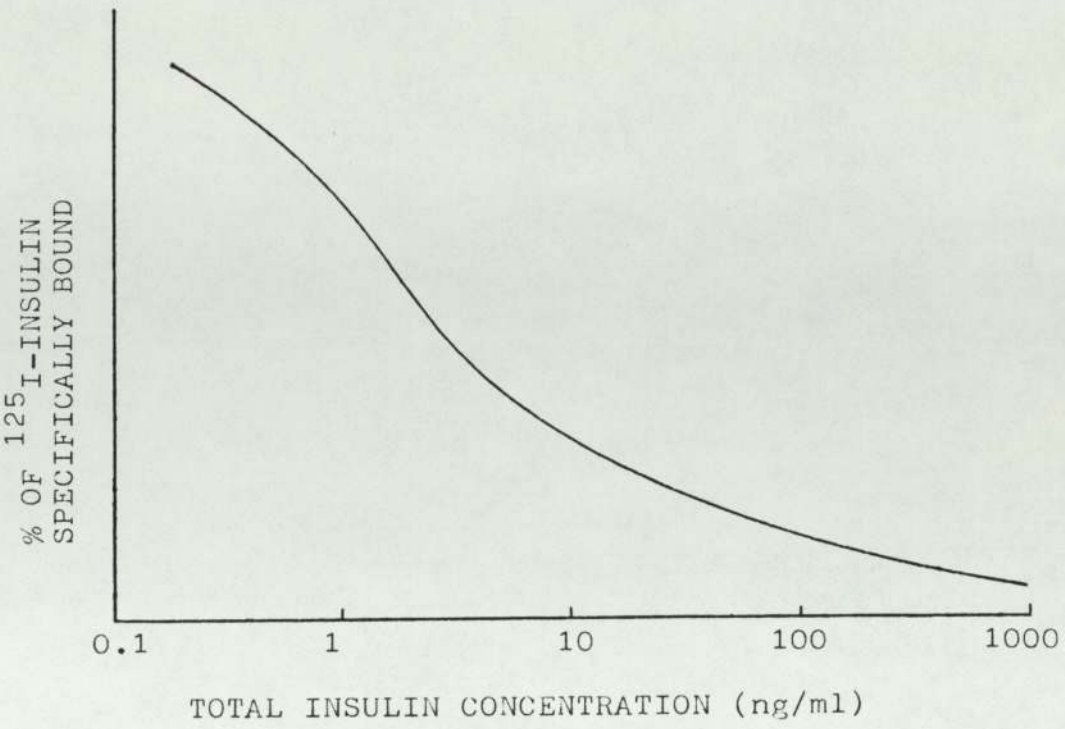

Figure 5. Competition or displacement binding curve of ^{125}I-insulin binding to a receptor population. The percentage of total ^{125}I-insulin bound is plotted as a function of the total insulin concentration.

Affinities of the ligand for the receptor are given by;

$$K_A = \frac{Ka}{Kd} = \frac{[HR]}{[H][R]} \qquad K_D = \frac{Kd}{Ka} = \frac{1}{K_A}$$

where :- K_A is the equilibrium association or affinity constant (M^{-1})

K_D is the dissociation constant (M)

It follows that the total ligand concentration ($[Ho]$) and total receptor site concentration ($[Ro]$) are given by the expressions :-

$$[Ho] = [HR] + [H] \quad \text{and} \quad [Ro] = [HR] + [R]$$

The fraction of receptors occupied ($\bar{Y}$) is thus given by $[HR]/[Ro]$ and varies from 0 to 1.0. Hence the fraction of free receptors is equal to $[R]/[Ro]$ or $(1-\bar{Y})$.

$$\text{Since} \quad (1-\bar{Y}) = \frac{[R]}{[Ro]} \qquad [R] = (1-\bar{Y})[Ro]$$

$$\text{and} \quad K_A = Ka[H][R]$$

$$\text{then} \quad K_A = Ka[H](1-\bar{Y})[Ro]$$

$$\text{Similarly} \quad K_D = Kd[HR]$$

$$\text{and} \quad K_D = Kd\ \bar{Y}[Ro]$$

At equilibrium, association and dissociation occur at equal rates, therefore :-

$$Kd\ \bar{Y}[Ro] = Ka\ [H]\ (1-\bar{Y})[Ro]$$

Dividing through by $[Ro]$ and $\bar{Y}$ gives :-

$$\bar{Y} = \frac{Ka\,[H]}{Kd + Ka[H]}$$

Dividing through by Ka :-

$$\bar{Y} = \frac{[H]}{Kd/Ka + [H]}$$

and as

$$K_D = \frac{Kd}{Ka}$$

$$\bar{Y} = \frac{[H]}{K_D + [H]} \qquad (1)$$

Dividing through by K_D gives :-

$$\bar{Y} = \frac{[H] \;/\; K_D}{1+([H]/K_D)}$$

and as,

$$K_A = 1/K_D$$

$$\bar{Y} = \frac{K_A\,[H]}{1 + K_A\,[H]} \qquad (2)$$

Equations (1) and (2) are the binding equivalents of the classical Michaelis-Menten equation for the description of enzyme kinetics (200).

A plot of binding site fractional occupancy ($\bar{Y}$) as a

function of total ligand concentration produces a saturation plot or binding isotherm. In practice, however, saturation plots are generally obtained by plotting the concentration of bound hormone $[HR]$, against the concentration of free $[H]$ (ie B vs F). Such a plot is a rectangular hyperbola passing through the origin (Figure 6). The maximum binding capacity (Ro) may be determined from the horizontal asymptote ($\bar{Y} = 1.0$), as $[HR] = Ro$. The dissociation constant, K_D, may also be evaluated as $[HR] = Ro/2$, $[H] = K_D$.

However, saturation plots allow only the approximation of Ro at excess insulin concentrations. Thus to enable more precise determination of receptor affinities and binding capacities, various methods have been described to linearise binding function plots, namely; Lineweaver-Burk plots (201), Hill plots (202) and Scatchard plots (203,204).

1.6.3.iii. Scatchard analysis.

Lineweaver-Burk and Hill plots have been used very little for the analysis of insulin-receptor interactions and undoubtebly the most popular form of analysis over the past decade has been that described by Scatchard (203,204).

$$\text{Since } \bar{Y} = \frac{[HR]}{[Ro]} = \frac{[H]}{K_D+[H]}$$

$$\text{Then } [HR](K_D+[H]) = [H][Ro]$$

Developing:-

$$[HR]K_D = -[HR][H] + [H][Ro]$$

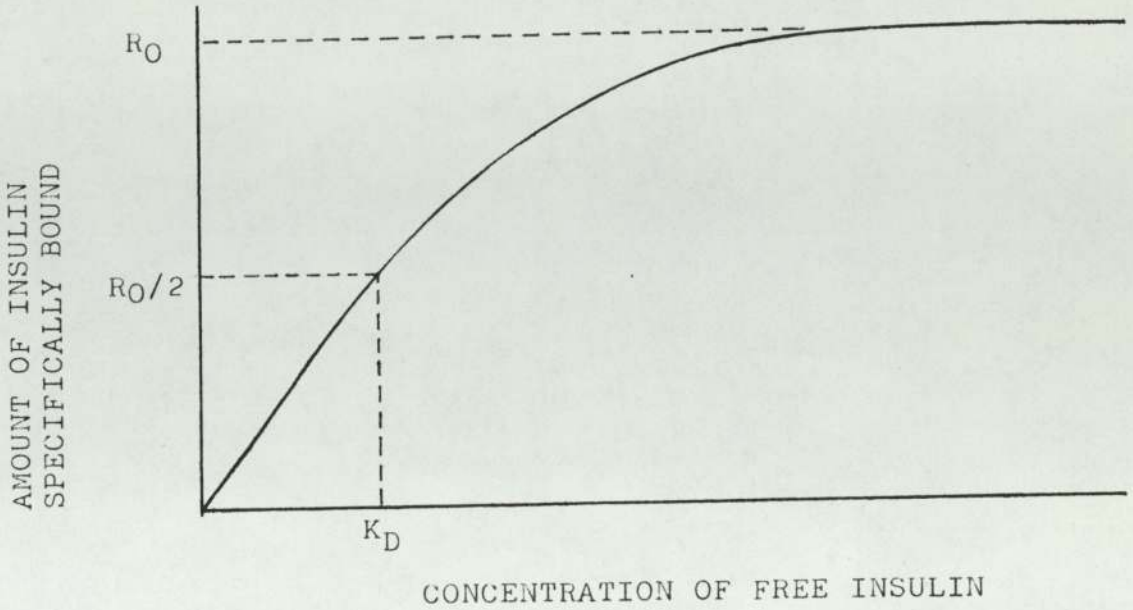

Figure 6. Saturation plot of specific insulin binding to a receptor population, revealing maximum binding capacity (R_O) and the dissociation constant (K_D).

Dividing through by $[H]$ and K_D:-

$$\frac{[HR]}{[H]} = -\frac{1}{K_D}[HR] + \frac{[Ro]}{K_D} \qquad (3)$$

Thus mathematical transformation of the law of mass action produces an equation for a straight line (eqn.3) of general form y= ax + b. A plot of $[HR]/[H]$ versus $[HR]$(in practice B/F vs B) should produce a straight line of slope $-K_A$, ordinate intercept $[Ro]/K_D$ and an abcissa intercept $[Ro]$ (193).

The analysis of certain hormone-receptor steady state binding data by the method of Scatchard does indeed yield linear plots, for example human growth hormone binding to cultured human lymphocytes (IM-9) (204). However, analysis of insulin-receptor binding data generally produces a curvilinear plot with an upward concavity (Figure 7). Such curvilinearity has been suggested to be a product of technical artifacts including errors in separation of bound and free hormone (206) or differences in the binding affinities of labelled and unlabelled insulin (207).

It must be noted that Scatchard analysis and other methods of linearising binding data may result in a loss of information, distort the weight given to individual points, present difficulties in the distinction of non-specific binding and be subject to insensitivity at high ligand or receptor concentrations (196,208). It is apparent, however, that no one method of binding data analysis is ideal (193,194)

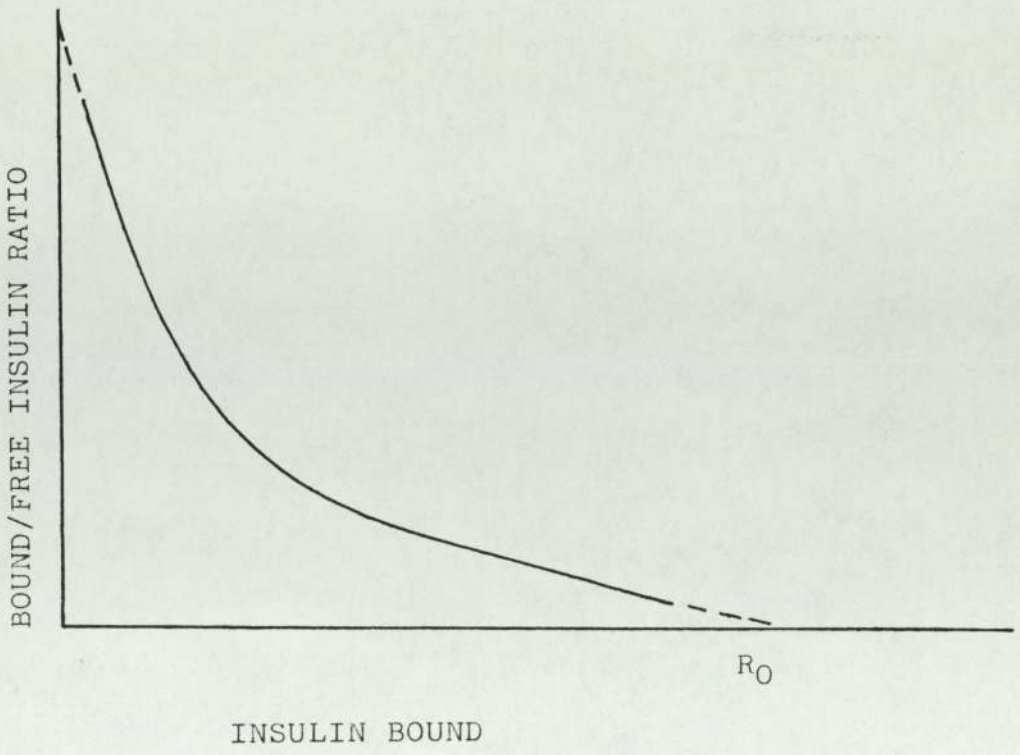

Figure 7. A typical curvilinear Scatchard plot for insulin binding to a receptor population. The bound/free ratio of ^{125}I-insulin is plotted as a function of the bound insulin after the non-specific binding of ^{125}I-insulin has been subtracted. R_0, derived by extrapolation of the curve to the abcissa intercept, represents the maximum binding capacity of the receptor population.

and although further criticism has recently been raised against Scatchard analysis (209), its use has also recieved renewed support (210). Thus, provided one is aware that Scatchard analysis is not without its critics and limitations, at present, it provides a most suitable method of analysing binding and allowing quantitative assessment of binding parameters.

1.6.3.iv. The interpretation of curvilinear Scatchard plots.

Currently the curvilinearity of Scatchard plots of insulin binding data are thought not to be due to technical artifacts but to represent particular properties of the insulin-receptor interaction. Interpretation can be based on a receptor population heterogeneous with respect to affinity (204,211-213). The minimum model which best fits such data consists of two classes of binding site with differing but fixed affinities (213,214). The affinity constant of the low affinity binding site is given by the shallow part of the curvilinear plot and the abcissa intercept of this line (Ro) (found by extrapolation) represents the maximum binding capacity of the system (Figure 8). Thus the total number of binding sites per cell (or by calculation binding sites/unit area of membrane) can be estimated, as follows

$$\text{Number of sites/cell} = \frac{\text{Ro (moles)} \times 6.022 \times 10^{23}}{\text{number of cells/litre}}$$

where 6.022×10^{23} is the number of molecules in one mole

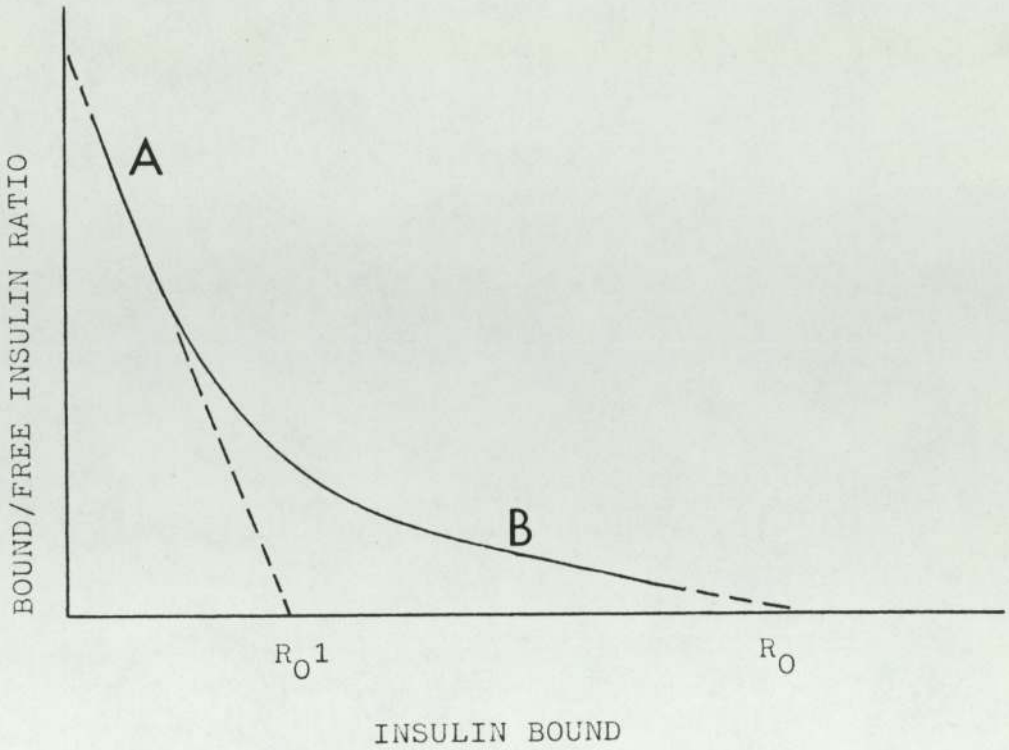

Figure 8. Interpretation of a curvilinear Scatchard plot revealing two classes of insulin receptors with differing but fixed affinities. The steeper portion of the curve (A) represents receptors of high affinity (given by the slope of A) and low binding capacity (given by R_01). The less steep portion of the curve (B) reveals receptors of low affinity (given by the slope of B) but high binding capacity. R_0 represents the combined binding capacity of both sets of receptors.

(Avagadro's number),(193). Similarly the high affinity constant and binding capacity of the high affinity receptors may be estimated from the slope and intercept respectively of the steeper portion of the plot (Figure 8). It has been emphasized that for the precise quantification of these parameters the slope of the low-affinity site must be first subtracted from the curve (192,213,215) although accurate fitting is now possible by iterative computer analysis (216).

As an alternative to the 'two site model' it has been argued that the curvilinearity of Scatchard plots is not due to heterogeneous receptor populations but due rather to a single population of receptors exhibiting negative cooperativity (217). This implies that as the amount of bound insulin increases, the average affinity of the remaining receptor population decreases. Individual empty binding sites may exist in one of two interconvertable states, high affinity (Ke) or low affinity (Kf). It may be that due to site-site interactions the affinity of empty binding sites will be determined by the occupancy of binding sites within close proximity, but the precise nature of this phenomenon is unclear.

The abcissa intercept of a Scatchard plot based on the negative cooperativity interpretation of the curvilinearity is indicative of the total insulin binding capacity of the receptor population (Figure 9). The total number of binding sites can therefore be calculated as previously described. Furthermore, the average affinity ($\bar{K}$)

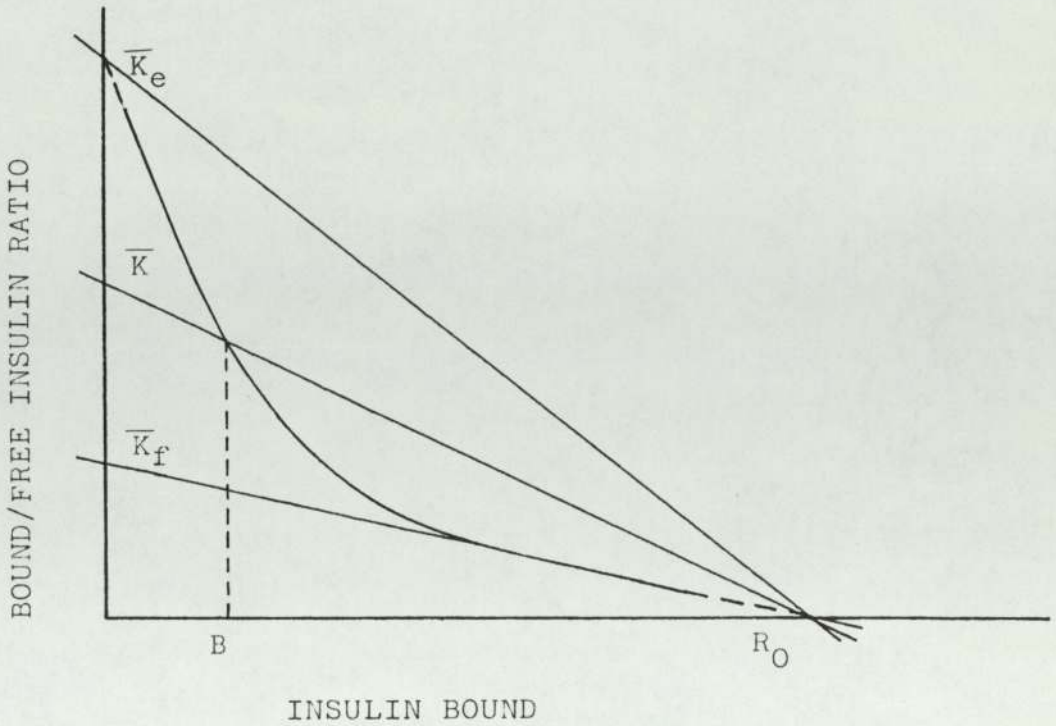

Figure 9. Interpretation of a curvilinear Scatchard plot suggesting negatively cooperative interactions between insulin receptors. Thus, the average affinity of the remaining receptors decreases as the number of occupied receptors decreases - for full explanation see text (page 47). $\overline{K}e$= maximum average affinity, $\overline{K}f$= minimum average affinity, $\overline{K}$= average affinity of unoccupied receptors when the amount of insulin bound = B, R_O= maximum binding capacity of receptor population.

of the unoccupied receptor population for any amount of bound insulin will be given by the slope of the line through the curve at that point and passing through the abcissa intercept (Figure 9) and $\bar{K}$ is therefore mathematically equivalent to (B/F)/Ro-B. Thus the average affinity ($\bar{K}$) of the unoccupied receptors may vary from a maximum affinity ($\bar{K}_e$) when the majority of receptor sites are empty to a minimum affinity ($\bar{K}_f$) when the majority of receptor sites are full. If receptor affinities do indeed alter according to the fractional occupancy of the receptor population the significance of traditional affinity constants for example K_A and K_D, is somewhat obscure and $\bar{K}$ represents their logical successor.

Aligned to the concept of negative cooperativity De Meyts and Roth (218) proposed analysis of binding parameters obtained from Scatchard plots in terms of an average affinity profile (Figure 10). This permits rapid visual determination of the average affinity ($\bar{K}$) of unoccupied insulin receptors for any given fractional occupancy of receptors ($\bar{Y}$). The average affinity profile gives therefore not only the maximum and minimum average affinities ($\bar{K}_e$ and $\bar{K}_f$ respectively) but also the fractional occupancy of receptors at which both states exist ($\bar{Y}_e$ and $\bar{Y}_f$ respectively).

It would, however, be unjust to leave the reader with the impression that the interpretation of curvilinear Scatchard plots suggesting negatively cooperative inter-

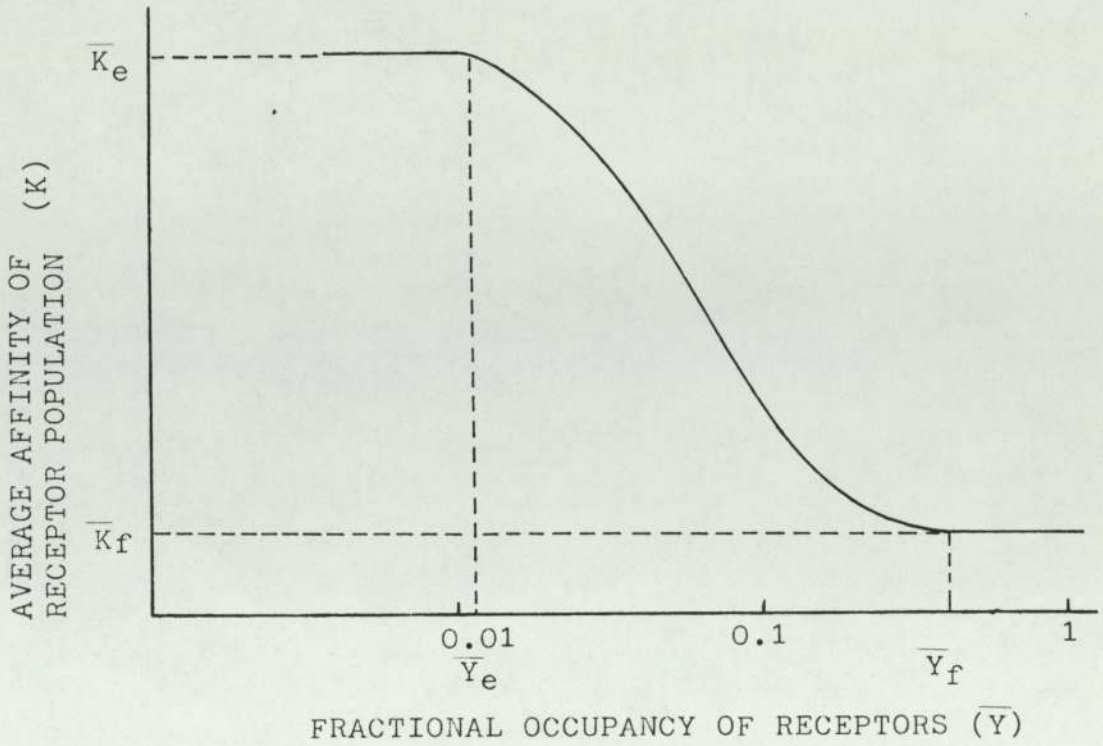

Figure 10. Average affinity profile of ^{125}I-insulin binding to an insulin receptor population. The plot shows both the maximum and minimum average affinities of unoccupied receptors, $\overline{K}_e$ and $\overline{K}_f$ respectively, and the fractional occupancy of receptors ($\overline{Y}$) at which both states exist.

actions between insulin binding sites has gone unchallenged.

Several authors, have indeed removed the need for such an interpretation of curvilinear Scatchard plots by considering only the initial steeper portion of the curve, the remaining binding component being non-specific, at least in a biological sense (175,219). In such an interpretation, the binding of insulin is to a homogeneous class of non-interacting receptors. This approach is, however, questionable for a number of reasons (193). It has also been reported (220) that insulin binding to adipocytes is best explained by a model consisting of functionally heterogeneous sites, one site of low affinity and high capacity which shows negative cooperativity but does not degrade insulin and another site of high affinity and low capacity not showing negative cooperativity but mediating insulin degradation.

Recently, new evidence has been presented in favour of a two receptor site model (221). It was shown that the binding profile of ^{125}I-insulin to human erythrocytes fitted a two site model. More importantly, the use of concanavalin A which competes for insulin binding sites linearised Scatchard plots of ^{125}I-insulin binding due to the preferential binding of concanavalin A to the high affinity site. However, the erythrocyte population is not a homogenous cell population, and it is possible that the high and low affinity receptors may reside in different cell types. Furthermore, it has been reported that concanavalin A and insulin may interact differently with the insulin receptor

as tryptic digestion decreases the ability of insulin but not concanavalin A to stimulate glucose oxidation in adipocytes and glucose transport in both fat and liver cells (222,223). Thus, in line with the model proposing negative cooperativity between insulin binding sites, the apparent blocking of the high affinity component of insulin binding by concanavalin A may be simply reflective of affinity changes due to receptor conformational alterations brought about by concanavalin A binding. Indeed it has also been argued that the ability of concanavalin A to block insulin binding may only be due to the close association of the two types of receptors and the large size of concanavalin A, its charge, or induced conformational changes in the glycocalyx (224).

Further evidence which does not comply with a cooperative model arises from studies of insulin degradation. Intact hepatocytes degrade ^{125}I-insulin into fragments which show lowered binding, and if this degradation is taken into account, a linear Scatchard plot results (225). However, these findings cannot be applied either to purified membranes or IM-9 lymphocytes which do not degrade insulin but produce curvilinear Scatchard plots (226,227). Also, insulin degradation is reduced at low temperatures (228) and by the addition of protease inhibitors, and under both conditions the resultant Scatchard plots of insulin-receptor binding are curvilinear.

Clear distinction of the most suitable model for the

description of insulin-receptor interactions is not possible by means currently available for the analysis of steady state binding data. Many studies have therefore been carried out on the kinetics of insulin binding in an effort to establish more precisely the nature of the interaction.

1.6.4. Kinetic studies of insulin-receptor interactions.

Negative cooperativity suggests that with increasing fractional occupancy of the receptor population there is a decrease in the apparent affinity of the remaining unoccupied receptors. This concept also suggests that the binding of hormone to receptors may destabilize the binding of already bound hormone. Such an effect is commonly due to site-site interactions (193) classically explained by ligand-induced 'sequential' conformational changes (229). Kinetic studies considering the rate of receptor bound hormone dissociation in the presence and absence of excess unbound hormone should therefore allow detection of such cooperative interactions. Indeed the dissociation of receptor bound ^{125}I-insulin is enhanced in the presence of excess cold insulin after 'infinite' dilution of the receptor population compared to dilution alone. Such evidence is therefore further suggestive of negative cooperativity between insulin receptors (217).

It must, however, be emphasized that enhanced dissociation of ^{125}I-insulin in the presence of excess unlabelled insulin after dilution of the receptor population, is not unambiguous proof of negative cooperativity. Such enhanced

dissociation has for example been shown in systems yielding linear Scatchard plots (226), in systems using biologically inert substances in place of receptor populations (230) and has also been reported to be completely independent of binding site occupancy (231).

It has also been argued that although negative cooperative interactions may occur, dimerization of the insulin molecule itself is responsible (230,232). Thus, non-dimerizing insulin analogues failed to show enhanced dissociation in kinetic studies, but it has subsequently been argued that alterations in the structures of the insulin analogues affect their ability to induce negative cooperative effects between binding sites. Indeed, the last 8 residues of the B chain and the A21 residue of the insulin molecule appear to be especially important in the induction of negative cooperativity (233), while the receptor binding region itself is located predominantly at A1,A5,A19,A21,B12,B16,B24,B25 and B26 of the insulin molecule (234).

The physiological significance of negative cooperativity between insulin receptors has also been questioned due to the apparent lack of the ability of insulin to enhance the dissociation of bound ^{125}I-insulin at physiological temperatures (219,235). Such arguments have, however, been countered by De Meyts (236) the initial and chief proponent of negative cooperativity. De Meyts suggested that the fractional occupancy of receptors required to induce negative cooperativity is temperature sensitive and at

higher temperatures enhanced dissociation is induced by the tracer hormone alone. Furthermore, it is apparent that on a physiological basis, negative cooperativity would provide a rapid buffering effect in conditions of hyperinsulinaemia while maintaining sensitivity of the cell at low insulin concentrations.

At present, therefore, the cooperative model provides a useful means of interpreting insulin binding data, and accepts considerable support from experimental and theoretical sources (226,237,238,239).

1.7. Insulin receptor structure.

Recent advances in affinity labelling and affinity purification techniques have contributed immensely to the identification of the insulin receptor, and to an understanding of its subunit composition (240-242). Specific immunoprecipitation of insulin receptors using anti-insulin receptor antibodies has also provided valuable information (243,244). The insulin receptor appears to comprise of a minimum of four glycoprotein subunits (Figure 11). These subunits are linked by disulphide bonds to form a large globular complex with a molecular radius of about 7nm, and an apparent Mr of 300-350K (245,246). There are two larger subunits (α), which are linked together by one or more disulphide bonds (class I S-S bonds), and two smaller subunits (β) which are disulphide linked (class II S-S bonds) to the α-subunits. This subunit composition is designated the $(\alpha\beta)_2$ complex. There may also be further subunits which

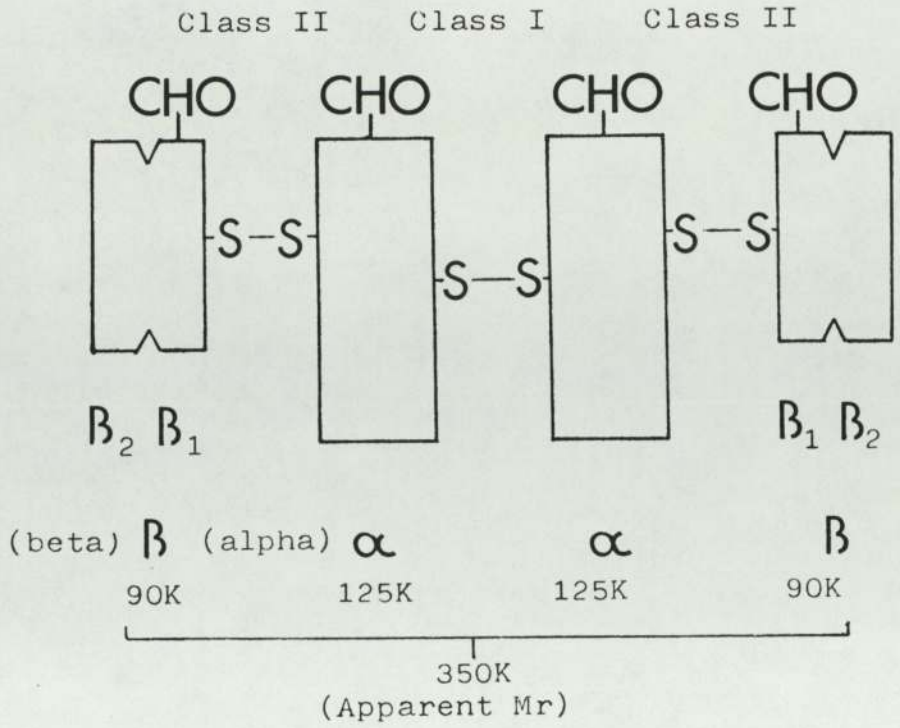

Figure 11. Representation of the subunit organisation of the insulin receptor as described by Czech, Massague and Pilch (242). The two larger subunits (α) are linked together by one or more disulphide bonds (class I S-S bonds), and the two smaller subunits (β) are disulphide linked (class II S-S bonds) to the α- subunits. The apparent Mr of the receptor complex assessed by polyacrylamide gel electrophoresis (SDS-PAGE) is lower than expected from the subunit composition. This may result partly because interchain disulphide bonds maintain a compact structure during SDS-PAGE purification.

are not covalently linked to the complex. Oligosaccharide side chains appear to be attached to both the α and β subunits (247). Indeed, a sialylated glycosidic moiety has been shown to participate in the interaction with insulin , involving D-galactose, N-acetyl-D-glucosamine and D-mannose residues (248). Although the exact location of the insulin binding sites on the receptor complex has not been precisely established, recent evidence suggests that the α subunit may be the predominant insulin binding fraction while the β subunit may have a kinase activity responsible for the mediation of insulin action (150).

Several investigators have identified an active receptor moiety with a molecular radius of about 4nm, suggesting that the high molecular weight receptor complex (Mr 300-350K) may comprise two active components (238,249,250). Consistent with these observations, selective reduction of class I S-S bonds dissociates the $(\alpha\beta)_2$ receptor complex into two symetrical $(\alpha\beta)$ halves, each of which will continue to bind insulin (242). Proteolytic fragmentation of the β-subunits of the $(\alpha\beta)_2$ receptor complex yields two additional forms of the insulin receptor, $(\alpha\beta)(\alpha\beta_1)$ and $(\alpha\beta_1)_2$, as shown in Figure 12. (242,246). Partially purified membranes from several insulin sensitive tissues have been shown to contain all three receptor forms: $(\alpha\beta)_2$, $(\alpha\beta)(\alpha\beta_1)$ and $(\alpha\beta_1)_2$ (242). Thus, different forms of the insulin receptor may exist on the same cell type, and this may account in part for some discrepancy over the size of insulin receptor subunits

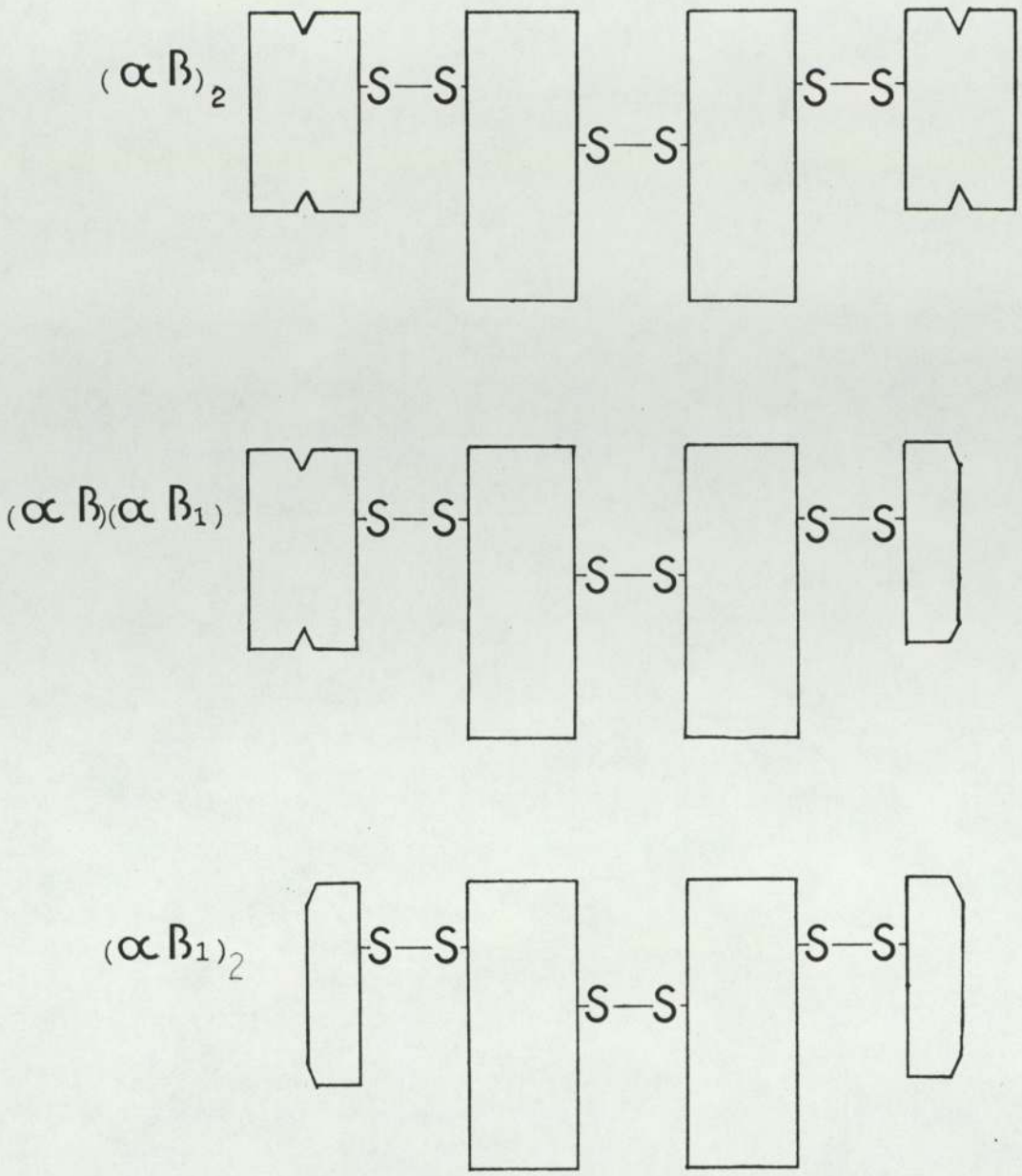

Figure 12. Proteolytic fragmentation of the $(\alpha\beta)_2$ insulin receptor complex into two additional forms of the insulin receptor, $(\alpha\beta)$ $(\alpha\beta_1)$ and $(\alpha\beta_1)_2$, as described by Massague, Pilch and Czech (246).

(244). Recent evidence suggests that different cell types may exhibit variations in insulin receptor structure (251). Estimates of the number of insulin receptor sites suggest considerable variations between cell types. For example, expressed per μm^2 of plasma membrane, estimates range from 14 sites in human erythrocytes, to 24 in cultured human lymphocytes, and 63 in rat hepatocytes (182).

1.8. Insulin receptor internalization, degradation and biosynthesis.

The following section is given over to a consideration of the cellular turnover of insulin receptors. This, it is hoped, will allow such concepts as negative cooperativity and down regulation to be explained in molecular terms and permit the presentation of mechanisms for the pathophysiological regulation of insulin receptor status (ie. receptor number and/or insulin binding affinity).

Specific insulin binding has been demonstrated and characterised in all its principal target tissues, namely fat, muscle and liver, and additionally in many tissues that are traditionally not considered to be bona fida insulin target tissues (252). Autoradiographic studies have shown that ^{125}I-insulin associates initially with the plasma membrane of cells of these tissues (253,254). In addition, the use of photoreactive ^{125}I-insulin analogues which can be covalently linked to the insulin receptor, have indicated that the insulin binding is localized at specific areas of the cell surface (224,255). This receptor aggregation appears to be a dynamic process mediated by the binding of

insulin to diffusely located receptors (256). However, aggregation may not be limited to occupied receptors, and the movement through the fluid mosaic of the plasma membrane, of both occupied and unoccupied insulin receptors, may induce binding site-binding site interactions resulting in a decreased binding affinity of the empty sites (239,257). Apart from this mechanistic explanation of negative cooperativity it has also been proposed that changes in the tertiary and/or quaternary structure of oligomeric receptors and the reversible repositioning and/or interaction of receptor subunits may be responsible for reductions in the insulin binding affinity of unoccupied receptors (236,258, 259).

Subsequent to the binding of insulin to its receptor, aggregates of receptors are taken into the cell ('internalization'). The majority of evidence for receptor internalization comes from the autoradiographic detection of photoreactive ^{125}I- insulin analogues covalently linked to receptors. Such studies reveal that insulin-receptor complexes are internalized by the invagination and pinching off of receptor coated fragments of plasma membrane, a process described as 'adsorptive endocytosis' (260,261).

Although it has been suggested that insulin-receptor complexes internalized into endocytotic vesicles associate immediately with lysosomes thus producing secondary lysosomes (256,262), it has also been suggested that endocytotic vesicles fuse initially with Golgi vesicles with

the subsequent conversion of the Golgi vesicles, or transfer of insulin-receptor complexes, to lysosomal type structures (263,264), (Figure13). The fate of these internalized complexes is not entirely clear, although the lysosomal degradation of the receptor appears to represent the end point of the binding site's functional role.

It is apparent, however, that at least in insulin target tissues,all of the internalized receptors are not degraded and consequently many are recycled and reinstated into the plasma membrane (265,266) possibly by an exocytotic-like mechanism, (Figure 13).

New receptors are synthesized on components of the endoplasmic reticulum, and, after Golgi processing are inserted into the plasma membrane, possibly as insulin receptor precursors (267). This transfer and insertion of receptors to the plasma membrane is likely to be an active process requiring microtubule involvement (268), (Figure 13).

Autoradiographic studies have shown that labelled insulin binds not only to the plasma membrane (253,254) but also to Golgi fractions (269) and the endoplasmic reticulum (257). It is unclear whether such binding serves to mediate specific biological functions or merely represents the insulin receptor in various stages of its cellular processing. Furthermore, insulin binding sites have been located on the nuclear envelopes of insulin target cells in some (270) but not all (271) studies. If present, their role,

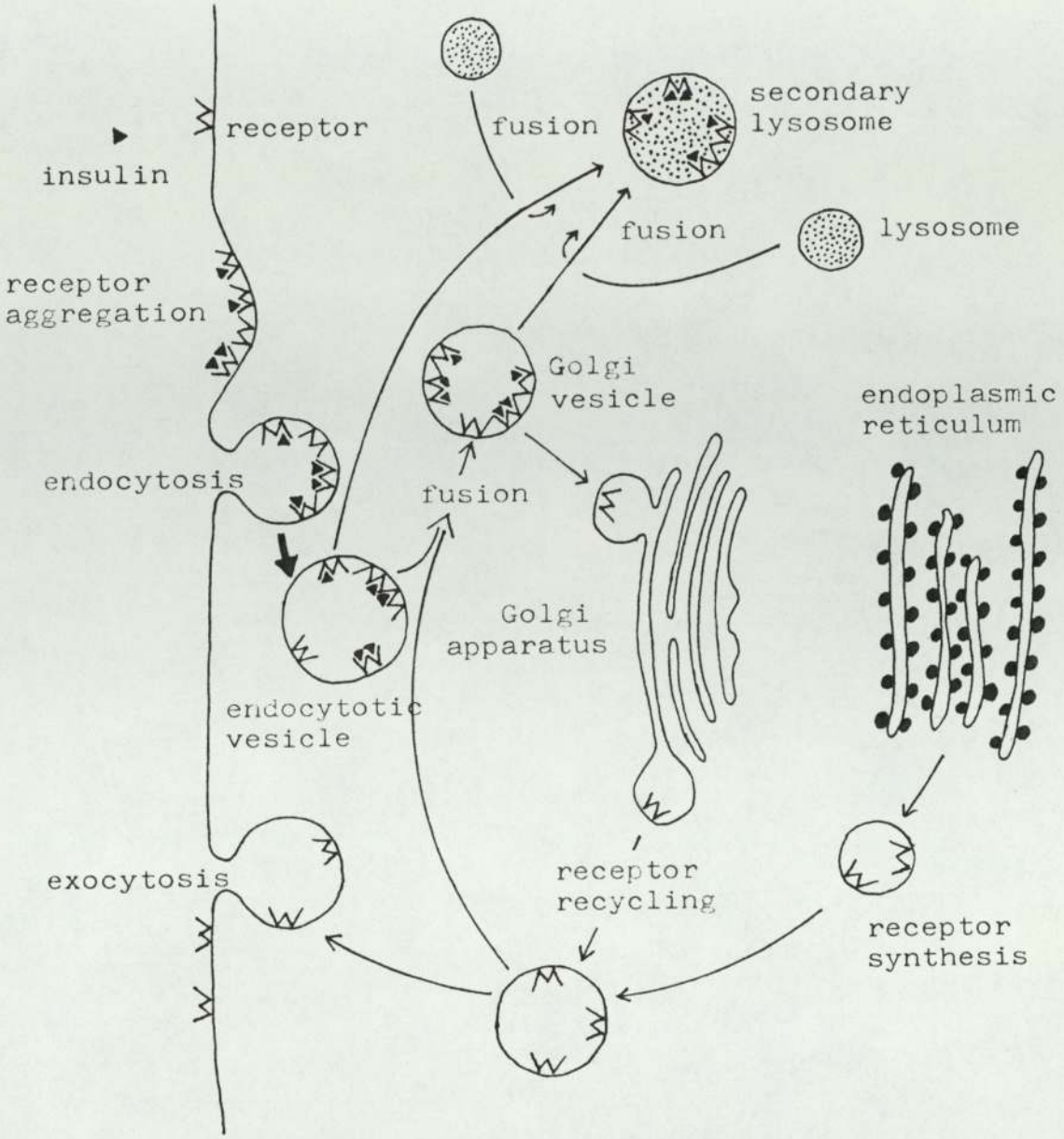

Figure 13. Generalised scheme showing the mechanisms underlying the cellular turnover of insulin receptors. For full explanation see text.

especially in a scheme of receptor metabolism is unclear although it has recently been reported that they serve to directly mediate regulatory effects of insulin on the nucleus (272).

Insulin, bound to receptors has been demonstrated to be the substrate for cell mediated insulin degradation in most (273-275), but not all (276) cells. The final lysosomal degradation of internalized insulin-receptor complexes provides for the receptor mediated degradation of the insulin molecule itself. There is, however, no general agreement as to the proportion of total insulin degradation that is mediated by this pathway and separate, distinct, protease systems have been implicated for the degradation of insulin receptors and insulin itself (277). Thus, although the lysosomal degradation of the insulin receptor appears to be the major route for receptor degradation, specific protease systems have been implicated in the degradation of the insulin molecule itself, namely glutathione-insulin transhydrogenase and insulin specific protease (275). The precise cellular locations and relative activities of these enzymes and their dependence upon insulin-receptor binding remain, however, unclear (275).

As discussed previously, alterations in insulin binding have been implicated in the induction of many insulin resistant states in Man and other species. Such alterations may be brought about by changes in either the affinity for insulin of individual binding sites or an alteration in the

concentration of sites available for insulin binding or indeed both.

Besides the rapid alteration in receptor affinities (negative cooperativity) proposed to account for the curvilinearity of Scatchard plots, other relatively short-term (hours) and rapid (minutes-hours) alterations in receptor affinities have been reported for both monocytes (278,279) and erythrocytes (280). In certain cases (278) a serum component(s) has been implicated in the induction of affinity changes. Whether this serum factor is insulin and whether these relatively rapid alterations in receptor affinity may be mediated by factors other than insulin requires further investigation. The molecular basis for the alterations in affinities have yet to be established although mechanisms previously considered to be responsible for the induction of negative cooperativity are likely candidates.

The concentration of receptors located in the plasma membrane and available for insulin binding will also determine the cellular sensitivity to insulin. Thus insulin action will also be determined by the relative rates of the various stages involved in the cellular turnover of insulin receptors (Figure 13).

Indeed, reductions in the concentration of receptors available for insulin binding at the plasma membrane have been reported in cells exposed to insulin (179,281,282). Furthermore, it has been reported that insulin induces an

internalization of its own receptors (283,284) through an accelerated endocytosis (265). Thus, insulin induced down regulation and the fate of internalized receptors involved in this phenomenon are governed by the extent of exposure to insulin in terms of both time and concentration. For example, it has been reported that in rat hepatocytes after exposure to insulin, the ability of plasma membranes to bind insulin is reduced but the total binding capacity of the cell remains the same (283,285). These changes occur over a period of minutes and remain unaltered for 40 minutes but the initial insulin binding capacity of the plasma membrane was restored within 2-3 hours. The changes also appear to be independent of alterations in the rates of receptor synthesis and degradation and probably occur as a result of the rapid, specific and reversible translocation of insulin receptors to discrete Golgi fractions. It is, however, noteworthy that rapid apparent down regulation (insulin desensitization) induced by insulin has been reported elsewhere (286) and suggested to be due to the formation of insulin-receptor complexes which dissociate at a slow rate.

Chronic exposure of insulin receptor bearing cells to insulin, however, results in an absolute decrease in the total number of extractable receptors (287) due to their enhanced degradation (288).

Bearing in mind the two possible mechanisms for insulin induced down regulation of its own receptor illustrated

above it is therefor expedient to classify down regulation in terms of the mechanism responsible. Thus, the terms 'translocational down regulation' and 'degradative down regulation' may be useful aids in describing more precisely the mechanisms underlying specific instances of insulin receptor down regulation.

It is apparent that through negative cooperativity and other undefined mechanisms of changes in insulin receptor affinity and also alterations in the cellular turnover of insulin receptors, that insulin itself plays a major role in the regulation of insulin receptor status. It is noteworthy, however,that the proposed scheme for the cellular turnover of insulin receptors (Figure 13), and also the various mechanisms by which insulin receptor affinity may be affected, provide scope for the regulation of insulin receptor status by factors other than insulin itself.

1.9. The effects of oestrogens and progestogens on insulin receptor status.

Literature, reviewed earlier (pages 1-20), indicated a more than casual relationship between sex hormones and carbohydrate metabolism. Indeed, several of the studies reviewed indicated that the natural ovarian steriods influence carbohydrate metabolism, at least in part, by altering insulin action in peripheral target tissues (pages 15-20). On this basis, the way in which insulin mediates its biological action have been subsequently reviewed (pages 20-30) in an effort to distinguish the mechanisms

by which the ovarian steroids may regulate insulin action.

Although the ovarian sex hormones are able to induce alterations in the activities of certain key glucoregulatory enzymes in insulin target tissue (pages 17-18), the full extent of such alterations, their physiological importance and indeed whether or not they represent the primary sites of sex steroid induced alterations in insulin action, remains to be established.

The fundamental role of the insulin receptor in mediating the metabolic effects of insulin suggests that the regulation of insulin receptor status is an ideal point for control of insulin action. The apparent role of alterations in insulin binding in the aetiology of many conditions of compromized insulin action (174) further emphasize the importance of receptor status in the regulation of insulin action. For these reasons a great deal of attention has been directed in the introduction to insulin-receptor interactions. Is there, however, any evidence to suggest that the ovarian steroids are capable of affecting insulin receptor status?.

The majority of studies concerning the influence of sex steroids on insulin-receptor interactions have centred around investigations of insulin receptor status in tissues from Man and animals under conditions of naturally occurring variations in sex steroid titres.

Although androgens appear to be diabetogenic in nature information presently available precludes assessment of their effects on insulin receptor status. It is apparent, however, that insulin binding to both monocytes and erythrocytes from men remains constant when monitored over monthly periods, where as the same cells from women show cyclical changes in insulin binding capacity in parallel with their menstrual cycles. Specific insulin binding capacities are greater during the follicular phase than in the luteal phase of the cycle due to alterations in the concentrations of insulin receptor sites (289,290, 291). Thus, insulin binding capacities, at least of monocytes and erythrocytes appear to be higher under conditions of low sex steroid titres. Indeed, an inverse relationship has been reported between insulin binding to monocytes and levels of 17B-oestradiol, progesterone and 17α-hydroxyprogesterone (290). However, the use of tissues not traditionally regarded as insulin target tissues, and the questioned ability of these cells, especially erythrocytes, to respond rapidly to factors influencing insulin receptor status (182,187,188) casts doubt on the validity of generalising such results.

Several studies seeking to compare insulin binding to both erythrocytes and monocytes from men and women (292,293) have failed to account for the cyclical variations in insulin binding to cells from premenopausal women. However, a comprehensive study (290) has shown that monocytes from women in the follicular phase of the menstrual

cycle have equivalent insulin binding to monocytes from men, but erythrocytes from follicular phase women show significantly lower insulin receptor concentrations compared to the same cells from men. Although it was suggested that factors exist during the follicular phase that lower insulin binding to erythrocyte insulin receptors in women, it must be emphasized that insulin binding to erythrocytes decreases exponentially with cell age (189-191) and the possibility of an altered cell age distribution within male and female erythrocyte populations can not be excluded.

Besides these studies of insulin receptor status under conditions of naturally occurring variations in sex steroid titres, there have been a limited number of reports describing the effects on insulin receptor status of the administration of compounds having sex steroid activity. For example, the insulin receptor status of both erythrocytes and monocytes has been determined in women undergoing oral contraceptive therapy, while insulin binding has been determined in men undergoing treatment with cyproterone acetate (an anti-androgen with progestogenic and glucocorticoid activity). In addition the influence of conjugated oestrogens on insulin receptor status have been determined in men and female rats.

The use of oral contraceptives (combined oestrogenic and progestogenic compounds)has been associated with the

removal of the cyclical changes in insulin receptor status observed during the menstrual cycle. Indeed the peak of insulin binding observed during the follicular phase in both erythrocytes and monocytes was not seen in 'pill' users (274,295),and it has been suggested that insulin receptors may play a role in the glucose intolerance and insulin insensitivity often described in 'pill' users (295).

Five days or one year of cyproterone acetate administration to men, at doses used in the management of prostatic cancer, did not alter the insulin receptor status of monocytes (196). However, cyproterone acetate produced a decrease in the insulin receptor number of adipocytes from female rats (297). The reason for the apparent discrepancy between these results is unclear. It may be due to differences between either Man and rat or the tissues involved or sex differences in the species.

The long term administration of conjugated oestrogens (chiefly sulphates) to male rats (10 days) and men (1 year) has also been associated with a reduced insulin receptor concentration in adipocytes and monocytes respectively. However, short term treatment with conjugated oestrogens in rats (3 days) and men (5 days) showed no effects on insulin receptor status in these tissues (296,298).

It must be emphasized, however, that the use of

synthetic ovarian hormones in oral contraceptives and the administration of cyproterone acetate and conjugated oestrogens far from mimic physiological conditions. Also taking into account sex differences and the use of cells not traditionally regarded as insulin target tissues for the determination of insulin receptor status in Man, the physiological interpretation of the results is extremely complicated.

Studies investigating the effect of human pregnancy (a condition of high sex steroid titres) on insulin receptor status have confirmed the diabetogenic stress of the condition. These studies revealed an increase in plasma insulin in fasting (299,300), in response to a glucose challenge (294,299,301) and associated with the maintainance of blood glucose levels during insulin infusion (302). Insulin binding to both erythrocytes and monocytes of pregnant women has been reported to be equivalent to binding in the luteal phase of the menstrual cycle (294,299), suggesting a reduction in insulin receptor concentration compared to the follicular phase. Similarly, a reduction in insulin receptor numbers in monocytes from pregnant women compared to non-gravid women has been noted (300) although it is unclear which phase of the menstrual cycle the control group represented. However, similar studies have reported an increased insulin binding capacity in both erythrocytes (291,302) and monocytes (301) of pregnant women to levels in excess of those seen in the luteal phase but similar to those levels seen in the

follicular phase of the cycle (291,302). These changes were a result of an altered insulin receptor site concentration.

Where as considerable thought has been given to the effect of pregnancy on insulin binding to various tissues from experimental animals, it should be emphasized that the possible influence of the oestrous cycle on insulin binding to tissues from control animals has not yet been considered in detail. There is at present a single report that indicated that the insulin receptor status of mouse mammary epithelial cells is unaltered throughout the oestrous cycle (303). However, factors governing insulin receptor status in mammary tissue may differ from those governing other insulin target tissues. Thus, tissue and species differences in the effect of the oestrous cycle on insulin binding require further investigation.

Studies with rat adipocytes (304), rat hepatocytes (305), mouse mammary epithelial cells (303), guinea pig liver and kidney membranes (306) and hepatocytes of Yucatun miniature swine (307) have indicated an increased insulin binding capacity during pregnancy due to an increase in receptor site concentrations. It was also noted that binding site concentrations on both rat adipocytes and hepatocytes decrease to levels seen in virgin rats at parturition. Tissue differences were, however, apparent. Thus insulin binding to mammary epithelial cells of pregnant rats actually increases both at parturition (308) and after

ovariectomy (309). These alterations were attributed to a direct effect of progesterone on the epithelial cells and it was suggested that reduced insulin binding to adipocytes and hepatocytes, and increased binding to mammary epithelial cells at parturition may be responsible for the coordinated changes during the perinatal period, allowing maximum glucose uptake and metabolism for milk production (309).

However, pregnancy is not only a condition of elevated sex steroid levels. Indeed pregnancy is associated with the production of chorionic gonadotrophin, relaxin and significant increments in the plasma levels of aldosterone, cortisol, parathormone, prolactin, insulin and protein bound thyroxine (310). In the face of doubts over the suitability of erythrocytes and monocytes of Man in reflecting the insulin receptor status of traditional insulin target tissues and the alterations in so many variables, interpretation of the combined or individual effects of sex steroids is almost impossible. It is apparent, however, in both human and animal pregnancy, that gross reductions in insulin binding capacity do not occur and the well documented insulin resistance during pregnancy must be mediated at the post-receptor level. It is also significant that in the face of hyperinsulinaemia, insulin receptors are not subject to the down-regulation usually associated with elevated insulin concentrations, indicating that factors might be present during pregnancy that are capable of opposing the down-regulatory effect of insulin on its own receptor.

1.10.1. Experimental aim and design.

From the literature reviewed in the introduction it is apparent that the ovarian sex steroids regulate carbohydrate metabolism and insulin action, although the role of changes in insulin receptor status in mediating such alterations are unclear. Data presently available suggests that there may be a relationship between female sex steroid titres and insulin receptor status although interpretation of such results is extremely complicated due to the presence of many experimental variables. There is therefore considerable requirement for more controlled studies to define precisely the effects of oestrogens and progestogens both alone and in combination on insulin receptor status. Furthermore, the role of any such changes in mediating alterations in insulin action require confirmation.

The present work was therefore undertaken to define the physiological role of oestradiol and progesterone both alone and in combination, in regulating carbohydrate metabolism and insulin action. In addition the role of alterations in insulin receptor status in the mediation of any regulatory activity has been assessed. This has been determined through the correlation of alterations in insulin receptor status with changes in selected parameters of carbohydrate metabolism and insulin action (for parameters selected see pages 76 - 79).

Glucose tolerance and insulin secretion in response to

a glucose challenge alter throughout the oestrous cycle in rats (96). Glucose tolerance and insulin secretion have been shown to be greatest at oestrus and lowest at dioestrus. On this basis, in the present study, selected parameters of carbohydrate metabolism and insulin action have been determined at these two stages which are representative of high and low sex steroid titres respectively.

Although oestrogens and progestogens are produced in the adrenal cortex and androgens may be converted to oestrogens in the plasma, these routes of production are minimal. Ovariectomised mice therefore have very low circulating levels of sex steroids in contrast to the levels seen in intact mice (311). For the purpose of the present study, ovariectomised mice provide ideal models for assessment of the physiological effects of endogenous ovarian steroids on carbohydrate metabolism and insulin action.

The cyclical variations in plasma concentrations of both oestrogens and progestogens throughout the oestrous cycle precludes assessment of the effects of individual endogenous steroids on either carbohydrate metabolism or insulin action. Administration of a selected oestrogen (17 β-oestradiol) and progestogen (progesterone) both alone and in combination in gonadectomised female mice therefore provides a suitable model for the assessment of both the individual and combined effects of the ovarian steroids on carbohydrate metabolism and insulin action in mice.

It was considered that such studies of the selected parameters of carbohydrate metabolism and insulin action in mice under conditions of naturally occuring variations in sex steroid titres (during the oestrous cycle), sex steroid depletion (ovariectomy) and sex steroid replacement regimes to ovariectomised mice, would provide data to enable more precise interpretation to be made of the effects of ovarian steroids on carbohydrate metabolism, insulin action and insulin receptor status.

1.10.2 Parameters selected for the determination of carbohydrate metabolism and insulin action.

Plasma glucose concentrations and the hypoglycaemic action of exogenous intraperitoneally administered insulin have been determined in female mice with differing sex steroid titres to indicate the overall, whole body effects of the sex steroids on glucose metabolism and insulin action respectively. However, for more precise determination of the effects of the ovarian hormones on carbohydrate metabolism and insulin action selected parameters were considered in skeletal muscle, represented by the soleus muscle, and liver, two major sites responsible for producing the hypoglycaemic action of insulin through appropiate alterations in glucose uptake and metabolism. Furthermore, the liver was considered a most suitable insulin target organ for study because insulin degradation and the metabolism, transformation and degradation of most steroids occurs in hepatic tissue (11). This property ideally predisposes the liver for the mediation of the effects of sex steroids on

carbohydrate metabolism and insulin action. Thus, the glycogen contents of both muscle and liver were determined as indicators of the overall carbohydrate balance within these tissues. Insulin-receptor binding was studied in both tissues in order to establish the role played by altered insulin binding in the mediation of changes in carbohydrate metabolism. Such changes have been indicated by both tissue specific effects (glycogen contents) and inferred effects (plasma glucose concentrations and the hypoglycaemic action of exogenous insulin).

In addition, insulin sensitive aspects of carbohydrate metabolism were determined in muscle, namely the rates of $^{14}CO_2$ production and ^{14}C-glycogenesis from ^{14}C-glucose to allow direct comparison of insulin action and insulin binding in an insulin sensitive tissue. Such comparisons could not, however, be made in the liver, as although the liver is an important target organ for insulin, the biological effects of insulin on the liver are not easily measured *in vitro*. Indeed very few quantitative studies comparing insulin binding and biological effects in liver have been performed (312). Hepatic insulin receptor status was therefore compared indirectly with insulin action via a consideration of hepatic glycogen content, the hypoglycaemic action of exogenous insulin and plasma glucose concentrations (generally of freely fed animals).

The parameters selected as indicators of carbohydrate balance and metabolism are influenced by the nutritional

status of the animals. It is apparent that dietary influences regulate insulin receptor status probably through alterations in the concentration of circulating insulin (313). As the ovarian sex steroids have been reported to influence both food and fluid consumption in rodents (102, 314), food and fluid consumption was monitored during both acute (2 weeks) and chronic (10 weeks) alterations in ovarian sex steroid titres.

The qualitative and quantitative biological effects of sex steroids show a time dependency ranging from days to months (69,315,316). Furthermore, the effects of oestrogens on experimental diabetes show a biphasic, time dependent action (69). The time dependency of sex steroid actions on insulin-receptor binding were therefore considered by the determination of insulin-receptor binding after both acute (2 weeks, 3-5 oestrous cycle equivalents) and chronic (10 weeks, 17-23 oestrous cycle equivalents) ovariectomy and hormone replacement regimes.

It is apparent that the outcome of sex steroid administration may depend not only on the duration of administration but also on the type of steroid employed (natural or synthetic), the route of administration, dosage and the existing status of carbohydrate tolerance and endocrine pancreatic reserve (11). Although the current work was unable to consider all of these parameters, consideration was also given to the dose dependency of sex steroid actions, specifically the dose dependency of the effects of oestradiol

(5μg/kg body weight/day versus 500μg/kg body weight/day) on the selected parameters of carbohydrate metabolism and insulin action.

2. Materials and Methods.

2.1. Chemicals.

The following chemicals were used in the studies presented in this thesis. N-2-hydroxyethylpiperazine-N^1-ethanesulphonic acid (HEPES), N-tris hydroxymethyl methyl-2-aminoethanesulphonic acid; 2-(2-hydroxy-1,1-bis(hydroxy methyl)-ethyl aminoethanesulhonic acid) (TES), Tricine, rabbit liver glycogen - Type V, collagenase Type II and Type IV, pyruvate and bacitracin were purchased from Sigma Chemical Co. Ltd., Dorset. Bovine serum albumin Fraction V (Lot numbers 330 and 335, insulin free) was purchased from Miles Laboratories Ltd., Slough. Monocomponent Porcine insulin (Lot number S8311160) was purchased from Novo Industria A/S, Copenhagen, Denmark. Na ^{125}I, U-^{14}C-glucose were purchased from Amersham International, Amersham. Micellar scintillant NE260 was purchased from New England Nuclear, Aberdeen. Sephadex G50 was obtained from Pharmacia Fine Chemicals AB, Uppsala, Sweden and sodium pentobarbitone (Sagatal) was purchased from May and Baker Ltd., Dagenham. Insulin antiserum (guineapig RD12, Lot number K4454) was obtained from Wellcome Reagents Ltd., Beckenham.

All other reagents were of analytical grade, purchased from British Drug Houses Ltd., Dorset. Double distilled water was used throughout.

2.2.1. The radioiodination of insulin.

A radioactively labelled tracer must fulfill two major requirements to be of use in the study of the interaction of the hormone with its receptor. Firstly the preparation must be of high specific activity to allow the use of low (physiological) concentrations and secondly the labelled hormone must retain its full biological activity.

For such preparations, γ-emitting isotopes offer many advantages over their β-emitting counterparts. Time consuming and expensive sample preparation is not required with γ-emitting isotopes, which also offer a relatively high number of detectable counts compared to β-emitting species. For the production of hormones of high specific activity the comparative ease with which the γ-emitting isotopes of iodine, ^{125}I, ^{129}I and ^{131}I can be substituted into the tyrosine residues of proteins and peptides has made them the isotopes of choice in many preparations.

The incorporation of one radioiodine atom per molecule of protein is the minimum conceivable substitution for the production of labelled hormone. At any level of substitution there may be problems of reduced biological activity and immunoreactivity particularly if the labelled residues are closely associated with the binding region(s) of the hormone. The probability of hormone damage due to radiation also increases. An absence of di-iodoinsulin is also essential in labelled insulin preparations, particularly

those to be used in receptor studies, due to a much reduced binding affinity towards these molecules and the possible production of monoiodinated products of unknown biological fate (317). Theoretically, monoiodoinsulin labelled with ^{131}I would be of a higher specific activity than the equivalent ^{125}I preparation. However, due to the relatively low isotopic abundance of ^{131}I (30%) compared to ^{125}I (virtually 100%) the latter produces a high specific activity tracer and is therefore used preferentially. Other factors favouring this choice of isotope include the longer ½ life of ^{125}I compared to ^{131}I (60 vs. 8 days) and the greater counting efficiency in most γ counters of ^{125}I decay products.

Several procedures for protein iodination have been developed during the last 20 years, which essentially involve the oxidation of ^{125}I or ^{131}I in the presence of the protein. The radioiodide is incorporated principally into the tyrosine residues although some iodine may also react with histidine, tryptophan or sulphydryl groups (318). For the production of labelled hormones of high specific activity without the use of large amounts of radioiodide small quantities of protein have to be used. Methods developed as a consequence of this include the use of iodine monochloride , chemical oxidants and electrolytic and enzymatic iodination although no one method has proved to be completely satisfactory and reliable.

Procedures employed today in most laboratories involve the use of the chemical oxidant chloramine-T or the

enzymatic method using lactoperoxidase. Both methods are relatively easily carried out, produce very little diiodo-insulin and at the level of substitution routinely achieved approximately 80% of the radioactivity is associated with the A14 tyrosine of the insulin molecule. The remaining activity is accounted for chiefly by the iodination of A19 tyrosine with very little iodination of the B16 and B26 tyrosines (319). Preferential labelling of the A14 tyrosine has important implications to receptor studies as this mono-iodoinsulin has a binding affinity and biological potency indistinguishable from that of cold insulin while both these characteristics are reduced to approximately 50% for A19 monoiodoinsulin (320).

The extent of A19 labelling is dependent on the percentage incorporation of ^{125}I into insulin during the iodination procedure. Providing the incorporation from iodination to iodination is within reasonable limits the level of iodinated tyrosines other than A14 will be similar. Indeed, in the present study the level of ^{125}I incorporation was fairly consistent, being a mean of 60.2% ±1.48 SEM. However, to ensure a pure A14 labelled hormone, techniques are now available for the rapid separation from other labelled products by purification of the labelled product on DEAE cellulose, HPLC (321) or polyacrylamide gel electrophoresis (322).

The chloramine-T method used initially by Hunter and Greenwood (323) for the ^{131}I-labelling of human growth

hormone has been developed for the routine laboratory production of ^{125}I-monoiodinated insulin of suitable purity, integrity and specific activity in sufficient quantities for use as a tracer (176,177). This technically simple and rapidly performed technique has been carried out routinely in the present work and is described in detail below.

2.2.2. The chloramine-T method for the iodination of insulin

Chloramine-T, the sodium salt of the N-monochloro derivative of p-toluene sulphonamide breaks down in solution to form mildly oxidizing hypochlorous acid. At pH 7.5 in the presence of chloramine-T Na ^{125}I will be oxidized forming $^{125}I^{+}$, while tyrosine molecules will only be very slightly ionized due to a pK of greater than 10 of the phenolic side chain. The iodination reaction probably proceeds through this small proportion of ionized groups, the iodine atoms being substituted in the ortho position to the hydroxyl group in the phenolic ring of tyrosine (Figure 14).

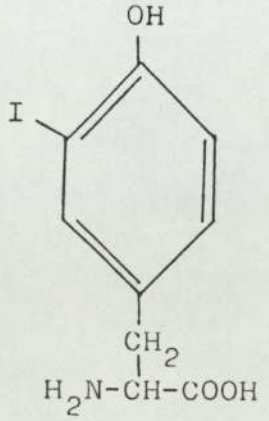

Figure 14. Monoiodotyrosine.

Although the monoiodotyrosine has a lower pK of aproximately

8.5, and thus would be ionized to a greater extent, the residue is less reactive after monosubstitution.

The level and position of substitution also depends on the microenvironments of individual tyrosine residues. Due to their position, outer residues would be iodinated more readily than inner residues and may also be important in the binding and biological activity of insulin, thus highlighting the need for reactivity testing of the products. For the production of monoiodoinsulin, labelled preferentially at the A14 tyrosine residue, the time of reaction and concentration of reactants must be strictly controlled.

2.2.3. Procedure for the radioiodination of insulin.

1mCi batches (10μl) of $Na^{125}I$ were supplied by Amersham International Limited (Amersham, Buckinghamshire) and iodinations were carried out one or two days after batch synthesis. Iodinations using label older than 3-4 days tended to yield preparations containing an increased percentage of damaged insulin (>10%). Although the reason for this is not clear, it may be due to an increase in the amount of irradiation products.

Iodinations were carried out in the standard, conical glass ampoules in which the label was supplied. All solutions were then added using automatic pipettes with disposable tips and thus uncapping and capping of the reaction vessel was required several times during the iodination procedure. Even with care, such action may produce aerosols (droplets

$<2x10^{-3}\mu Ci$) and the reactions were therefore carried out in a fume cupboard. Lead shielding was placed around the reaction vial and surgical gloves and a face mask worn as further precautions.

$Na^{125}I$ was supplied in a dilute sodium hydroxide solution (pH 7.11) and was first buffered to pH 7.4 by the addition of 100μl 0.5M phosphate buffer. Insulin was then added followed quickly by freshly prepared chloramine-T to initiate the reaction. The quantities of insulin and chloramine-T used depended on the age of the $Na^{125}I$ and are given in Table 1 . After 2-3 gentle inversions of the reaction vial in 20 seconds, freshly prepared sodium metabisulphite (20μl,10μg) was added to stop the reaction and after a further 15 seconds, incorporating one further inversion, 200μl of 0.05M phosphate buffer was added. This buffer contained 2.5% bovine serum albumin (BSA fraction V) to reduce adsorption of the reaction products to the reaction vial and the Sephadex column and to facilitate the separation of the reaction products on the Sephadex column.

The reaction quantities of insulin, chloramine-T and other reactants employed are given in Table 1 along with the reaction procedure. 10μl of $Na^{125}I$ employed for the iodination of insulin had a calculated activity of 1mCi on the given activity reference date but batches were, however, synthesized 14 days prior to this date. Thus the activity of the ^{125}I was in excess of 1mCi when used in the iodination procedures. Generally the $Na^{125}I$ preparations were used 13

Table 1. The procedure for the iodination of insulin with ^{125}I.

Reaction procedure 13 days before the ^{125}I-activity reference date.

Reactants were added in the following order;

10μl $Na^{125}I$ (1.162mCi)

100μl 0.5M phosphate buffer (8.9%Na_2HPO_4,1.404%NaH_2PO_4, pH7.4)

11.62μl insulin (2.905μg)

23.24μl chloramine-T (5.81μg)

20 seconds, two gentle inversions of reaction vial.

20μl sodium metabisulphite (10μg)

15 seconds, one gentle inversion of reaction vial.

200μl 0.05M phophate buffer (0.89%Na_2HPO_4,0.1404%NaH_2PO_4, 2.5%BSA,pH7.4).

The following alterations were made for iodinations carried out 12 days before the activity reference date;

10μl $Na^{125}I$ (1.149mCi - aproximately 1% decay since day 13).

11.49μl insulin (2.873μg)

22.98μl chloramine-T (5.745μg).

or 12 days before the activity reference date. The concentrations of reactants, specifically insulin and chloramine-T had therefore to be adjusted in accordance with the specific activity of the $Na^{125}I$ preparation (Table 1).

2.2.4. Separation of ^{125}I-monoiodoinsulin.

Immediately after the reaction a 10μl sample was removed from the rection vial and later used to assess the percentage of ^{125}I incorporation into insulin. The contents of the vial were removed, the vial washed once with 180μl of 0.05M phosphate buffer (2.5% BSA) and the combined volume transfered to a Pharmacia column (K15/30) packed with Sephadex G50 (fine) to facilitate the separation of the labelled insulin from other reaction products and free ^{125}I.

The column was preflushed with 5ml of 0.05M phosphate buffer (pH 7.4) containing 2.5% BSA to purge the column of material remaining from previous iodinations and to prevent the adsorption of the freshly prepared labelled insulin to the Sephadex and the column itself.

The mixture of reactants were eluted through the column with 0.05M phosphate buffer (pH 7.4) containing 0.5% BSA and 0.1% sodium azide (to prevent bacterial contamination of the column) at a flow rate of approximately 1ml/minute. Sixty four,1 minute fractions were collected in disposable polystyrene tubes (LP3, Luckhams Ltd.) and counted for ^{125}I-activity (3 seconds) on a Gamma counter (ICN,Tracer Lab)

with a counting efficiency of 57%.

By examination of the elution profile (activity versus fraction number) the tubes containing the maximum activity associated with insulin could be identified. This peak fell typically in the region of fractions 30-40, after the peak representing aggregated iodination products and before the peak representing free, unreacted ^{125}I (Figure 15).

Five to six fractions representing the apex of peak II (iodinated insulin) were pooled and a 10μl sample was taken for the assessment of the amount of damaged insulin present. The remainder was distributed into 50μl aliquots (typically 2μCi/tube) and stored at -20°C until required.

2.2.5. Determination of the specific activity and integrity of ^{125}I-insulin preparations.

The 10μl sample removed from the reaction vial immediately after the iodination procedure was used to assess the specific activity of the preparation. After counting the radioactivity of this sample, 0.5ml of 0.05M phosphate buffer (0.5% BSA, pH7.4) was added,followed by 0.5ml of 10% trichloroacetic acid (TCA) to precipitate the undamaged intact protein. After centrifugation (10 minutes, 840 g) the supernatant containing ^{125}I-activity, predominantly as free ^{125}I, was aspirated and the ^{125}I associated with the precipitate was determined. Thus the percentage of ^{125}I associated with intact, undamaged insulin could be calculated. Since the amount of ^{125}I and insulin in the

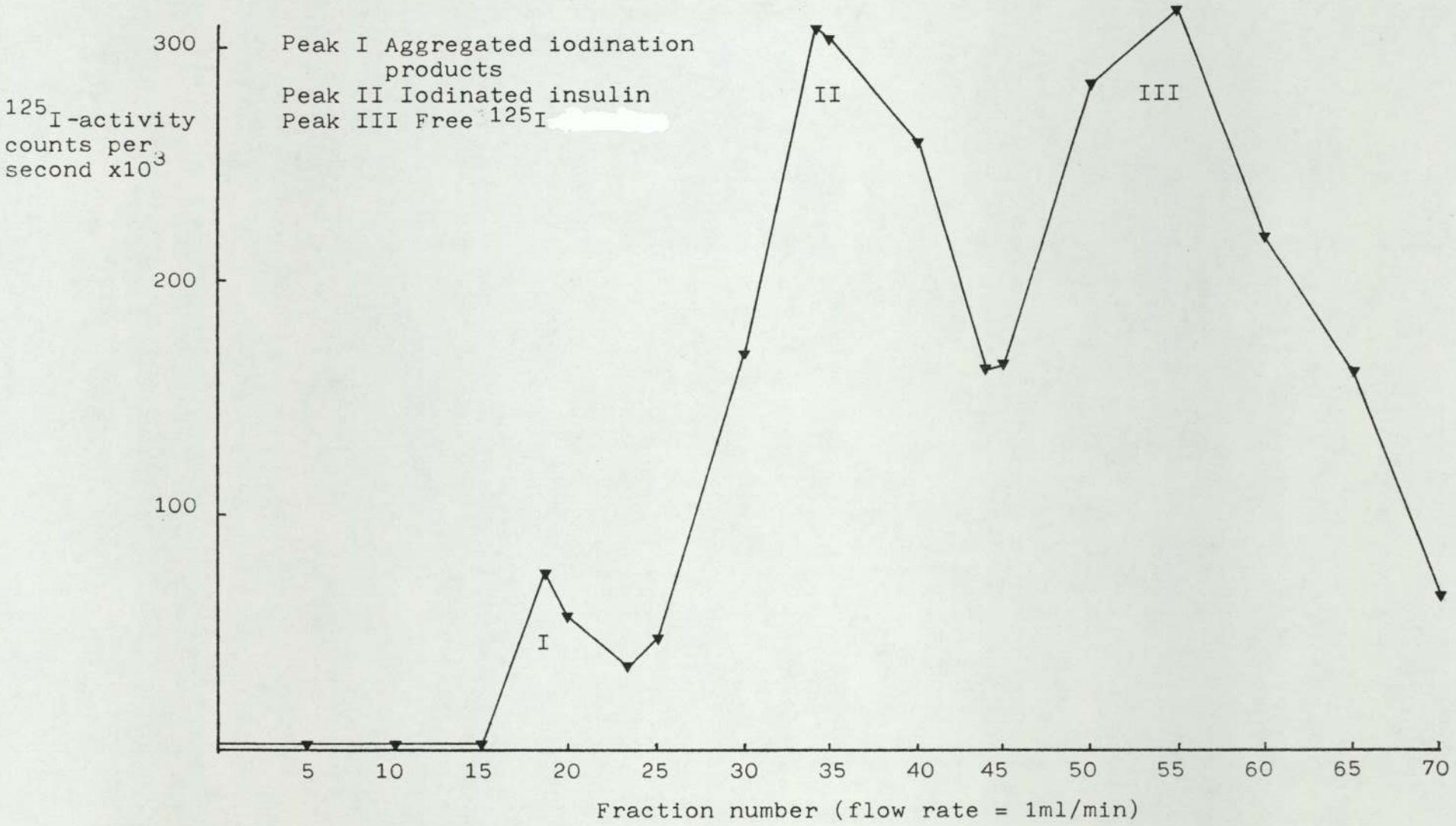

Figure 15. ^{125}I-activity profile of fractions after elution on Sephadex G50(fine) of chloramine-T iodinated insulin.

reaction and the percentage incorporation of ^{125}I into insulin were known the specific activity could be calculated from the following expression;

$$\text{Specific activity } (\mu Ci/\mu g) = \frac{\text{fraction } ^{125}I \text{ incorporated} \times ^{125}I\text{-activity (mCi)}}{\text{insulin present } (\mu g)}$$

The average incorporation of ^{125}I was 60.2 ±1.48% (mean ±SEM, n=32), yielding an average specific activity of 247.6 ±5.46μCi/μg (mean ±SEM, n=32).

In preliminary studies, damage to the radioiodinated insulin was assessed by TCA precipability, adsorption to dextran coated charcoal and binding to excess guinea pig insulin antiserum.

TCA precipitability was assessed by the addition of 0.5ml 0.05M phosphate buffer (0.5%BSA, pH7.4) and 0.5ml of 10% TCA to 10μl of the pooled ^{125}I-insulin preparation of known specific activity. After centrifugation (10 minutes, 840 g), the supernatant was aspirated and the activity of the precipitate counted.

Adsorption to charcoal was determined by the addition of 25μl of the pooled ^{125}I-insulin preparation to 400μl of 0.5M phosphate buffer (pH7.4) followed by 10mg of dextran coated charcoal in cold buffer (324). After mixing and centrifugation (10 minutes, 840 g) the supernatant

was aspirated and the precipitate ^{125}I-activity determined.

The immunological competence of the preparation was assessed by the addition of 0.5ml of 0.05M phosphate buffer containing 0.5%BSA (pH7.4) to 10μl of the pooled ^{125}I-insulin preparation. After a 48 hour incubation at 4°C in the presence of excess antiserum (1:40,000, GPBL/3), the bound and free activity were separated by centrifugation (10 minutes, 840 g) through dextran coated charcoal and the bound ^{125}I-activity determined.

The preparation was judged to be suitable for use with a TCA precipitability > 90%, charcoal binding > 90% and antiserum binding > 85%. Samples which were pooled from peak II satisfied these criteria. Subsequently TCA precipitibility was the routine method used for the assessment of damage and was usually > 95%, but never below 92%, giving an average damage of 5.16% ±0.48%,(mean ±SEM, n=32).

Freezing the ^{125}I-insulin had no apparent effect on TCA precipitability. ^{125}I-insulin preparations once thawed were never refrozen for further use. Aliquots of the ^{125}I-insulin preparations were used for both insulin radioimmunoassay and insulin receptor studies for two weeks after synthesis at which time TCA precipitability had generally increased by a further 2-3%, possibly due to self irradiation damage. The specific activity of the preparation on the day of use was calculated from the original specific activity of the ^{125}I-insulin assuming a decrease of 1% per day.

2.3. Animals

Female Theiller Original albino mice (Bantin and Kingman, Hull) were used throughout the study and were housed 6 per cage in an air conditioned room at 22 ±2°C with a 12 hour lighting schedule (09.00 to 21.00 hours light). The mice were fed a standard pellet diet (Mouse breeding diet, Haggate and Sons, Northamptom) and allowed drinking water ad libitum.

The various stages of the oestrous cycle in intact animals were determined by the vaginal smear technique (Appendix pages 218-219).

The parameters to be assayed in the present work are greatly influenced by the nutritional status of the animal. For example, insulin receptor status is greatly dependent upon the prevailing plasma insulin concentration and therefore nutritional status (313). Starving animals (for example overnight) prior to use might reduce some of the inherent variability but may also either mask or exaggerate the effect of sex hormones under normal physiological conditions. Hence animals were not starved allowing a greater understanding of the physiological roles of any observed changes. However, to minimize diurnal variation all experimental procedures were carried out on animals between 10.00am and 12.00am.

2.4. Ovariectomy of mice and administration of oestradiol and progesterone replacement regimes.

Mice were bilaterally ovariectomised under sodium

pentobarbitone anaesthesia (Sagatal, 45mg/Kg body weight, intraperitoneally), supplemented with ether when required, at approximately 7 weeks of age. The surgical procedure was based on that of D'Amour and colleagues (325). Sex steroid replacement regimes of either 17β -oestradiol (5μg/Kg/day) or progesterone (1mg/Kg/day) or the two in combination were initiated one week after ovariectomy. The hormones were dissolved in 1ml of 98% ethanol and made up to the required concentrations in the drinking water based on an intake of 20ml /100gm body weight/day. Where ovariectomised and intact animals served as controls, drinking water was supplemented with equivalent volumes of 98% ethanol. Oestradiol was also administered at a dose of 500μg/Kg/day to allow the dose dependency of sex steroid actions to be considered.

Subcutaneous injections of the above hormone concentrations (5μg/Kg/day oestradiol and 1mg/Kg/day progesterone) have been used to produce sex steroid titres equivalent to those seen in mice in oestrus (78,98,99). The conversion of a proportion of oestradiol to oestrone in the gut (326) coupled with the hepatic removal and degradation of orally administered oestradiol and progesterone reduces the estimated peripheral concentrations and are thus the probable causes of the reduced but still apparent efficacy of orally administered sex hormones noted elsewhere (327). Recent work from our laboratory has also shown extensive effects of orally administered sex steroids on a range of parameters associated with glucose metabolism in skeletal muscle (116)

and it was therefore considered that the oral route would be the most suitable to use, particulary for extended periods of sex hormone administration.

2.5. Blood sample collection for determination of plasma insulin, glucose and oestradiol concentrations. Insulin hypoglycaemia tests.

Blood samples from the cut tip of the mouse tail were milked into heparinized 200μl Beckman polyethylene microfuge tubes (Beckman Ltd., High Wycombe). After centrifugation (1 minute, Beckman Microfuge, Type B; Beckman Ltd., High Wycombe) plasma was separated and either stored at -20°C for insulin or oestradiol assay later or used immediately for glucose determination. Plasma insulin and oestradiol concentrations were determined by radioimmunoassay techniques (Appendix pages 220-226). Plasma glucose concentrations were determined by an automated glucose oxidase procedure (Beckman Glucose Analyzer 2, Beckman Ltd., High Wycombe).

The net insulin sensitivity of mice was determined by the extent of the reduction in blood glucose after intraperitoneal administration of insulin.

A dose of insulin was selected (0.35U (13.6mg)/kg) that produced a maximum reduction of approximately 50% of the initial blood glucose concentration. The mice were not allowed access to either food or water for the duration of the test. Blood samples for plasma glucose determinations were taken as described above and the results were expressed in terms of the area above the curve of plasma glucose for the one hour period following insulin administration. The initial blood glucose concentration was taken as the

reference point (Figure 16). This area, designated K_A, represented the total insulin induced plasma glucose disappearance during the 1 hour of the test (mM glucose/ hour) and is given by the formula:-

$$K_A = \frac{(a-a)+(a-e)}{2} + (a-b) + (a-c) + (a-d)$$

where a,b,c,d,e, represent blood glucose values at 0,15,30,45 and 60 minutes respectively after insulin injection (Figure 16).

2.6. The determination of food and fluid consumption.

Food and fluid consumption by cages of six mice were monitored daily for 10 consecutive days. The weights of food and fluid were recorded at 10.00am each day and consumption calculated by weight difference. Results were expressed in terms of gm/mouse/day and gm/kg body weight/day.

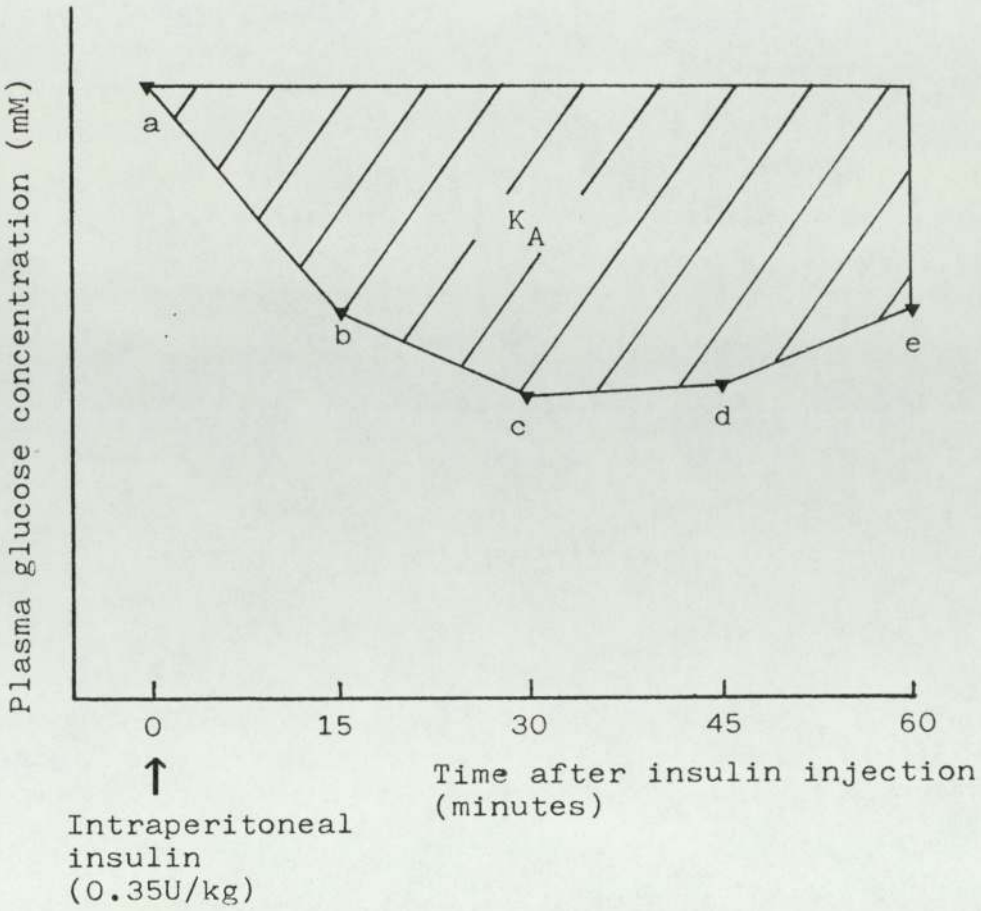

Figure 16. Diagramatical representation of K_A;- the total insulin induced plasma glucose disappearance during the 1 hour of the insulin hypoglycaemia test (mM glucose/hr.).

$$K_A = \frac{(a-a)+(a-e)}{2}+(a-b)+(a-c)+(a-d)$$

2.7. Isolation of mouse soleus muscle.

The soleus muscle in mice is a slow-twitch, red skeletal muscle composed predominantly of slow oxidative fibres (328) and the intact muscle can be isolated intact without damage from the hind limb (329). The biochemical properties of the soleus muscle have been examined (330) and the muscle has proved to be very satisfactory for *in vitro* studies on glucose uptake and metabolism.

Soleus muscle may be used to study insulin binding and a variety of post-receptor actions of insulin (331,332). It is also of significance that slow-twitch soleus muscle has been reported to represent a more important target site for the binding of insulin and the disposal of glucose than fast-twitch muscle (333). The study of insulin binding and post-receptor actions of insulin in soleus muscle is therefore very relevant to the present study.

Although only two soleus muscles are available from each animal, the two muscles provide ideal matched pairs in which one muscle serves as an experimental control for the other.

Soleus muscle excision was based on the technique of Maizels (329). The mouse was first killed by cervical dislocation and the two hind limbs removed by cutting above the knee joint. Skin and fur were removed by peeling upwards from the ankle. If not visable, the achilles tendon was exposed by peeling the remaining skin downwards towards the

foot. The limb was then secured, palm uppermost, to a cork board by inserting pins through the foot and knee joint. Using fine forceps, a ligature (4/0 capillary braided suture: Armour Pharmaceutical Co., Eastbourne) was passed around the achilles tendon and secured. The tendon was then attached closely to one end of a polyethylene horseshoe by the ligature (Figure 17a). The attachment of the achilles tendon to the ankle was then severed and the gastrocnemius and associated muscles were gently peeled back toward the knee joint thus exposing the soleus on the underside of the gastrocnemius (Figure 17b). After securely attaching a ligature around the freshly exposed upper tendon the tendon attachment to the knee joint was then cut and the soleus muscle gently separated from the gastrocnemius. The free end of the soleus muscle was next closely attached to the remaining end of the polyethylene horseshoe by the ligature (Figure 17c). The muscle was maintained under tension by the horseshoe to mimic the physiological situation.

The suture and horseshoe had been preweighed in a foil boat, and by weighing the muscle secured to the polyethylene horseshoe in the foil boat, the weight of the muscle could be determined by difference. Excess suture was cut off and the preparation was rinsed in 0.9% saline and used immediately for the experimental work.

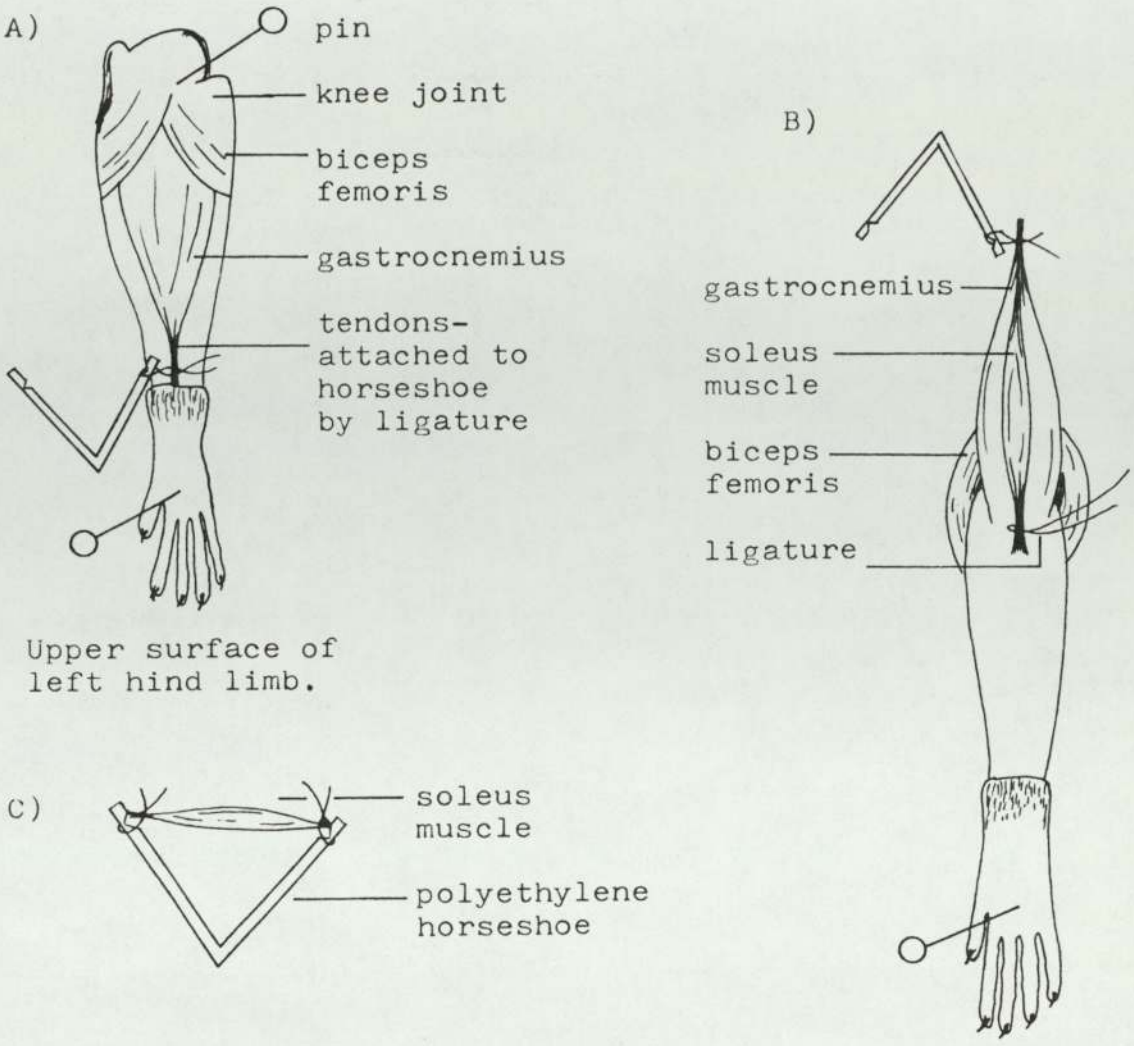

Figure 17. Procedure for the removal of the soleus muscle from the mouse hind limb.

A). Skin and fur of the leg have been removed to expose the gastrocnemius. A ligature was tied securely around the exposed tendons and then secured to a polyethylene horseshoe. Tendons were then cut distal to the ligature.

B). The gastrocnemius and associated muscles were peeled upward to reveal the soleus muscle on the underside of the gastrocnemius. A ligature was secured around the freshly exposed soleus muscle tendon and the attachment of the tendon to the leg was then severed.

C). The isolated soleus muscle was maintained under tension by securing to the polyethylene horseshoe.

2.8.1. The fate of glucose entering muscle.

Glucose entering muscle cells may either be anaerobically metabolized via pyruvate to lactate (glycolysis) or aerobically metabolized via the tricarboxylic acid (TCA) cycle or be stored as glycogen. Although glycolysis is able to produce energy rapidly and is the major form of energy production in type 2 (white) muscle fibres (334) glucose metabolism via glycolysis releases very little of the potential chemical energy of glucose.The complete oxidation of glucose is at least 10-fold more efficient than glycolysis in terms of energy production per glucose residue (334) and is the preferential pathway for glucose metabolism in type 1 (red-intermediate) slow oxidative fibres (334) of which the soleus muscle is predominantly composed (328).

Under aerobic conditions glucose is first oxidized to acetyl-CoA which enters the TCA cycle proper, whereupon its acetyl group is degraded to form two molecules of CO_2 and four pairs of hydrogen atoms. The subsequent transfer to and passage through the respiratory chain of the latter hydrogen atoms (or their corresponding electrons) results in the release of energy laden ATP molecules and the conversion of molecular oxygen to water.

Under conditions in which glucose supply exceeds the immediate cellular fuel demands, glucose is stored in a polymeric form as glycogen.

The rate of *in vitro* glycogen production by mouse

soleus muscle was estimated directly by the determination of ^{14}C-activity associated with glycogen extracted from muscles incubated with U-^{14}C-glucose. Assessment of the rate of $^{14}CO_2$ production from U-^{14}C-glucose served as an index of oxidative phosphorylation of TCA cycle intermediates and thus TCA cycle activity since muscle virtually lacks the ability to oxidize glucose by the pentose phosphate pathway (335).

2.8.2. Determination of the rate of glucose oxidation and glycogen synthesis by the isolated soleus muscle.

The method employed for the determination of the rate of glucose oxidation and glycogen synthesis by muscle was based on that of Chaudry and Gould (336), with modifications introduced by Cuendet (337).

Muscles were prepared as described (pages 99-101) and immediately preincubated at 37°C in flat bottomed glass vials (inner diameter 2cm) containing 2ml of pregassed (95% O_2;5% CO_2) incubation buffer (Krebs Ringer Bicarbonate (KRB), 2%BSA, pH7.4 at 37°C, 5mmol/l glucose). After 5 minutes gassing of the vials and a further 10 minutes pre-incubation period, muscles were removed and washed by submerging briefly in KRB at 37°C. The left leg muscle of each matched pair was placed in an Erlenmeyer flask containing 3 ml of incubation buffer and 0.5μCi/ml ^{14}C-glucose (D-U-^{14}C-glucose, specific activity 292mCi/mmol, Amersham International PLC) at 37°C (Figure 18). The muscle of each right leg was transferred to identical conditions except

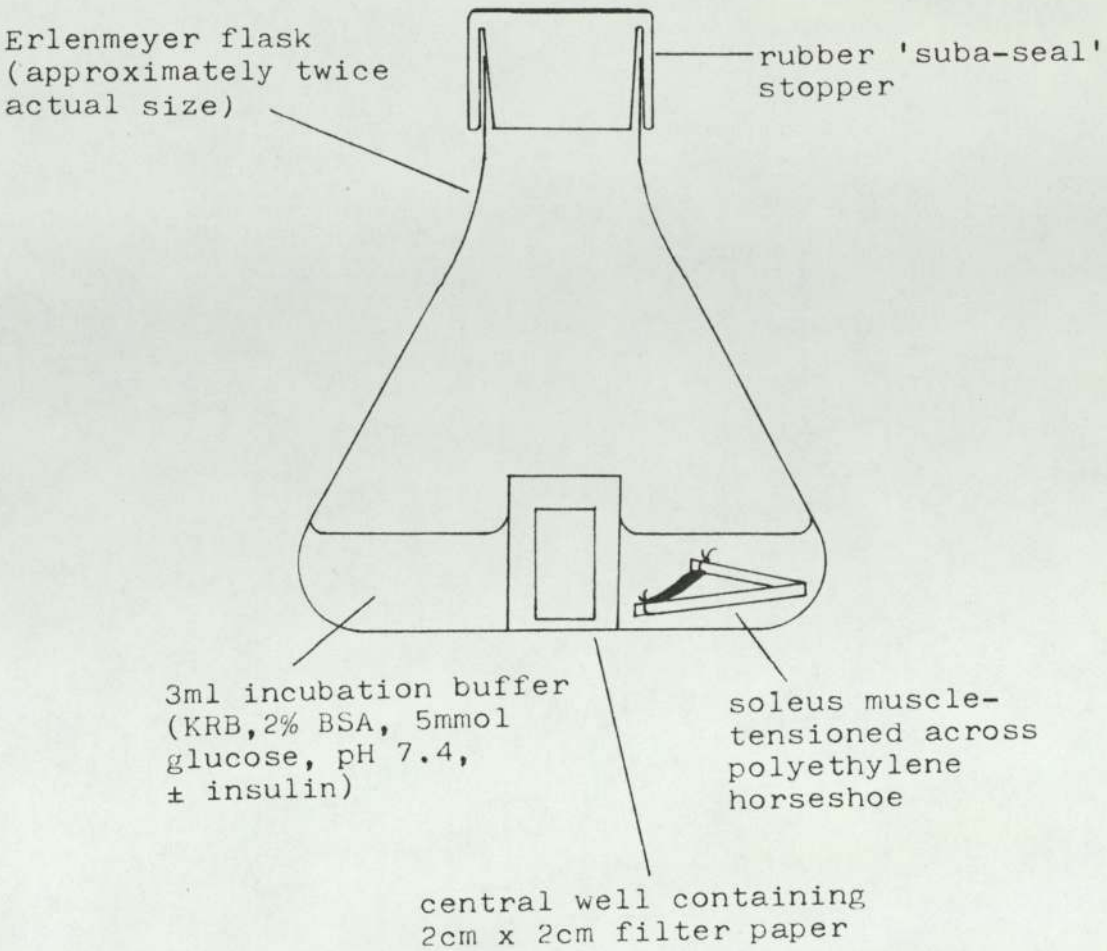

Figure 18. Incubation conditions of isolated soleus muscle for the determination of the rate of $^{14}CO_2$ production and ^{14}C-glycogen synthesis from ^{14}C-glucose. The flasks were placed in a shaking water bath (92 cycles/min) at 37°C.

for the presence of insulin at the required concentration (see below).

Thus, the left and right muscle of each matched pair, incubated in the absence and presence of insulin respectively were used for the simultaneous determination of the rates of ^{14}C-glycogen and $^{14}CO_2$ production.

After gassing for 5 minutes the flasks were sealed (suba-seals, Fisons Ltd.) and incubated for the required time (see below). All preincubations and incubations were carried out at 37°C in a shaking water bath (92 cycles/ minute).

At the end of the incubation period 0.1ml of 1M NaOH was injected through the seal of each flask onto a $2cm^2$ folded piece of filter paper located within the central glass well (inner diameter 1cm) of each flask. The flasks were then placed on ice for 4 minutes. Each muscle was then removed, frozen in liquid nitrogen, and stored at -20°C for subsequent determination of ^{14}C-glycogen and protein content. The flasks were immediately restoppered and the incubation media acidified to release dissolved $^{14}CO_2$ by the injection of 0.3ml of 3M perchloric acid through the seal of each flask. The $^{14}CO_2$ liberated from the medium was trapped by the NaOH during a further 1 hour shaking at 37°C. At the end of this time the filter paper was transferred to a scintillation vial containing 10ml of NE 260 scintillant (New England Nuclear, Aberdeen) which had been precounted

to ensure low ^{14}C-activity (<50dpm). After storing at room temperature for 1 week in the dark to reduce chemiluminescence, ^{14}C-activity was determined for 10 minutes on a Beta counter (Tricarb 2660, Packard Instruments).

Frozen muscles were thawed and digested in 0.5ml of 1M NaOH (10 minutes 100°C) containing 2mg/ml of carrier rabbit glycogen. After digestion a 50μl aliquot was removed from each digest for protein determination by the method of Lowry, Rosebrough, Farr and Randall(338) (Appendix, pages 227 -228). Glycogen was precipitated from the remaining digest by the addition of 1ml of 95% ethanol. Following centrifugation (30 minutes, 840 g, 5°C), the supernatant of each sample was aspirated and the glycogen precipitate redissolved in 1ml of distilled water. ^{14}C-activity associated with the glycogen was determined by counting 0.5ml of the sample in 10 ml of micellar scintillant (NE 260,New England, Nuclear Aberdeen) for 10 minutes on a Beta counter (Tricarb 2660, Packard Instruments).

The activities of ^{14}C-glycogen and $^{14}CO_2$ were counted at efficiencies of 88.8-90.3% and 93.1-94.8% respectively as determined by the external standard ratio method (339) and were provided automatically by the Beta counter in disintegrations per minute (dpm).

^{14}C-glycogen and $^{14}CO_2$ production have been expressed in terms of dpm/mg of muscle protein and dpm/muscle. Insulin stimulated ^{14}C-glycogen synthesis and $^{14}CO_2$ production have been considered to be the

differences between production in the absence and presence of insulin for each matched pair of muscles.

2.8.3. The time course of $^{14}CO_2$ production and ^{14}C-glycogen synthesis from ^{14}C-glucose by the isolated soleus muscle.

The production of ^{14}C-glycogen and $^{14}CO_2$ are dependent upon the period of incubation (329,340). Thus, the time course of both parameters was determined in the absence and presence of insulin (20ng /ml, 0.5mU/ml) over a total time of 3 hours. The incorporation of ^{14}C into glycogen and CO_2 was higher in the presence of insulin over the 3 hour incubation period (Figures 19 and 20). However, incubation for only 2 hours allowed a suitable discrimination between $^{14}CO_2$ and ^{14}C-glycogen production in the absence and presence of insulin and was the incubation period used in subsequent studies.

2.8.4. The effect of exogenous insulin concentration on the rate of $^{14}CO_2$ production and ^{14}C-glycogen synthesis.

This preliminary experiment was designed to assess the insulin dose dependency of ^{14}C-glycogen and $^{14}CO_2$ production to allow the determination of a suitable stimulatory insulin concentration for use in subsequent studies. Thus ^{14}C-glycogen and $^{14}CO_2$ production by mouse soleus muscle were determined after the incubation of muscles for 120 minutes in the presence of a range of insulin concentrations.(0-400ng /ml, 0-10mU/ml). After subtraction of the basal ^{14}C-glycogen and $^{14}CO_2$ production from their production in the presence of insulin, insulin stimulated production was

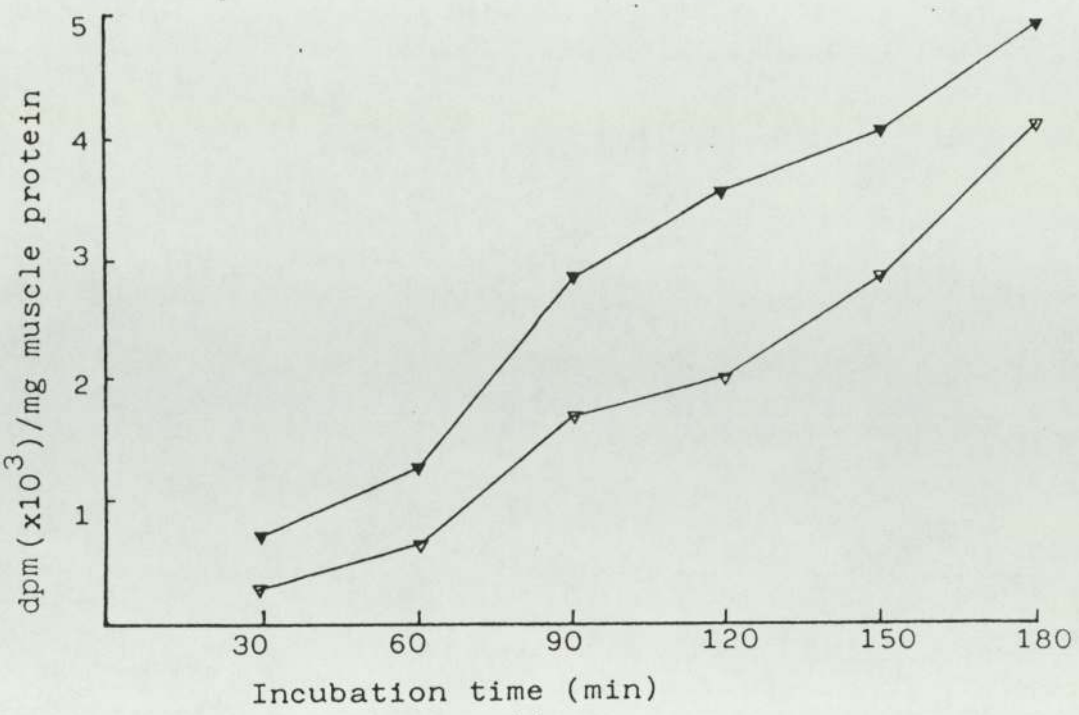

Figure 19. The production of ^{14}C-glycogen by isolated mouse soleus muscle incubated in the presence (▼) and absence (▽) of insulin (20ng/ml) with respect to time.

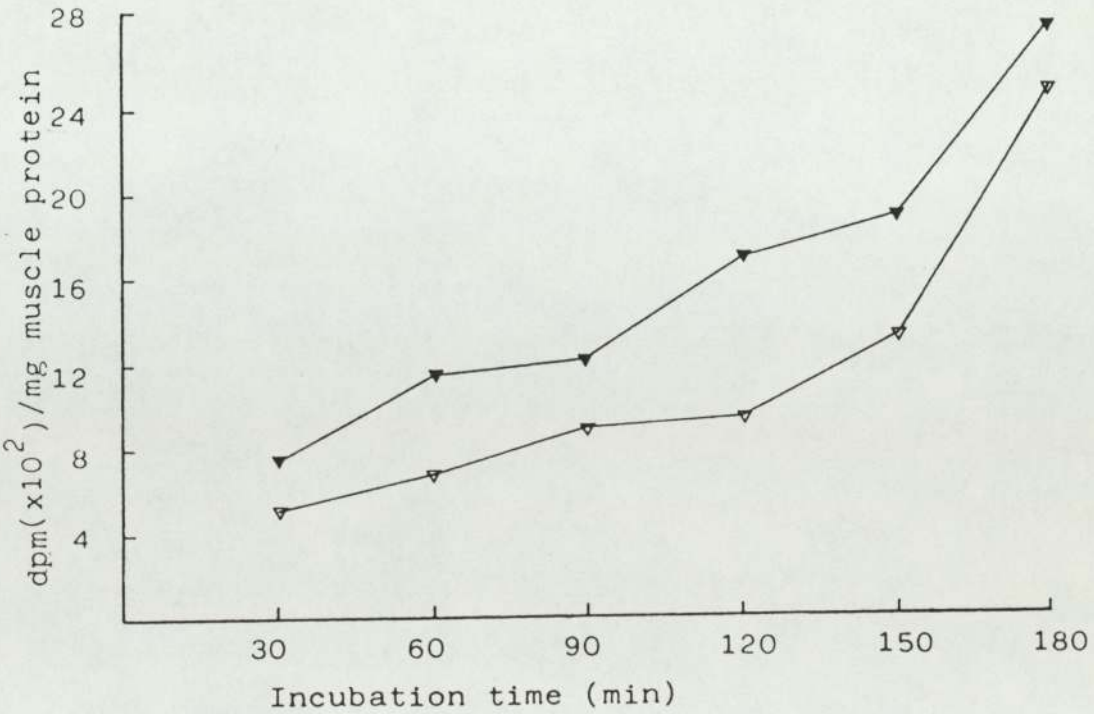

Figure 20. The production of $^{14}CO_2$ by isolated mouse soleus muscle incubated in the presence (▼) and absence (▽) of insulin (20ng/ml) with respect to time.

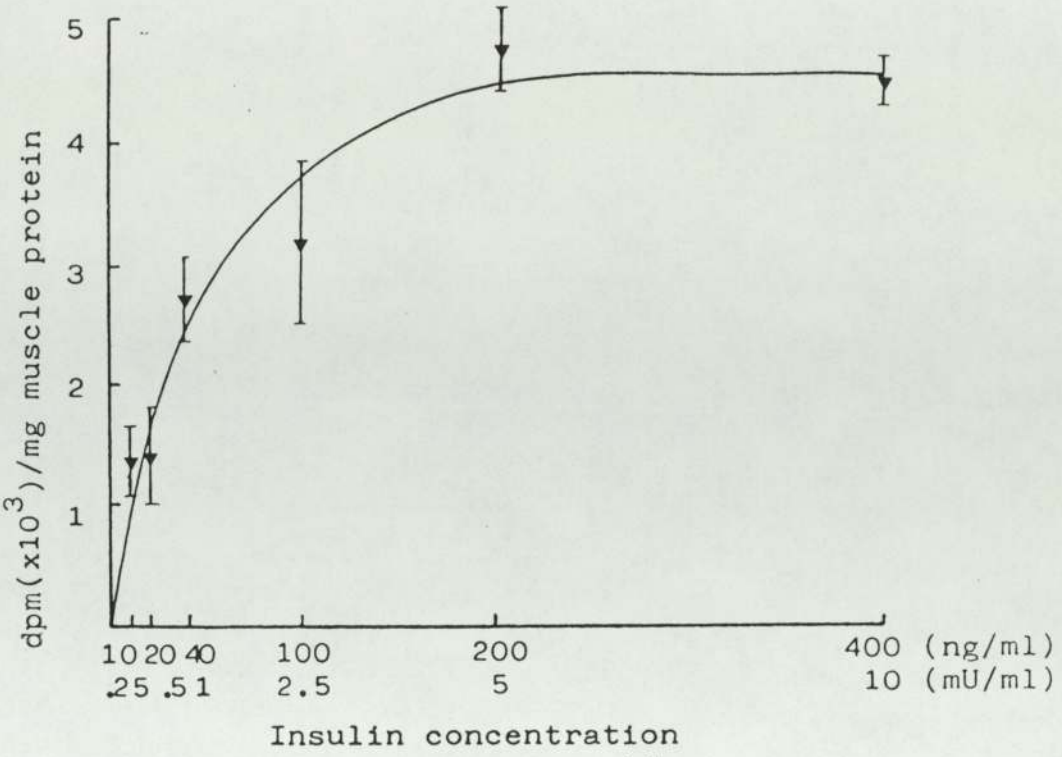

Figure 21. The increase above basal ^{14}C-glycogen production by isolated mouse soleus muscle incubated in the presence of increasing insulin concentrations. Mean values ± SEM (n=3).

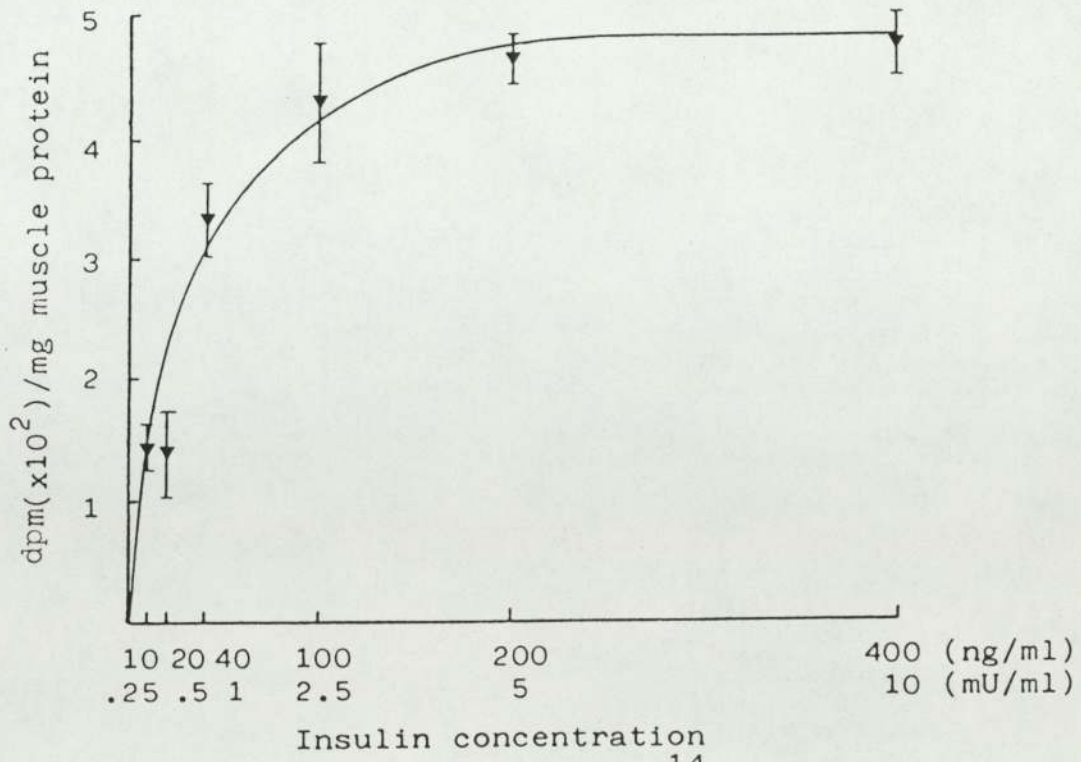

Figure 22 . The increase above basal $^{14}CO_2$ production by isolated mouse soleus muscle incubated in the presence of increasing insulin concentrations. Mean values ± SEM (n=3).

expressed in terms of dpm/mg of muscle protein.

Figures 21 and 22 illustrate that increasing concentrations of insulin stimulated both ^{14}C-glycogen and $^{14}CO_2$ production, reaching a maximum in the presence of approximately 200ng /ml, 5mU/ml insulin. Stimulation of ^{14}C-glycogen and $^{14}CO_2$ production was approximately linear over the range 0-40ng /ml (0-1mU/ml) insulin but much less sensitive to increments above this concentration range, typical of zero order saturation kinetics.

Double reciprocal (Lineweaver-Burk) (201) plots were constructed (Figures 23 and 24) in order to determine the insulin dose required for half maximal stimulation of ^{14}C-glycogen and $^{14}CO_2$ production (Km). The Km values were 0.64 mU/ml, (25.6ng/ml) and 0.59mU/ml, (23.6ng/ml) for ^{14}C glycogen and $^{14}CO_2$ production respectively. Thus, the rate of ^{14}C-glycogenesis appeared to be a little more sensitive to insulin than the rate of $^{14}CO_2$ production.

On the basis of this experiment it was considered that a dose of 20ng/ml (0.5mU/ml) insulin would be suitable for use in subsequent studies to allow the detection of alterationsin the insulin sensitivity of soleus muscle.

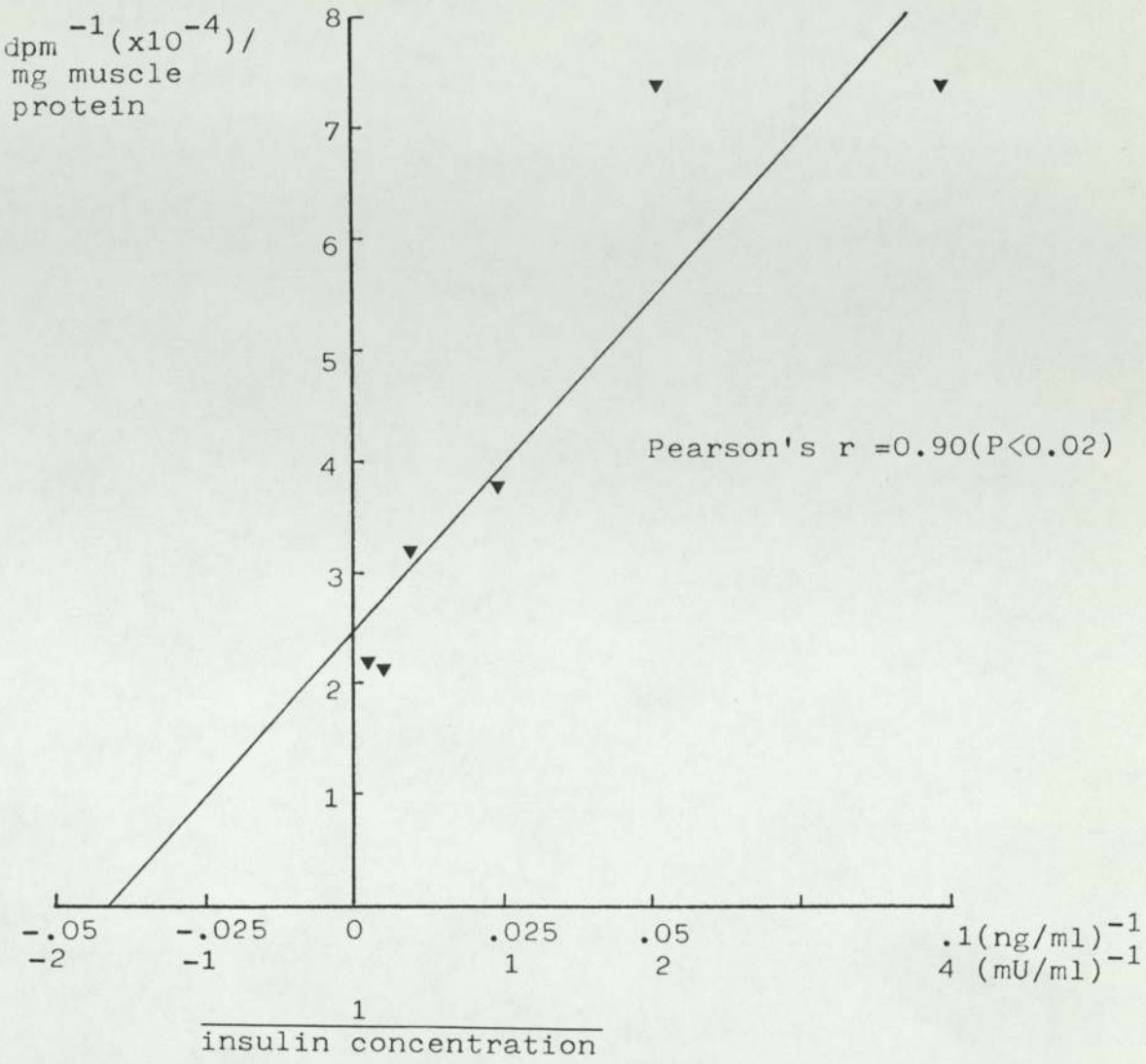

Figure 23. Double reciprocal (Lineweaver-Burk) plot of 1/V versus 1/[S] for ^{14}C-glycogen production above basal by isolated mouse soleus muscle in the presence of a range of insulin concentrations. Data from Figure 26.

1/V = 1/insulin stimulated ^{14}C-glycogen production

1/[S] = 1/insulin concentration

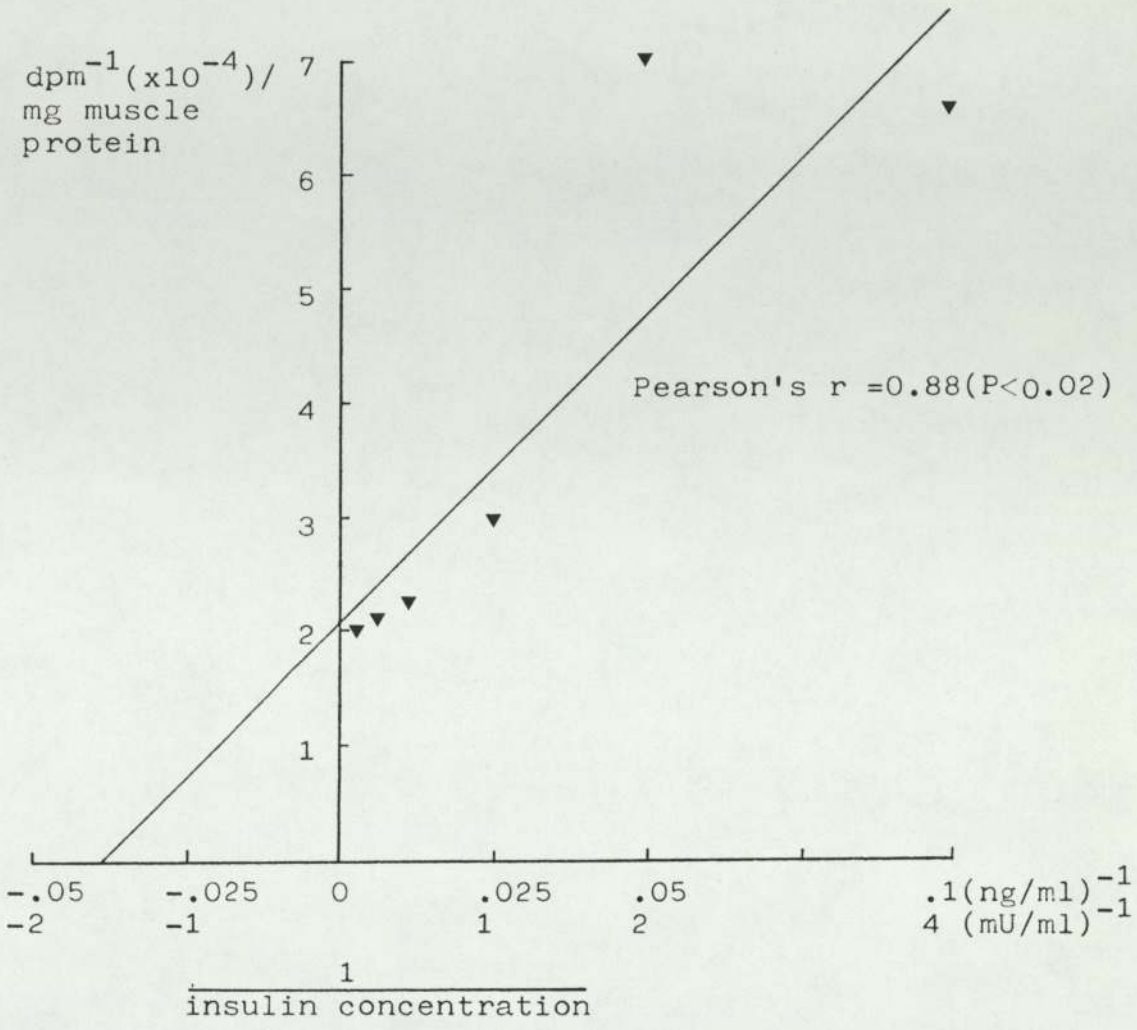

Figure 24. Double reciprocal (Lineweaver-Burk) plot of 1/V versus 1/[S] for $^{14}CO_2$ production above basal by isolated mouse soleus muscle in the presence of a range of insulin concentrations. Data from Figure 29.

1/V = 1/insulin stimulated $^{14}CO_2$ production

1/[S] = 1/insulin concentration

2.9 Determination of the glycogen content of soleus muscle and liver.

Mice were killed by cervical dislocation and the soleus muscles or the liver removed. Soleus muscle preparation was based on the procedure previously described (pages 99 -101) but the tendons were neither ligatured nor attached to a polyethylene horseshoe. Soleus muscles were weighed and then individually digested (0.5ml of 1M NaOH, 10 minutes, 100°C) after which 50μl aliquots were taken and stored frozen (-20°C) for subsequent protein determination by the method of Lowry, Rosebrough, Farr and Randal (338) (Appendix, pages 227 -228). The glycogen content of the remaining digest was then determined by the method of Lo, Russell and Taylor (341) (Appendix, pages 229 -230) and expressed in terms of mg glycogen/muscle, mg glycogen/mg muscle protein and mg glycogen/gm muscle wet weight.

Livers were weighed, samples (approximately 300-500 mg) taken from the largest lobe and digested (0.5ml of 1M NaOH, 10 minutes, 100°C). The glycogen content of the digests were then assayed by the method of Lo, Russell and Taylor (341) (Appendix, pages 229 -230) and expressed in terms of mg glycogen/liver and mg glycogen/gm liver wet weight.

2.10 The isolation of hepatocytes from mouse liver.

Techniques for the isolation of rat hepatocytes based on an *in situ* collagenase digestion (342-345) yield cell populations with generally greater than 90% viability as judged by standard trypan blue exclusion tests. Similar procedures employed for the isolation of mouse hepatocytes (165,345), however, yield cell populations with between 70-80% viability. In the present study hepatocytes were isolated by a modification of the method of Kahn et al (165).

The liver perfusion system employed in the present system is represented diagramatically in Figure 25.

Procedure.

Under anaesthesia (intraperitoneal sodium pentobarbitone (Sagatal) 0.5ml /Kg body weight) a frontal midline cut was made in skin and body wall of the mouse from the xyphis pubis to within 1cm of the vaginal opening. The contents of the abdominal cavity were displaced to the right and the hepatic portal vein (HPV) exposed. Ligatures (4/0 capillary braided suture: Armour Pharmaceutical Co., Eastbourne) were tied loosely around the HPV above the entry of the mesenteric artery and also distal to the point of cannulation just above the entry of the splenic artery. All connective tissue was teased away from the HPV. At this point the ligature distal to the point of cannulation was secured. An incision was then made in the HPV with a fine pair of cataract scissors. A fine polyethylene cannula (PP10, Portex Limited, Hythe, Kent) was inserted 0.5cm into the vein and secured with

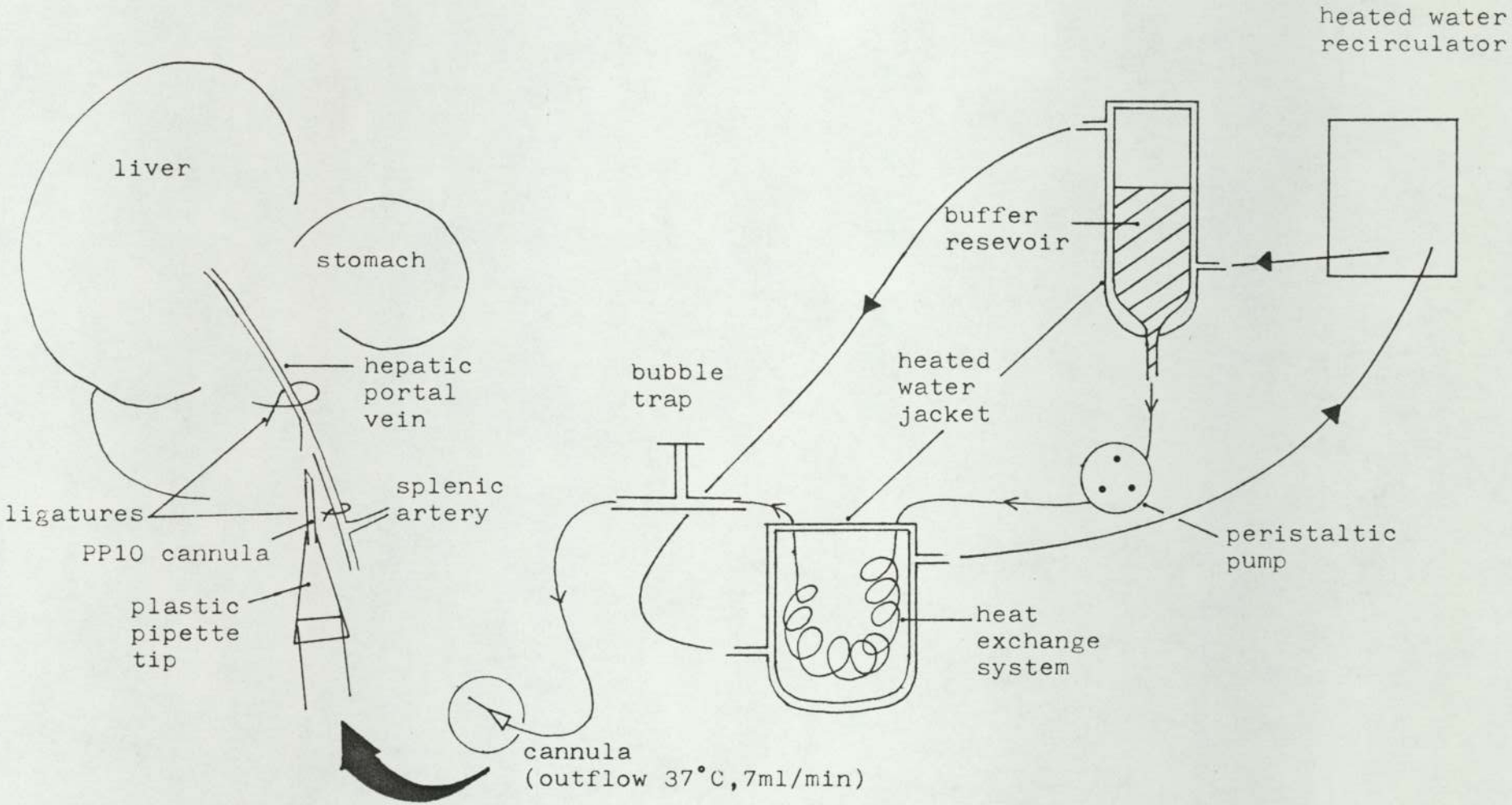

Figure 25. Mouse liver perfusion for the isolation of mouse hepatocytes.

the second ligature (Plate 1, Figure 25).

Perfusion was initiated with a Hepes based buffer containing heparin and EDTA (Buffer 1, Table 2) and after the liver had lost its maroon colour, the vena cava was cut through to allow drainage of perfusate into the body cavity (Plate 2). The optimum flow rate for comprehensive digestion of the liver and maximum cell viability was 7ml/minute. Faster flow rates increased the mechanical damage to hepatocytes and slower flow rates reduced the perfusion pressure thus reducing the flow of perfusate to the peripheral regions of the liver lobes. The heparin content (200U/l) of Buffer 1 successfully prevented blood clotting. Liver digestion was improved if Ca^{++} was removed from the liver before collagenase perfusion (343). This was probably due to a reduction in Ca^{++} dependent cell adhesion (343). For this reason the Ca^{++} chelating agent EDTA was included in Buffer 1 (0.49g/l).

The initial 25ml of Buffer 1 was followed by 25ml Ca^{++} Hepes based Buffer (Buffer 2, Table 2). The role of Buffer 2 was to remove all the EDTA from the liver since collagenase has been shown to be a Ca^{++} dependent enzyme (343). Finally the liver was perfused with a collagenase (0.5g/l) and Ca^{++} containing Hepes based buffer (Buffer 3, Table 2) until the liver was completely digested. During the period of collagenase perfusion the liver distended to twice its original size (Plate 3). Usually, 30-40ml of collagenase containing buffer was allowed to perfuse

	Perfusion buffers			
	Buffer 1 g/l	Buffer 2 g/l	Buffer 3 g/l	Buffer 4* g/l
NaCl	8.30	8.30	8.30	4.00
KCl	0.50	0.50	1.50	0.40
$CaCl_2.2H_2O$			1.70	0.18
$MgCl_2.6H_2O$				0.13
KH_2PO_4				0.15
Na_2SO_4				0.10
HEPES	2.40	2.40	2.40	7.20
TES				6.90
TRICINE				6.50
1M NaOH(ml /l)	7.50	7.50	8.00	50
Heparin (U/l)	200			
EDTA	0.49			
Collagenase			0.50	
pH	7.4	7.4	7.5	7.8

TABLE 2.

Composition of buffers used for hepatocyte isolation and insulin binding assays.

* After Seglen 1973 (343).

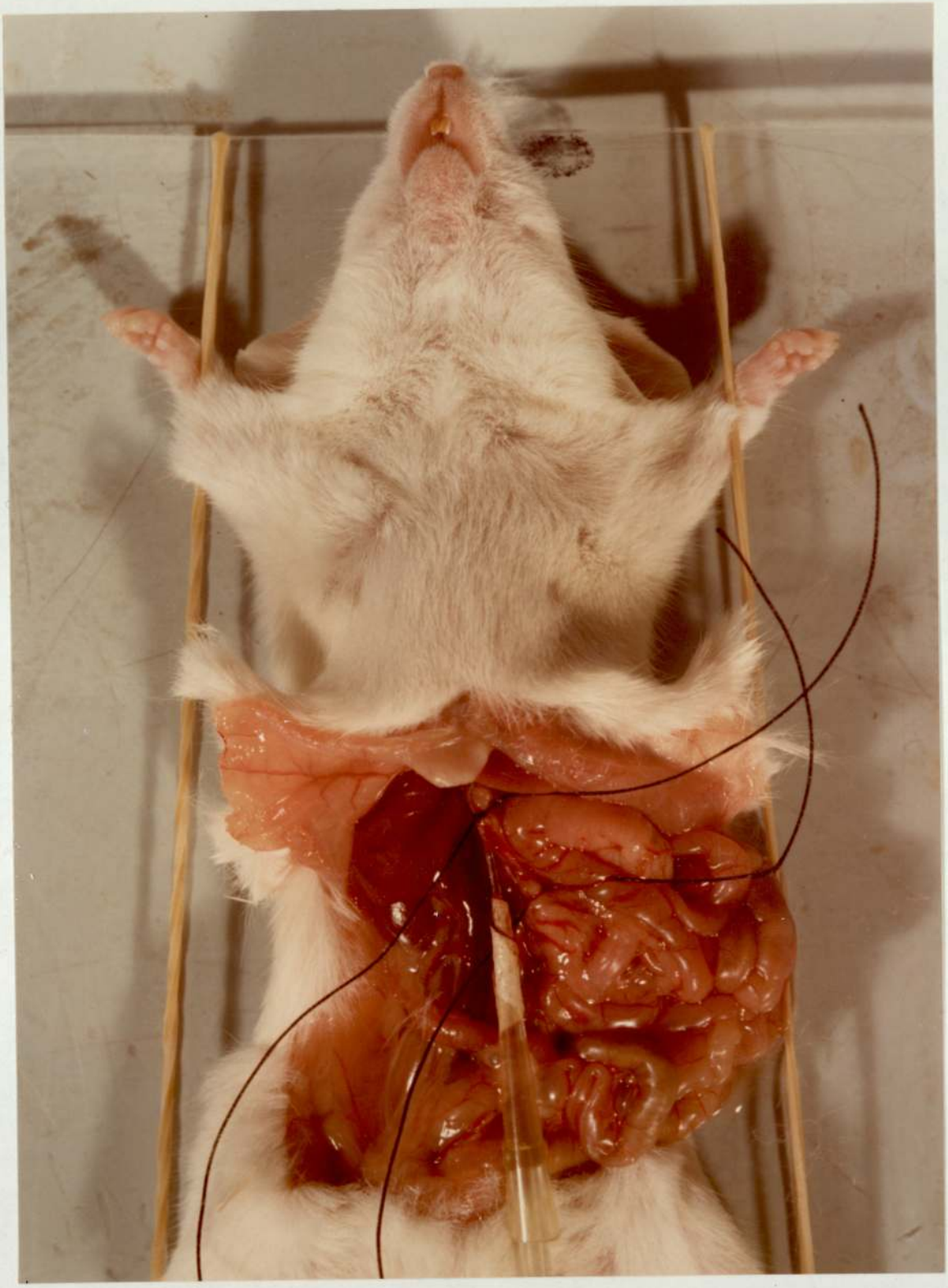

Plate 1

The hepatic portal vein has been ligatured just above the entry of the splenic artery at a point distal to the place of cannulation. The cannula has been secured in place but perfusion has not begun.

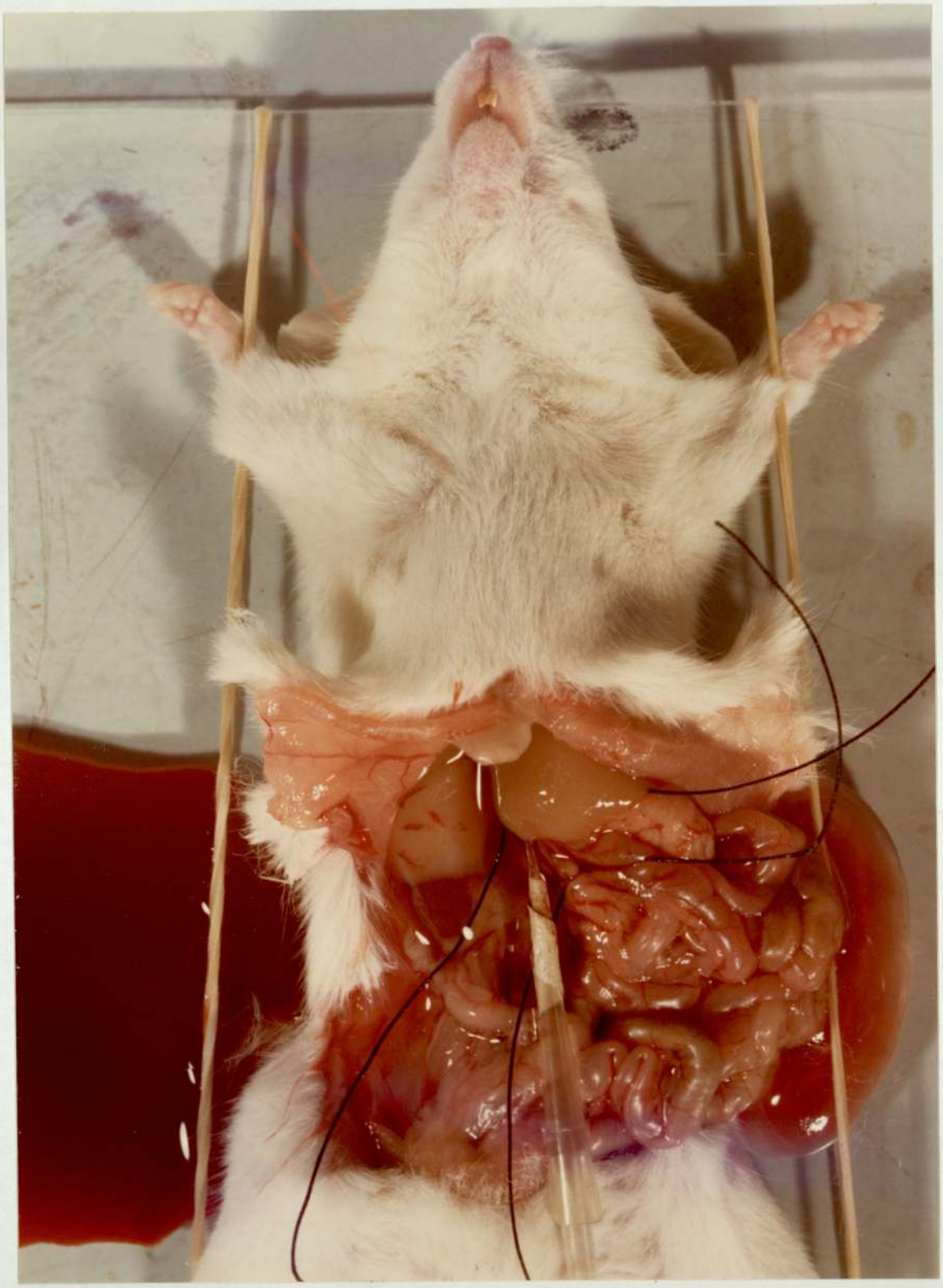

Plate 2

The liver has blanched due to its perfusion by buffer (7ml/min, 37°C) and the vena cava has been severed to allow drainage of the perfusate into the body cavity.

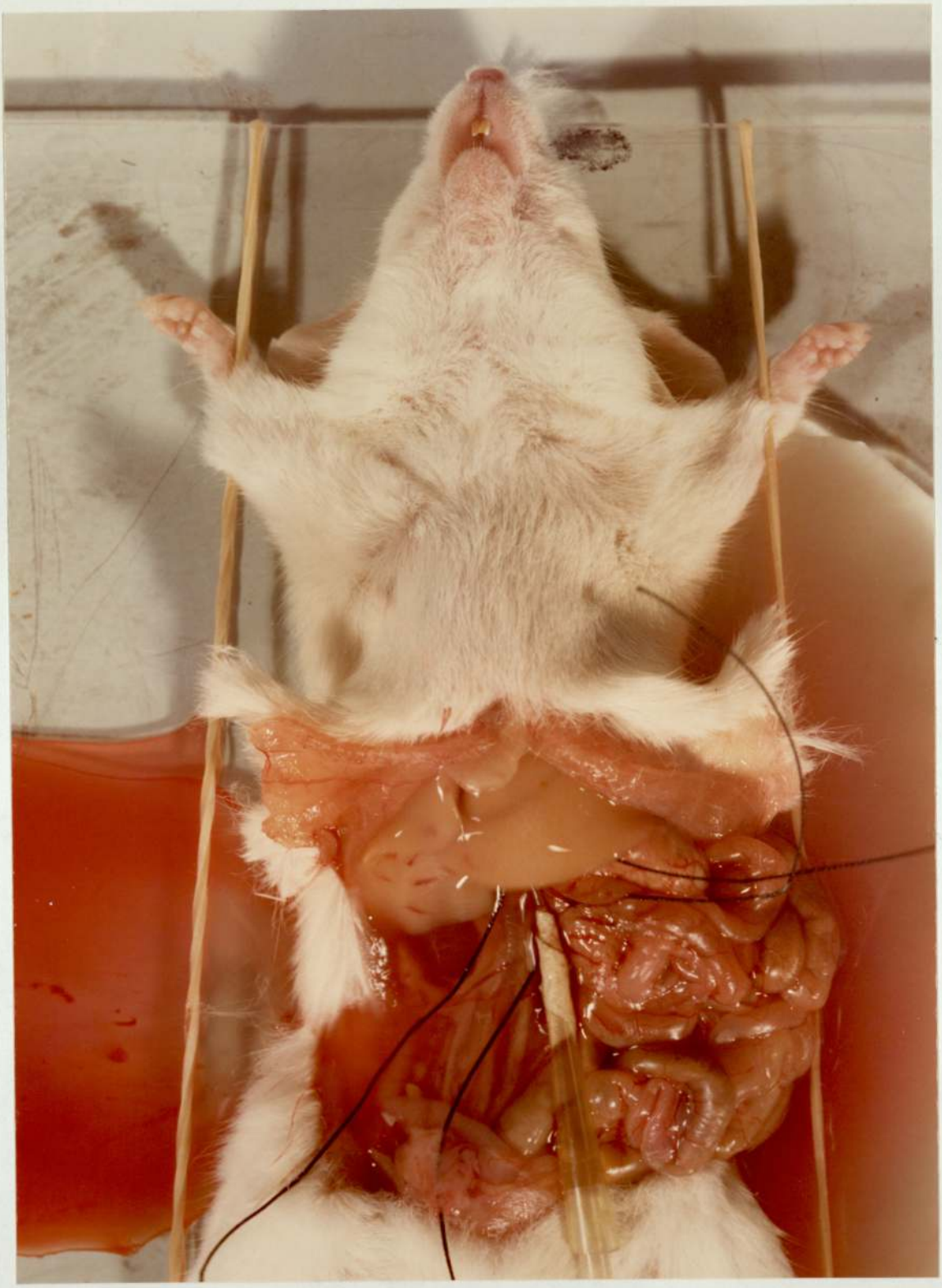

Plate 3

The liver has been digested by its perfusion with buffer containing collagenase (0.5g/l) and has been distended to approximately twice its original size.

through the liver after which time digestion was seen to be complete. This was determined by the extremely soft consistency of the liver which tended to disaggregate with the application of mimimal mechanical force. The temperature of all perfusates leaving the perfusion cannula was 37°C and was maintained by a slightly higher temperature in the two water jackets of the heat exchange system (Figure 25).

Hyaluronidase, used by other investigators (165) for the in situ digestion of livers was not included in the final perfusate. None of the reported (165) detrimental effects of the exclusion of hyaluronidase on cell viability were observed, possibly as a result of the decreased collagenase concentration employed in the present study, the increased flow rate and the presence of Ca^{++} in the final perfusion medium.

After digestion and removal of the cannula, the liver was removed, placed in a plastic petri dish and rinsed once in a Hepes based buffer having a greater buffering capacity than the buffers employed for the perfusion (Buffer 4, Table 2). Subsequent cell isolation and purification procedures were carried out in plastic ware to reduce cell adhesion.

After removal of the gall bladder and any loose connective tissue the liver was transferred to fresh Buffer 4, gently teased apart and the liver cells dispersed with blunt forceps using a minimum of mechanical force. Any regions of the liver that had not been adequately digested were discarded.

Careful manipulation of the cells at this stage was essential to produce a high yield of viable cells. The liver cells were then filtered through a 120μm nylon mesh (Sericol UK. Limited) to remove smaller undigested portions of liver and any remaining connective tissue.

After the cells had been gassed (95% O_2, 5% CO_2) for 1 minute and gently mixed by slow rotation of the plastic beaker into which they had been filtered, they were centrifuged at a slow speed (5 minutes, 4°C, 100 g, MSE Chilspin) in four aliquots of approximately 5ml each. Less dense non-viable hepatocytes and Kupffer cells remained in the resultant supernatant and were aspirated. After washing the sedimented cells with Buffer 4, the cell suspension was centrifuged as previously descibed and the resultant hepatocyte pellets suspended in 4ml of incubation medium (Buffer 4 +0.5%BSA +0.08% bacitracin). The aliquots were pooled and filtered through a 60μm nylon mesh (Sericol UK. Limited) to remove any clumps of hepatocytes.

Aliquots (100μl) of the cell suspension were taken for estimation of hepatocyte concentration, diameter and viability . Hepatocyte concentrations were determined by counting 1:20 dilutions on a haemocytometer (Improved Neubar). To reduce counting errors, hepatocyte concentrations were determined in 32, $1mm^3$ volumes (4 per grid, 8 per chamber). Where possible hepatocyte concentrations were adjusted to approximately $2.5x10^6$ cells/ml and recounted. Hepatocytes, at a dilution of 1:20 in the haemocytometer were also used

for the determination of cell diameters. For any one hepatocyte preparation the cell diameter was estimated by determining the average diameter of 100 cells using a precalibrated microscope eye-piece graticule. Cell viability was assessed routinely by the trypan blue exclusion test. A 20μl sample of a 1:20 dilution of the hepatocyte suspension was added to 400μl of incubation medium and 500μl of 0.4% trypan blue. After 2 minutes those cells that had taken up the dye and appeared blue were judged to be non viable, while those excluding the dye were considered viable. Cell viability was routinely assessed to be greater than 75%.

2.11.1. Insulin-receptor binding assays.

When an insulin receptor population is incubated for an optimum, predetermined length of time with insulin, steady state or equilibrium binding is achieved. That is the rates of insulin-receptor association and dissociation are equivalent. At this point free and receptor bound insulin are separated, generally by centrifugation. Provided a tracer amount of ^{125}I-insulin was present during the incubation the ^{125}I-activity associated with either the bound or free fraction can be determined and used to calculate the total free or bound insulin concentrations. For a fuller explanation of the theory behind this receptor binding assay the reader is referred to pages 35 -36.

The optimum conditions for steady state binding vary from tissue to tissue and several physical and chemical factors are important regulators of insulin binding *in vitro*, for example; time (Figure 26), temperature (Figure 27) and pH (Figure 28) of incubation (346). The albumin concentration of the incubation medium may also influence insulin binding by affecting the rate of insulin degradation (347).

Generally, insulin binding studies are carried out at subphysiological temperatures (10-20°C) since insulin binding shows an inverse correlation to temperature (348) (Figure 27). This may be due to an enhanced rate of insulin degradation *in vitro* at higher temperatures (213,273) and/or an increased rate of hormone receptor internalisation at higher temperatures (349).

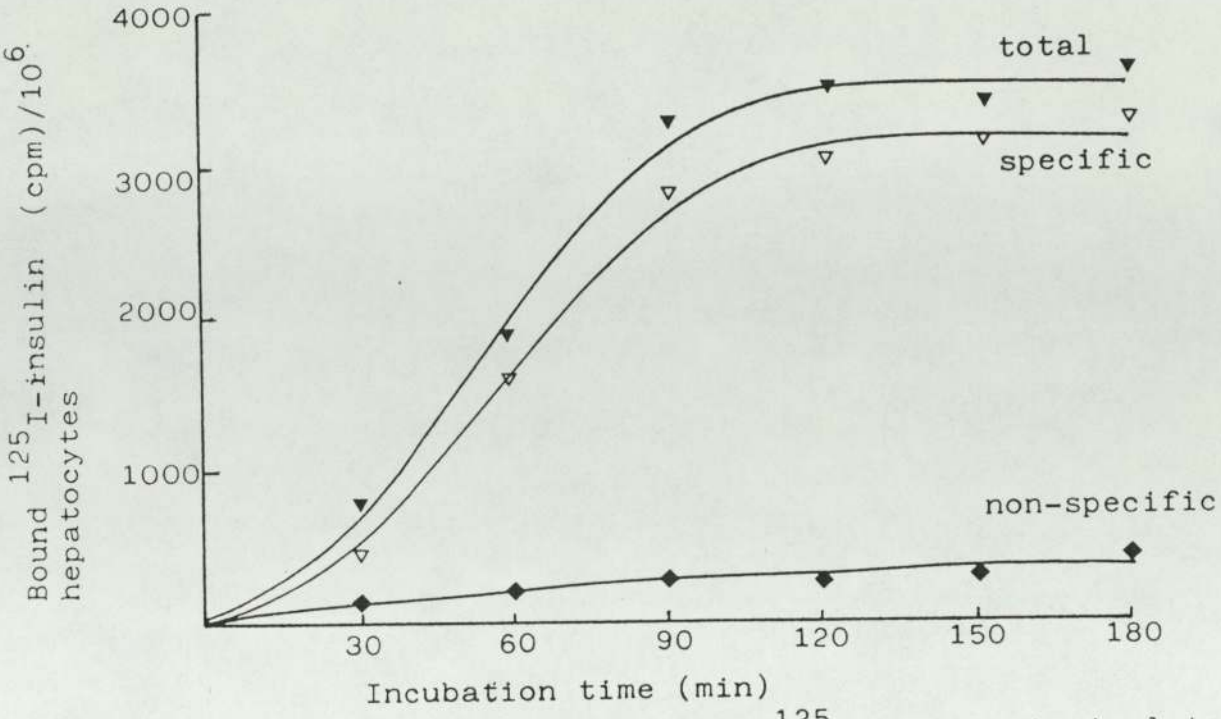

Figure 26. Time course of binding of ^{125}I-insulin to isolated mouse hepatocytes at 20°C.

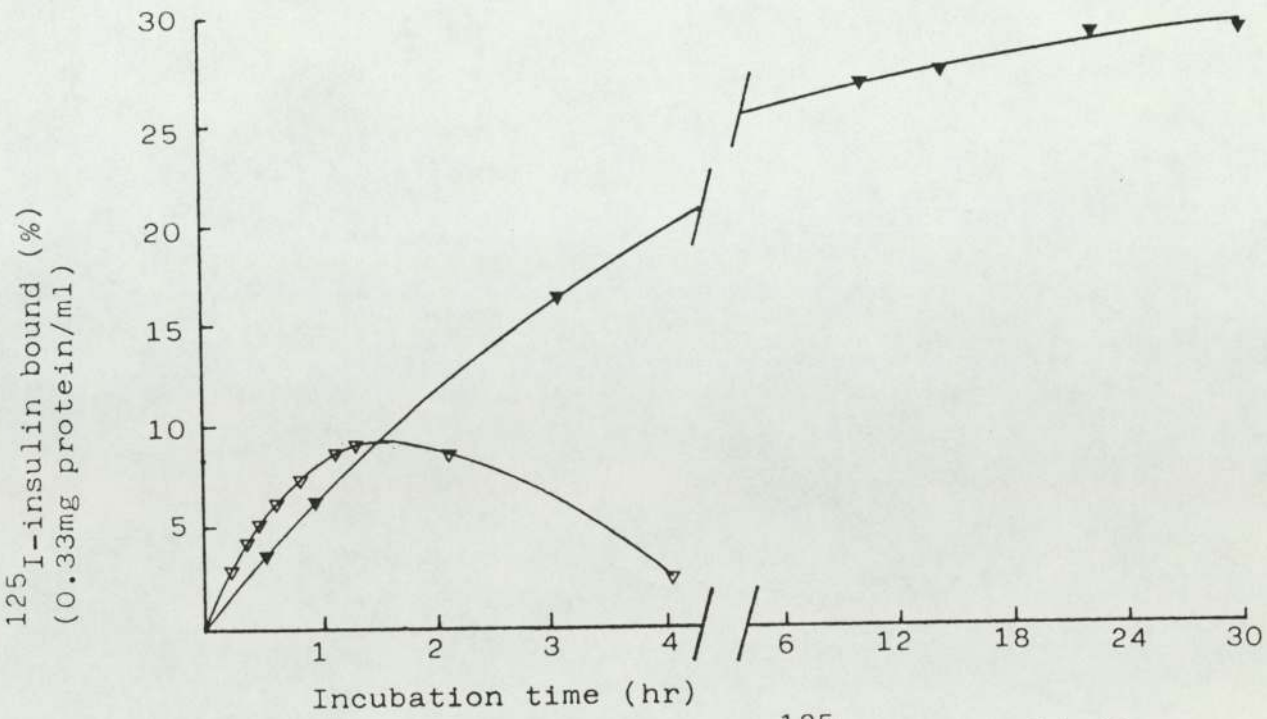

Figure 27. Temperature dependence of ^{125}I-insulin binding to liver plasma membranes at 4°C(▼) and 30°C(▽),(213).

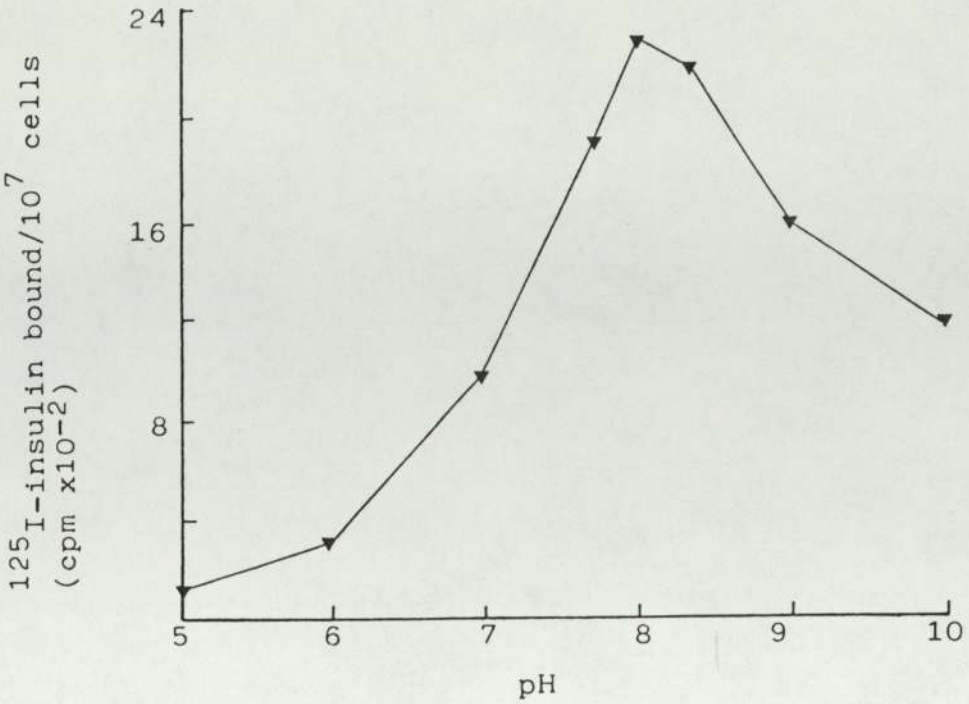

Figure 28. The effect of pH on the specific binding of ^{125}I-insulin to human cultured lymphocytes,(from 214).

Optimal conditions for insulin receptor binding were determined for both hepatocyte and soleus muscle preparations (Table 3).

2.11.2. Assay of insulin binding to isolated mouse hepatocytes.

The insulin binding assay was based on the method of Kahn et al (165).

400μl of cells at a known concentration in the region of $2.5x10^6$ cells/ml were aliquoted into 1.5ml polyethylene microfuge tubes (PPRIS, Beckman Limited, High Wycombe) containing 50μl of ^{125}I-monoiodinated insulin ($2.5x10^4$ cpm, approximately 0.08ng/tube) and 50μl of incubation buffer with a range of cold insulin concentrations. The final incubation volume was thus 0.5ml and contained concentrations of approximately $2.0x10^6$ cells/ml, 0.16ng/ml ^{125}I-insulin and a range of 17 cold insulin concentrations (0-1000ng/ml). Non-specific binding was considered to be that proportion of the total ^{125}I-insulin remaining bound in the presence of an excess of cold insulin (10^5ng/ml).

All tubes were set up in triplicate where possible although the use of younger mice often allowed only duplicate determinations because of the reduced cell yield.

After the tubes had been sealed they were inverted to ensure the contents were mixed and then transfered to a water bath at 20°C and left in an upright position. The

Cell or tissue	Incubation period (hrs)	Incubation pH	Incubation Temperature (°C)	Incubation buffer	Medium for separation of bound and free insulin
Mouse hepatocyte	2	7.8	20	buffer 4+ 0.5%BSA+ 0.08% bacitracin	ice cold buffer 4
Mouse soleus muscle	4	7.4	20	KRB+ 0.5%BSA	-

TABLE 3.

Optimal conditions for insulin-receptor binding with hepatocyte and soleus muscle preparations.

tubes were gently inverted at half hourly intervals and after 2 hours at which point equilibrium binding was reached (Figure 26). The tubes were placed on ice to stop the reaction. 0.5ml of ice cold incubation buffer was then added to each tube to dilute the cell suspension and thus reduce non-specific trapping of free insulin upon centrifugation. Following centrifugation at 9,000 g for 1 minute in a Beckman microfuge (Type B, Beckman Limited, High Wycombe) to separate the bound and free insulin fractions, supernatants were aspirated and a further 0.5ml of ice cold incubation buffer added to wash the cell pellet. After a similar centrifugation and aspiration procedure the tip of each microfuge tube containing the cell pellet was excised and the ^{125}I-activity in the pellet deterimined for 1 minute on a Gamma counter (ICN, Tracerlab) at a counting efficiency of 57%.

It is significant that during incubation of hepatocytes, non-viable cells release insulin degrading activity into the incubation medium with the subsequent degradation of cold insulin and ^{125}I-insulin into A and B chains and polypeptides of intermediate chain length. These fragments of insulin have varying affinities for the insulin receptor and thus severely impede the analysis of binding data (225).

The incorporation of bacitracin, an inhibitor of glutathione insulin transhydrogenase, greatly reduced the extracellular degradation of insulin. Indeed the maximum insulin degradation, assessed by TCA precipitation of ^{125}I-insulin

in the absence of cold insulin was found to be only 4-5% of the total amount of ^{125}I-insulin. It has, however, been reported (350) that bacitracin has effects on the mechanism of insulin processing by intact hepatocytes but the full significance of this is unclear.

BSA (fraction V, tested and found to be insulin free) was also added to the incubation medium to help maintain cell integrity during the incubation period and to reduce the non-specific binding of ^{125}I-insulin to the assay tube and hepatocytes. Under these conditions the non-specific ^{125}I-insulin binding was found to be a relatively small fraction (2-5%) of the total ^{125}I-insulin binding.

It is also noteworthy that insulin receptors have been shown to be subject to enzymatic cleavage, indeed trypsin is often used to strip cell membranes of insulin receptors(69,351).It is possible that collagenase used for the enzymatic digestion of the liver contained non-specific protease activity capable of damaging insulin receptors. Although the reported enzyme specifications were consulted to ensure this was minimal, it was difficult to assess the relative non-specific degrading activities of the different batches of collagenase. All binding assays in which results were to be quantitatively compared were therefore carried out on hepatocytes prepared with identical batches of collagenase. In addition, it was ensured that all experimental groups to be compared quantitatively, had equal numbers of hepatocyte binding assays carried out with any one ^{125}I-insulin

preparation. Thus, the possibility of biased results due to small differences in the specific activity and percentage damage in successive ^{125}I-insulin preparations was eliminated as far as possible.

2.11.3. Assay of insulin binding to isolated mouse soleus muscle.

The determination of insulin binding to isolated mouse soleus muscle was based on the method of Le Marchand et al (331).

Muscles were removed and tensioned on polyethylene miniature horseshoes as previously described (pages 99-101). Each muscle preparation was individually preincubated for 15 minutes at 20°C in 2ml of pregassed KRB (2% BSA, 2mM pyruvate, pH7.4) to wash out endogenous insulin. One of each pair of muscles was then transferred to a separate incubation vial. The muscle from the left leg was transferred to a vial containing 1.5ml of incubation medium (2% BSA, 2mM pyruvate, pH7.4) with 1ng/ml of ^{125}I-insulin for the determination of maximum total insulin binding. The muscle from the right leg was placed in an identical vial except for the presence of 50μg/ml of cold insulin to allow the determination of that proportion of ^{125}I-insulin bound non-specifically.

Sequential muscle isolations and incubations allowed the use of up to 6 animals per day generally comprising 1 or 2 animals from each experimental group.

During incubation the vials were gassed (95%O_2, 5%CO_2) for 1 minute at intervals of half an hour. After incubation each muscle was washed 5 times for a period of 5 minutes in 4ml of ice cold saline (+0.5% BSA). After washing, the muscles were removed from the horseshoes and each digested in 0.3ml of 1M NaOH (10 minutes, 100°C). 0.1ml of each digest was removed and stored frozen (-20°C) for the subsequent determination of protein content by the method of Lowry, Rosebrough, Farr and Randall (338) (Appendix, pages 227 - 228). The ^{125}I-activity associated with the remaining 0.2ml was then determined for 10 minutes on a Gamma counter (ICN, Tracerlab) at a counting efficiency of 57%.

Preliminary experiments indicated that maximum ^{125}I-insulin binding occurred after 4 hours incubation (Figure 29) and this incubation period was therefore used in subsequent studies.

2.11.4. Analysis of insulin-receptor binding data.

Data of insulin binding to isolated mouse hepatocytes was analysed throughout the present work by means of Scatchard analysis and competitive binding curves. Scatchard analysis allowed the determination of a range of affinity constants and receptor numbers which as previously explained (pages 45 - 49) were dependent upon the model of insulin-receptor binding adopted. To establish the correct molecular interpretation of the curvilinear Scatchard plots obtained from insulin binding data is beyond the scope of this thesis, but it was considered that a model based on a

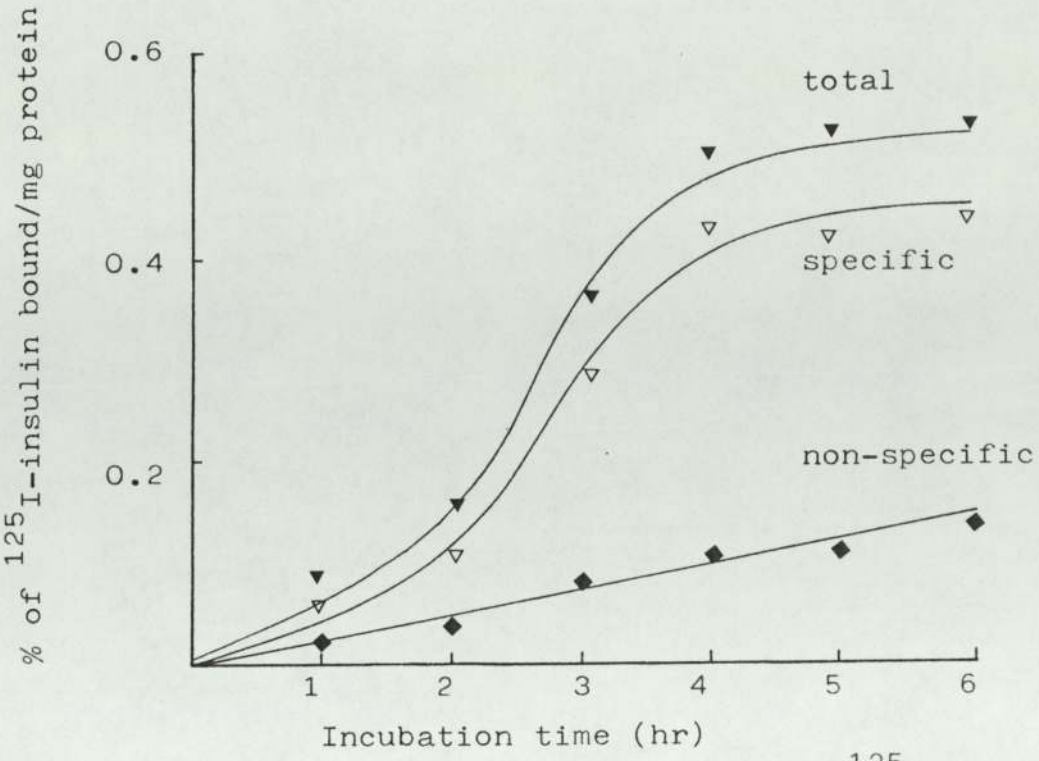

Figure 29. Time course for the binding of ^{125}I-insulin to isolated mouse soleus muscle at 20°C.

single class of insulin receptors exhibiting negative cooperativity provided a useful means for the quantitative comparison of insulin binding data. However, some consideration was given to a model comprising two classes of insulin receptors of fixed but differing affinities by presenting insulin receptor number in terms of 'high' and 'low' affinity components.

Hepatocyte insulin binding data were subjected to Scatchard analysis by means of a computer program written in Algol-68R (RECEPTORSK- Appendix, pages 232 - 233) for an ICL 1904S computer. Analyses were received on a printout along with a graph (Figure 30) prepared by a second program written in Algol-60 (RECEGRAFICA- Appendix, page 234).

Scatchard 'curves' were fitted by dividing the plot into three sections, each of which were ideal for linear regression analysis (Figure 30). It must be emphasized, however, that such a procedure makes no assumptions concerning the model used to interpret the 'curvilinearity' of Scatchard plots. The linear regression analysis thus allows the determination of abcissa intercepts for the determination of receptor binding capacities. Calculation of both total and low affinity receptor number, together with $\bar{K}e$ and $\bar{K}f$ require a knowledge of Ro obtained from extrapolation to the abcissa of the lower portion of the Scatchard plot (Figure 9). It is noteworthy that the accuracy of Ro determinations and consequently the derived binding parameters depended upon the accuracy with which the slope of the lower

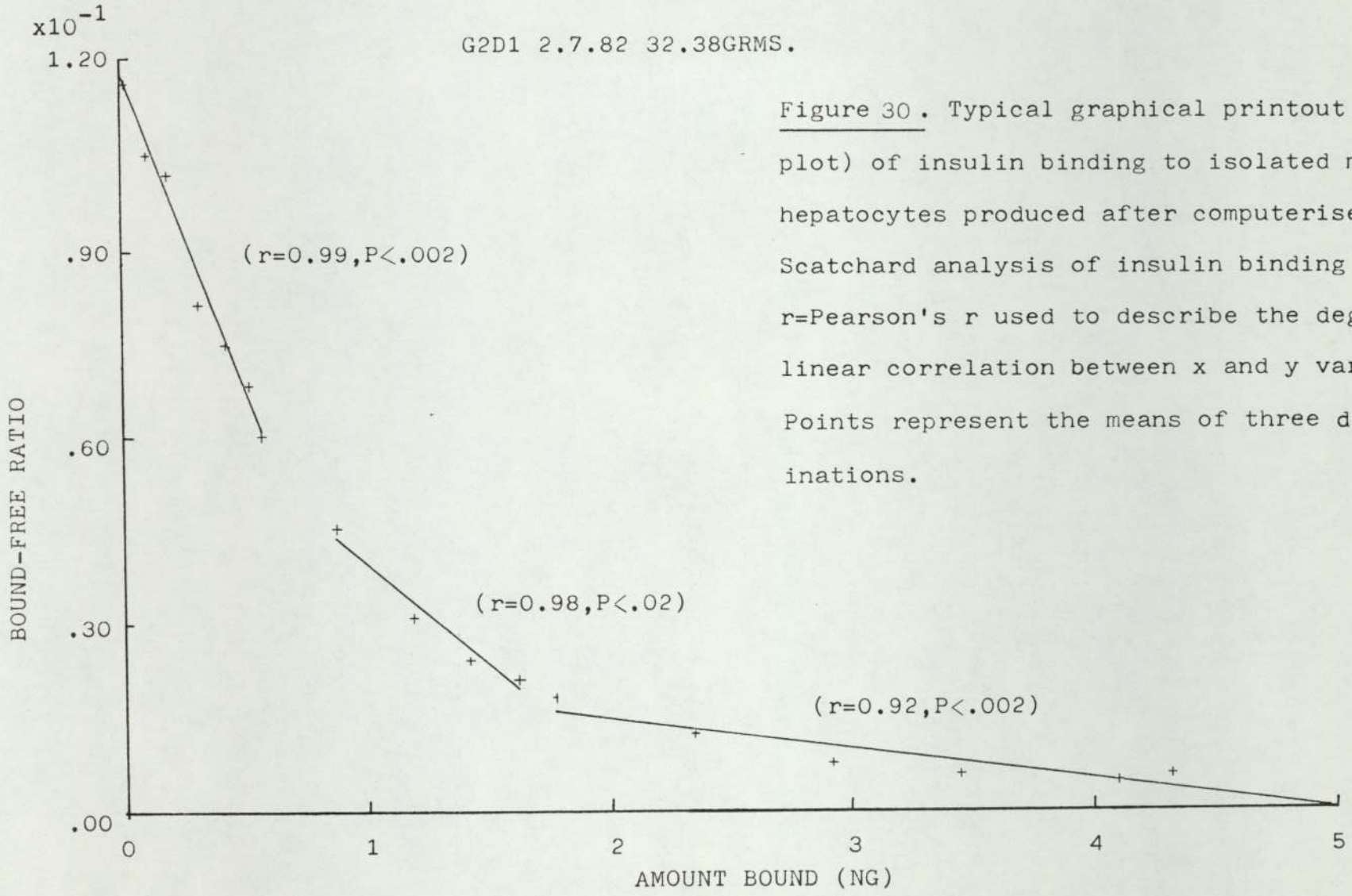

Figure 30. Typical graphical printout (Scatchard plot) of insulin binding to isolated mouse hepatocytes produced after computerised Scatchard analysis of insulin binding data. r=Pearson's r used to describe the degree of linear correlation between x and y variables. Points represent the means of three determinations.

portion of the Scatchard plot could be determined.If the displacement of ^{125}I-insulin from receptors is insensitive to increasing insulin concentrations, for example in erythrocyte insulin receptor assays (181) extrapolation to the abcissa is difficult and unreliable. However, hepatocyte ^{125}I-insulin binding was sensitive to increasing insulin concentrations and it was considered that Ro determinations were reliable. Furthermore the lower slopes of the Scatchard plots, based upon 6 points, showed excellent linear correlation (generally $P<0.02$, Figure 30), inferring accurate determination of the slope and thus the abcissa intercept.

Data from soleus muscle insulin binding assays could not be subjected to Scatchard analysis because of the limited number of binding determinations allowed by the two soleus muscles available from each mouse. Analysis of these binding studies were limited therefore to the determination of total and non-specific ^{125}I-insulin binding, and thus, by difference, the maximum specific ^{125}I-insulin binding.

2.12. Statistical analysis.

Straight lines were fitted by the method of least squares and linear correlation was assessed by Pearson's correlation coefficient (Pearson's r). Estimation of significance of differences between mean values was by Student's t test, unpaired unless otherwise stated. Data in this thesis are presented as the mean ±SEM (standard error of the sample mean).

3. Results.

For the sake of clarity the results of the present study have been divided into three sections.

Section 3.1.

This section describes several parameters of carbohydrate metabolism and insulin action in intact female mice during the oestrus and dioestrus phases of the oestrous cycle. The parameters selected for study include the hypoglycaemic action of exogenous insulin, the rate of glucose oxidation and glycogenesis by isolated soleus muscle, the glycogen content of soleus muscle and liver and ^{125}I-insulin binding to both isolated soleus muscle and hepatocytes.

Section 3.2.

This section describes the effect of ovariectomy of 10 weeks duration on selected parameters of carbohydrate metabolism and insulin action. In addition the effect of ovariectomy of 2 weeks duration on insulin binding to isolated soleus muscle and hepatocytes has been described.

Section 3.3.

This final section describes the results of investigations involving the effect of ovarian sex steroid replacement regimes of 10 weeks duration in ovariectomised mice on the selected parameters of carbohydrate metabolism and insulin action. In addition the effect of 2 week hormone replacement regimes on insulin binding to isolated soleus muscle and hepatocytes has been described.

Glossary of abbreviations used in Tables 4 - 33

O oestrus stage of the oestrous cycle.

D dioestrus stage of the oestrous cycle.

E oestradiol administration at a dose of 5μg/kg/day to ovariectomised mice.

EHD oestradiol administration at a dose of 500μg/kg/day to ovariectomised mice.

E+P oestradiol (5μg/kg/day) and progesterone (1mg/kg/day) administration to ovariectomised mice.

P progesterone administration at a dose of 1mg/kg/day to ovariectomised mice.

3.1. Insulin-receptor binding and insulin action during the oestrus and dioestrus stages of the oestrous cycle in mice.

The assay of plasma oestradiol concentration confirmed the presence of greater circulating levels of this hormone in mice in the oestrus phase of the oestrous cycle (167 pg/ml) compared to levels at dioestrus (<25pg/ml), (Table 4). Although plasma progesterone concentrations were not monitored in the present study, it was assumed that plasma progesterone would also be elevated at oestrus with a reduction to minimum at dioestrus (Appendix, pages 218-219). These natural cyclical alterations in sex steroid titres were not however, accompanied by alterations in freely fed or starved (12 hours) plasma glucose and insulin concentrations (Table 4).

The hypoglycaemic action of intraperitoneally administered insulin (0.35U/kg body weight) was significantly reduced at dioestrus compared to oestrus. Thus plasma glucose concentrations were greater in dioestrus mice compared to oestrus mice for the 60 minute period following the injection of insulin (Figure31). The area above the glucose curve during insulin hypoglycaemia tests (K_A) (the total insulin induced plasma glucose disappearance) was therefore significantly reduced on the day of dioestrus compared to the day of oestrus (8.13 ±1.06 versus 16.96 ±0.82 mM glucose/hour respectively, $P<0.01$), (Table 5).

The rate of glucose oxidation by isolated soleus muscle

	n	Oestradiol (pg/ml)	Plasma glucose (mmol/l)		Plasma insulin (ng/ml)	
			Fed	12hr starved	Fed	12hr starved
Oestrus	6	167	11.26 ±0.49	6.05 ±0.453	2.20 ±0.13	1.29 ±0.01
Dioestrus	6	25*	10.92 ±0.48	6.45 ±0.377	2.36 ±0.15	1.37 ±0.16

TABLE 4.

Plasma oestradiol, glucose and insulin concentrations in fed and 12 hr starved mice at the oestrus and dioestrus stages of the oestrous cycle. * indicates $P<0.05$ for dioestrus compared to oestrus mice.

	n	K_A
Oestrus	6	16.96 ±0.82
Dioestrus	6	8.13 ±1.06***

TABLE 5.

The total insulin induced plasma glucose disappearance (K_A) during the 1 hour period following the injection of insulin at a dose of 0.35U/kg body weight in mice at the oestrus and dioestrus stages of the oestrous cycle.

*** indicates $P<0.01$ for dioestrus compared to oestrus mice.

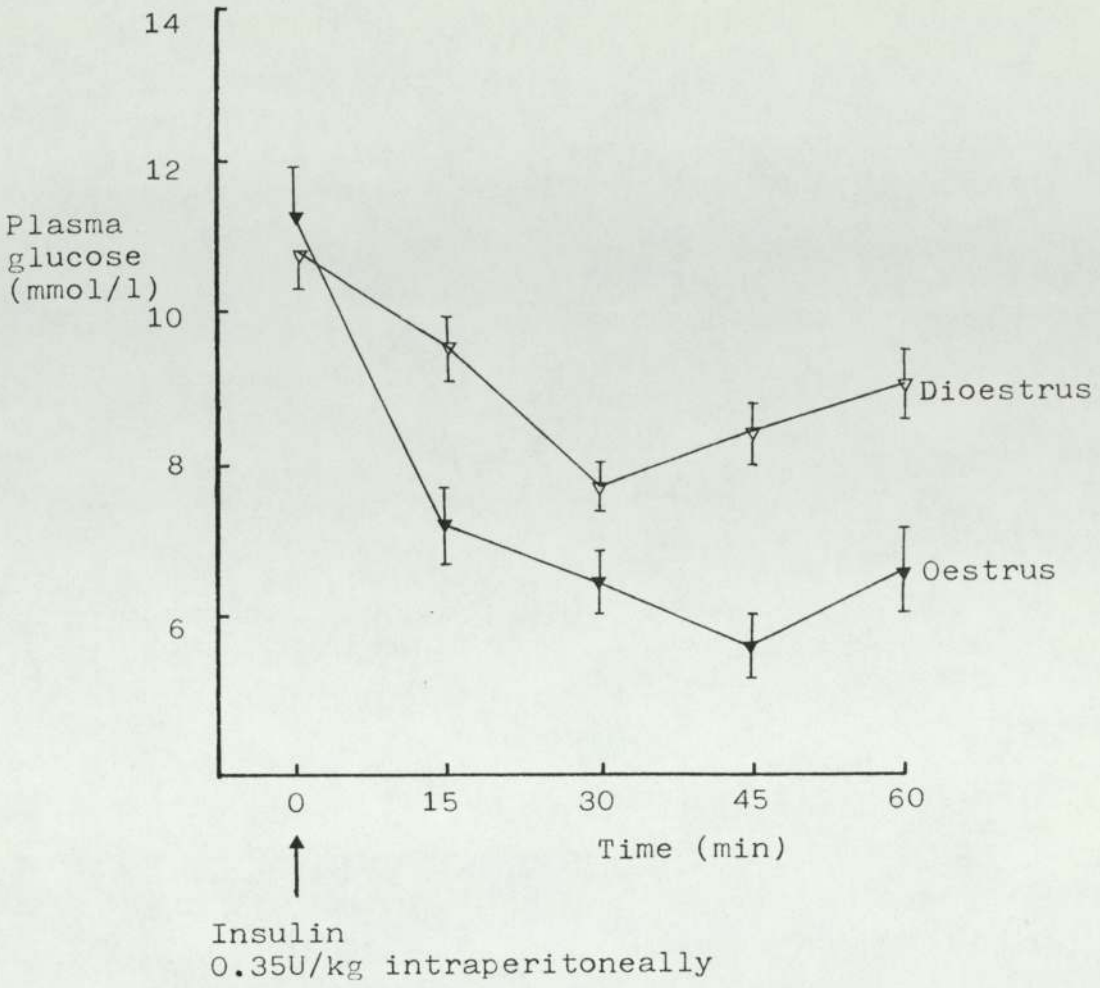

Figure 31. Insulin hypoglycaemia tests in freely fed mice at the oestrus and dioestrus stages of the oestrous cycle. Points are means of 6 values ± SEM.

as assessed by the rate of $^{14}CO_2$ production from ^{14}C-glucose, was not significantly different at oestrus compared to dioestrus either in the absence or presence of insulin (20ng/ml (Table 6). This was true whether the results were expressed in terms of the rate of $^{14}CO_2$ production/ muscle (dpm/ muscle/2hr) or the rate of $^{14}CO_2$ production per mg of protein (dpm/mg of muscle protein/2hr). Insulin stimulated $^{14}CO_2$ production by isolated soleus muscles from oestrus and dioestrus mice, defined as the calculated difference between production in the presence and absence of insulin (page 106) were not therefore significantly different.

Table 7 indicates that the basal rates of ^{14}C-glycogen synthesis from ^{14}C-glucose in isolated soleus muscles of oestrus and dioestrus mice were not significantly different. There was a suggestion of a reduced rate of ^{14}C-glycogen synthesis by isolated soleus muscles of dioestrus mice, both in the presence of insulin and when the data were expressed in terms of the rate of insulin stimulated ^{14}C-glycogen synthesis. These reductions were not generally significant with the one exception of a 34% reduction in the rate of ^{14}C-glycogen production per muscle in the presence of insulin. It is noteworthy that such a significant reduction ($P<0.05$), was only observed when the results were expressed in terms of the rate of ^{14}C-glycogen production per muscle but not when expressed in terms of the rate of ^{14}C-glycogen production per mg of muscle protein. This was because of a slightly lower protein content of soleus muscles from dioestrus mice compared to muscles from oestrus mice although

		$^{14}CO_2$ production (per 2hr incubation)					
		Basal		In the presence of insulin (20ng/ml)		Insulin stimulated	
	n	dpm/mg protein	dpm/ muscle	dpm/mg protein	dpm/ muscle	dpm/mg protein	dpm/ muscle
Oestrus	6	4327 ±482	7842 ±880	4593 ±667	8272 ±499	475 ±534	366 ±1120
Dioestrus	6	5892 ±842	9346 ±1341	5924 ±1081	9628 ±821	31.2 ±494	129 ±876

Table 6.

The rate of $^{14}CO_2$ production from ^{14}C-glucose in the absence and presence of exogenous insulin (20ng/ml) by isolated soleus muscle of mice at the oestrus and dioestrus stages of the oestrous cycle.

		^{14}C-glycogen production (per 2hr incubation)					
		Basal		In the presence of insulin (20ng/ml)		Insulin stimulated	
	n	dpm/mg protein	dpm/ muscle	dpm/mg protein	dpm/ muscle	dpm/mg protein	dpm/ muscle
Oestrus	6	1497 ±195	2947 ±442	3777 ±414	6997 ±353	2280 ±445	4060 ±428
Dioestrus	6	1448 ±215	2366 ±200	2690 ±379	4610* ±797	1242 ±327	2254 ±718

TABLE 7.

The rate of ^{14}C-glycogen synthesis from ^{14}C-glucose in the absence and presence of exogenous insulin (20ng/ml) by isolated soleus muscle of mice at the oestrus and dioestrus stages of the oestrous cycle. * indicates $P<0.05$ for dioestrus compared to oestrus mice.

this reduction was clearly not significant (Appendix, Table 33, Panel 1). These data indicated that the protein content of mouse soleus muscle was not determined by the stage of the oestrous cycle. Furthermore, this suggested that data should be expressed not only in terms of muscle protein content but also per muscle.

The glycogen content of soleus muscle from dioestrus mice was significantly reduced compared to soleus muscles from oestrus mice (Table 8). This reduction was significant when expressed either in terms of the glycogen content per mg of muscle protein (41% reduction) or the glycogen content per gram wet weight of muscle (40% reduction) although the reduction in the total muscle glycogen (22%) was not significant. The total glycogen content of the liver and indeed the wet weight of the liver were, however, not significantly different in dioestrus and oestrus mice (Table 8).

There was no significant difference between the maximum specific ^{125}I-insulin binding to isolated soleus muscles from mice at the dioestrus and oestrus stages of the oestrous cycle (Table 9). The various parameters of hepatocyte insulin receptor status (receptor numbers and affinity constants) were also not significantly different at the two selected stages of the oestrous cycle (Table 10). Furthermore, hepatocyte size was not significantly different at the two selected stages of the oestrous cycle, the diameters being 20.48 ±0.58μm (n=9)

	n	LIVER			MUSCLE			
		Wet weight (gm)	Total glycogen content (mg)	Glycogen/ gm wet weight (mg)	Wet weight (mg)	Total glycogen content (mg)	mg Glycogen/ mg muscle protein	mg Glycogen/ gm wet weight
O	6	1.6698 ±0.0865	18.55 ±4.38	11.02 ±2.35	7.6 ±0.47	0.0137 ±0.0011	0.0092 ±0.0011	1.843 ±0.183
D	6	1.6839 ±0.0857	28.28 ±7.14	16.37 ±3.94	8.03 ±0.48	0.0107 ±0.0014	0.0054* ±0.0008	1.103★ ±0.172

TABLE 8.

Wet weights and glycogen content of liver and muscle from mice in the oestrus and dioestrus stages of the oestrous cycle. * indicates $P<0.05$ for dioestrus compared to oestrus mice.

	n	% of total CPM ^{125}I-insulin/mg protein	% of total CPM ^{125}I-insulin/ muscle	insulin bound (ng x10^{-3})/mg protein	insulin bound (ngx10^{-3})/ muscle
O	6	0.154 ±0.037	0.248 ±0.051	2.080 ±0.588	3.548 ±0.723
D	6	0.167 ±0.026	0.325 ±0.055	2.392 ±0.364	4.651 ±0.784

TABLE 9 .

The specific binding of ^{125}I-insulin to isolated soleus muscle of freely fed adult mice in the oestrus and dioestrus stages of the oestrous cycle.

	n	High affinity receptor number/ cell	Low affinity receptor number/ cell	Total receptor number/ cell	$\bar{K}e_{nM^{-1}}$	$\bar{K}f_{nM^{-1}}$
O	9	111459 ±10627	314166 ±30518	426540 ±38375	0.179 ±0.021	0.0323 ±0.0021
D	11	85481 ±9270	344786 ±23733	430313 ±27487	0.226 ±0.036	0.0261 ±0.003

TABLE10.

Insulin receptor status of hepatocytes isolated from freely fed adult mice in the oestrus and dioestrus stages of the oestrous cycle.

and 20.73 +0.24μm(n=11) for hepatocytes from mice at oestrus and dioestrus respectively.

No significant differences in either body weight or soleus muscle protein content were found in dioestrus compared to oestrus mice throughout this study (Appendix, Tables 32 and 33).

3.2. The effect of ovariectomy on insulin-receptor binding and insulin action in mice.

Investigation of the effects of ovariectomy on carbohydrate metabolism and insulin action has involved the determination of the parameters under study in both ovariectomised and intact mice. Where no significant difference had been observed for a particular parameter between mice in the dioestrus and oestrus stages of the oestrous cycle (see Section3 .1), values obtained for that parameter in dioestrus and oestrus mice have been combined and presented as data from intact mice unless otherwise stated. However, when a significant difference was observed between dioestrus and oestrus mice for a particular parameter, values for both stages of the oestrous cycle have been presented and have both been statistically compared to values observed for ovariectomised mice.

The plasma oestradiol concentrations of mice that had been ovariectomised for 10 weeks were reduced to approximately those concentrations seen in the dioestrus phase of the oestrous cycle (<25pg/ml) (Table11). After 10 weeks ovariectomy these mice had vaginal smears typical of dioestrus mice (not shown). Although plasma progesterone concentrations were not monitored in the present study it was assumed ovariectomy would result in a low physiological concentration of the hormone.

Although ovariectomy of 10 weeks duration was not accompanied by alterations in either fed plasma

	n	Oestradiol pg/ml		Plasma glucose mmol/l	Plasma insulin ng/ml
Oestrus	6	167	Intact Oestrus and dioestrus	8.56 ±0.34 (18)	2.56 ±0.47 (18)
Dioestrus	6	<25*			
OVX	6	<25*	OVX	8.02 ±0.24 (18)	2.31 ±0.38 (18)

TABLE 11.

The effect of ovariectomy for a period of 10 weeks on plasma concentrations of oestradiol, glucose and insulin in freely fed mice. Figures in brackets represent the number of determinations. * indicate $P<0.05$ compared to oestrus mice.

glucose or insulin concentrations (Table 11), ovariectomy was, however, associated with a significant reduction in the hypoglycaemic action of insulin compared to that seen in oestrus mice (Table12). Thus, ovariectomy of 3,6 and 9 weeks duration was associated with a significant reduction in the initial rate of insulin induced plasma glucose disappearance. This was indicated by a significantly higher plasma glucose concentration 15 minutes after insulin injection in ovariectomised mice compared to intact oestrus mice. Only long-term ovariectomy for 6 and 9 weeks significantly reduced the total plasma glucose disappearance (K_A) during the 1 hour insulin hypoglycaemia test compared to that observed in oestrus mice (Table 12).

The amount of food and water consumed over a 10 day period by intact mice (approximately 2 oestrous cycles) and ovariectomised mice (8-10 weeks duration) was not significantly different (Table13). Indeed the body weights of intact and ovariectomised mice were not significantly different at the time of sacrifice (Appendix, Table 32) and hence these animals were both age and weight matched.

Compared to intact mice, ovariectomy of 10 weeks duration significantly reduced the rate of ^{14}C-glucose oxidation by isolated soleus muscles in the absence of insulin (basal $^{14}CO_2$ production) (Table14). This reduction was significant whether the results were expressed either in terms of the rate of $^{14}CO_2$ production per mg protein or $^{14}CO_2$ production per muscle. Similarly, ovariect-

	n	Time from insulin injection					K_A
		0	15	30	45	60	
Intact (oestrus)	6	11.27 ±0.49	7.50 ±0.31	6.55 ±0.38	5.63 ±0.40	6.58 ±0.53	16.96 ±0.82
Intact (dioestrus)	6	10.92 ±0.48	9.60** ±0.45	7.68* ±0.23	8.37*** ±0.40	8.87** ±0.40	8.31*** ±1.06
3 weeks OVX	6	11.75 ±0.50	9.27** ±0.43	7.777 ±0.65	7.33 ±0.83	6.38 ±0.75	13.21 ±1.59
6 weeks OVX	6	10.60 ±0.28	9.10** ±0.20	7.48 ±0.27	6.62 ±0.32	5.97 ±0.35	10.92*** ±0.62
9 weeks OVX	6	11.30 ±0.48	10.00** ±0.50	7.97** ±0.26	8.43*** ±0.34	8.97** ±0.32	8.65*** ±0.90
		Blood glucose concentration mmol/l					

TABLE 12.

The effect of ovariectomy and its duration on the sensitivity of fed mice to exogenous insulin (0.35U/kg. body weight) administered intraperitoneally.

*P<0.05, **P<0.02 and ***P<0.01 when ovariectomised mice were compared with intact oestrus mice.

	Food consumption		Water consumption	
	gm/mouse /day	gm/kg body wt/day	Gm/mouse /day	Gm/kg body wt/day
Intact	5.58 ±0.45	153.7	7.17 ±0.70	197.5
8-10wks OVX	5.03 ±0.63	141.7	6.05 ±0.67	170.4

TABLE 13.

Food and water consumption over a consecutive 10 day period for intact and ovariectomised adult female mice. Mice were housed in cages of five and allowed free access to food and water.

		$^{14}CO_2$ production (per 2hr incubation)					
		Basal		In the presence of insulin (20ng/ml)		Insulin stimulated	
	n	dpm/mg protein	dpm/muscle	dpm/mg protein	dpm/muscle	dpm/mg protein	dpm/muscle
Intact (oestrus and dioestrus)	12	5110 ±532	8735 ±655	5258 ±663	8809 ±732	253 ±369	119 ±714
OVX	6	3082 ±507*	5404 ±1059**	3467 ±398	5813 ±801*	385 ±415	556 ±747

TABLE 14.

The effect of ovariectomy for a period of 10 weeks on the rate of $^{14}CO_2$ production from ^{14}C-glucose in the absence and presence of exogenous insulin (20ng/ml) by isolated soleus muscle.* $P<0.05$,**$P<0.02$ when ovariectomised mice were compared to intact mice

omy was associated with a reduction in the rate of ^{14}C-glucose oxidation by isolated soleus muscle in the presence of insulin (Table14). This reduction was not significant when expressed in terms of the rate of $^{14}CO_2$ production per milligram of muscle protein. There was a small although not significant reduction in the protein content of soleus muscles from ovariectomised mice compared to muscles from intact mice; 1.70 ±0.08 and 1.86 ±0.07mg protein per muscle respectively, (Appendix, Table33, Panel 1). This revealed a significant reduction (34%) in the rate of ^{14}C-glucose oxidation by isolated soleus muscles from ovariectomised mice when expressed in terms of the rate of $^{14}CO_2$ production per muscle. The parallel reduction (~40%) in the rate of ^{14}C-glucose oxidation, both in the absence and presence of insulin by isolated soleus muscle from ovariectomised mice meant that ovariectomy was not associated with a reduction in the calculated rate of insulin stimulated $^{14}CO_2$ production (Table14).

The rate of ^{14}C-glycogen synthesis by isolated soleus muscles in the absence of exogenous insulin was not significantly different in mice ovariectomised for a period of 10 weeks and intact mice in either the oestrus or dioestrus stages of the oestrous cycle (Table15). The rate of ^{14}C-glycogen synthesis in the presence of insulin and the calculated rate of insulin stimulated ^{14}C-glycogen production were, however, increased in isolated soleus muscles from ovariectomised mice compared to muscles from intact mice (Table15). These increases were significant when

		^{14}C-glycogen production (per 2hr incubation)					
		Basal		In the presence of insulin (20ng/ml)		Insulin stimulated	
	n	dpm/mg protein	dpm/muscle	dpm/mg protein	dpm/muscle	dpm/mg protein	dpm/muscle
Oestrus	6	1497 ±195	2947 ±442	3777 ±414	6997 ±353	2280 ±445	4060 ±428
Dioestrus	6	1448 ±215	2366 ±200	2690 ±379	4610 ±797	1242 ±327	2254 ±718
OVX	6	1712 ±518	2645 ±645	7165 ±1530*	11572 ±2253*	5452.5 ±1452*	8745 ±2178*

TABLE 15.

The effect of ovariectomy for a period of 10 weeks on the rate of ^{14}C-glycogen synthesis from ^{14}C-glucose in the absence and presence of exogenous insulin (20ng/ml) by isolated soleus muscle. * indicates $P<0.05$ for ovariectomised compared to dioestrus mice.

expressed either in terms of the rate of ^{14}C-glycogen production per mg muscle protein or per soleus muscle, but only when compared with mice in the dioestrus phase of the oestrous cycle (Table 15).

Concomitant with the observed increases in the rate of ^{14}C-glycogen synthesis, the glycogen content of soleus muscles from mice ovariectomised for a 10 week period was increased compared to soleus muscles from intact animals (Table 16). Again this increase was significant only when the glycogen content of soleus muscles from ovariectomised mice and mice in dioestrus were compared.

The wet weight of livers from mice ovariectomised for 10 weeks and from intact female mice were not significantly different (Table 16). However, contrary to the increases observed in soleus muscle glycogen content (see above), the glycogen content of the liver, whether expressed in terms of the total glycogen content or glycogen per gram wet weight of liver, was significantly reduced in ovariectomised compared to intact mice by 65% and 60% respectively (Table 16).

The binding of insulin to its receptors on both isolated hepatocytes and isolated soleus muscle was affected by the extent of the period of ovariectomy. The maximum specific binding of ^{125}I-insulin to isolated soleus muscles from mice ovariectomised for a short duration (2 weeks) and from intact mice was not significantly different (Table 17).

		LIVER					MUSCLE			
	n	Wet weight (gm)	Total glycogen content (mg)	mg Glycogen /gm wet weight		n	Wet weight (mg)	Total glycogen content (mg)	mg Glycogen/ mg muscle protein	mg Glycogen /gm wet weight
Intact O+D	12	1.6768 ± 0.0609	23.41 ±4.42	13.69 ±2.42	O	6	7.6 ±0.47	0.0137 ±0.0011	0.0092 ±0.0011	1.84 ±0.18
					D	6	8.03 ±0.48	0.0107 ±0.0014	0.0054 ±0.0008	1.10 ±0.17
OVX	6	1.5034 ± 0.0379	8.19 ±1.95†	5.52 ±1.34†	OVX	12	8.41 ±0.34	0.0182 ±0.0019*	0.0135 ±0.0014***	2.20 ±0.22**

TABLE 16.

The effect of ovariectomy for a period of 10 weeks on the wet weight and glycogen content of liver and soleus muscle from freely fed mice.

† indicates $P<0.05$ for ovariectomised compared to intact mice. *$P<0.05$, **$P<0.02$, ***$P<0.01$ when ovariectomised mice are compared to dioestrus mice.

	n	% of total cpm ^{125}I-insulin/ mg protein	% of total cpm ^{125}I-insulin/ muscle	Insulin bound (ngx10^{-3})/mg protein	Insulin bound (ngx10^{-3}/ muscle
Intact O+D	12	0.160 ±0.023	0.286 ±0.039	2.236 ±0.348	4.099 ±0.556
2 weeks OVX	5	0.173 ±0.032	0.278 ±0.050	2.477 ±0.429	3.995 ±0.687
Intact D	6	0.197 ±0.026	0.271 ±0.033	2.951 ±0.378	4.059 ±0.482
10 weeks OVX	6	0.134 ±0.015	0.169* ±0.013	2.017 ±0.223	2.543* ±0.210

TABLE 17

The effect of ovariectomy for periods of 2 and 10 weeks on the maximum specific ^{125}I-insulin binding to isolated mouse soleus muscle. * indicates $P<0.05$ for ovariectomised compared to intact mice.

Ovariectomy of 10 weeks duration did, however, reduce the maximum specific binding of ^{125}I-insulin to isolated soleus muscle. This reduction, although apparent when data were expressed in terms of the specific ^{125}I-insulin binding capacity per milligram of muscle protein, was significant (a reduction of 37%) only when the binding data were expressed in terms of the maximum specific ^{125}I-insulin binding capacity per soleus muscle (Table 17).

A significant reduction (30%) in the total number of hepatocyte insulin receptors was apparent after acute ovariectomy of 2 weeks duration compared to intact dioestrus mice (Figure 32). When the data were considered in terms of two classes of insulin receptor (pages 45 - 49), the decrease was largely accounted for by a reduction (37%) in the number of receptors designated as having a low affinity and a high capacity for insulin (Table 18). However, the affinity constants $\bar{K}e$ and $\bar{K}f$ were significantly increased by 61% and 77% respectively (Table 18). This suggested that the insulin binding capacity of hepatocytes from mice ovariectomised for a period of 2 weeks and hepatocytes from intact mice would not be significantly different particularly at insulin concentrations below those required to saturate the receptors. This was indeed established by plotting the two competition curves (Figure 33) which indicated an unchanged binding capacity for the hepatocytes from mice in both groups, at all insulin concentrations employed. Ovariectomy for a 10 week period was associated with a significant 23% reduction in the total number of hepato-

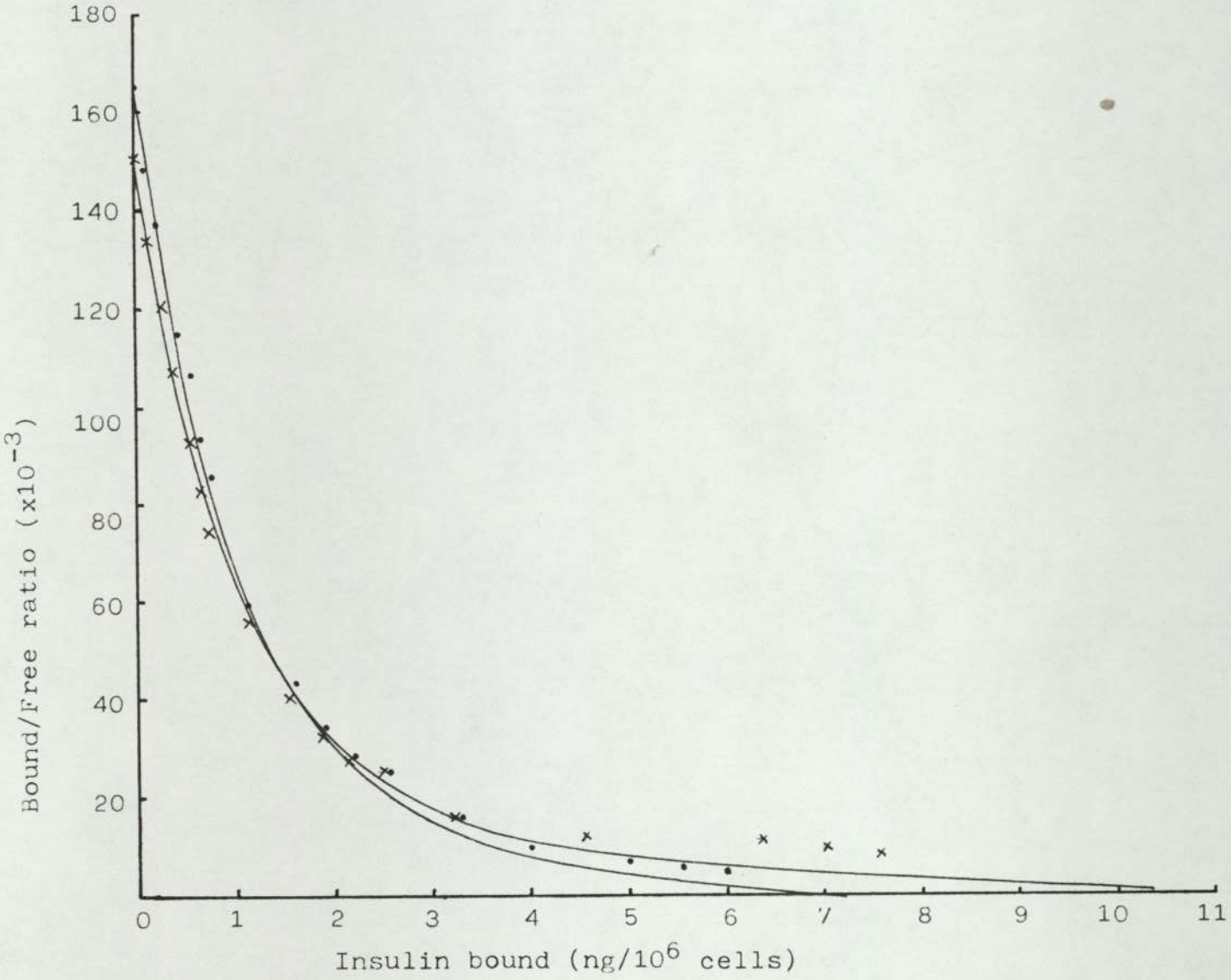

Figure 32. Scatchard plots of insulin binding to isolated hepatocytes of intact (dioestrus) mice(X) and mice ovariectomised for a period of 2 weeks(•). Each point represents the mean of 6 determinations Data derived from these plots are provided in Table 18.

	n	High affinity receptor number /cell	Low affinity receptor number /cell	Total receptor number /cell	$\bar{K}_e$ nM^{-1}	$\bar{K}_f$ nM^{-1}
Intact (dioestrus)	6	173,065 ±10,592	961,857 ±102,656	1,116,358 ±121,326	0.086 ±0.009	0.0150 ±0.0011
2 weeks OVX	6	154,501 ±12,765	604,830* ±82,434	777,895* ±91,216	0.183* ±0.017	0.0265* ±0.0037
Intact (dioestrus)	5	160,108 ±17,744	680,688 ±28,706	819,233 ±22,613	0.098 ±0.009	0.0199 ±0.0015
10 weeks OVX	5	145,119 ±9,594	498,593* ±55,146	630,777* ±61,141	0.118 ±0.012	0.0245 ±0.0034

TABLE 18.

The effect of ovariectomy for periods of 2 and 10 weeks on the insulin receptor status of hepatocytes isolated from freely fed adult mice. * indicates $P<0.05$ for ovariectomised compared to intact mice.

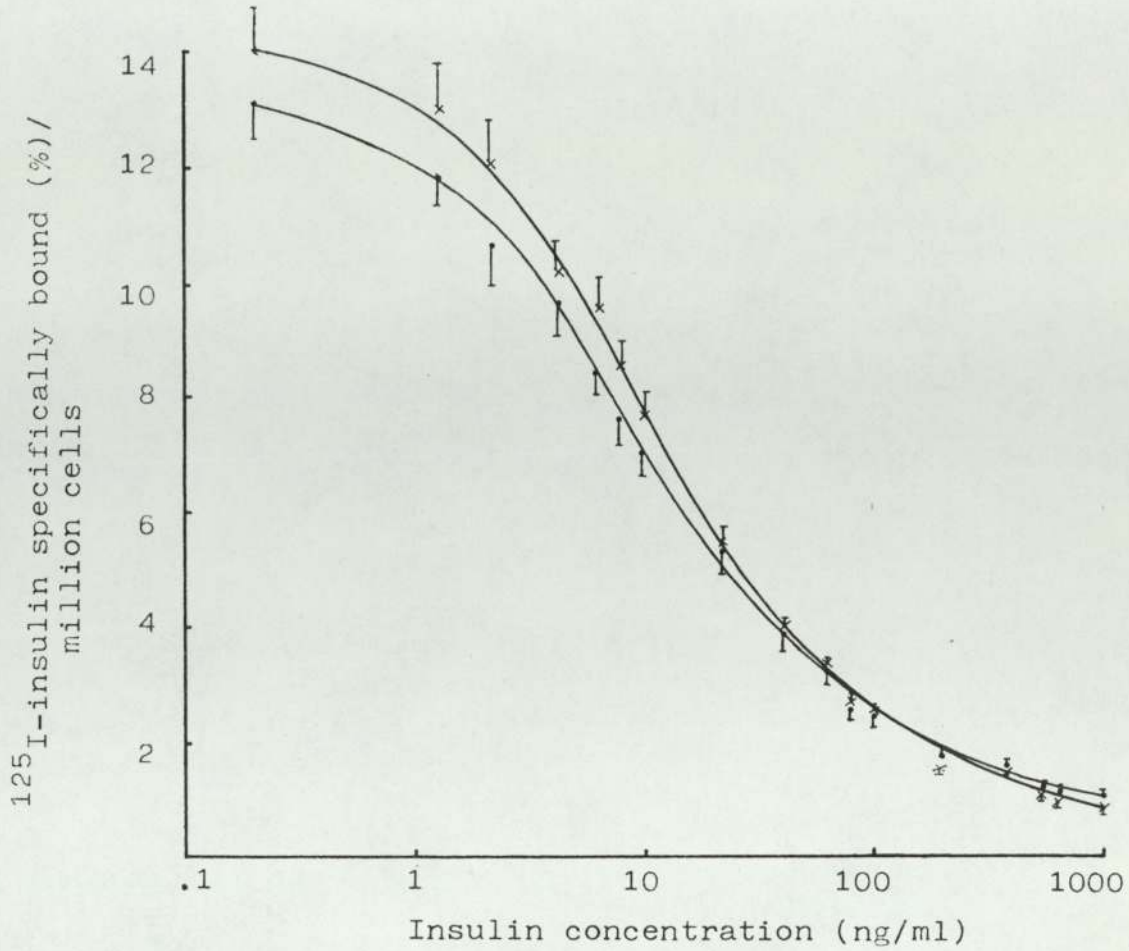

Figure 33. Competition curves of ^{125}I-insulin binding to isolated hepatocytes from intact mice (X) and mice ovariectomised for a period of 2 weeks (•). Each point represents the mean of 6 determinations, bars are ± SEM. No significant differences (Student's t test) were observed between the two curves at any of the insulin concentrations employed.

cyte insulin receptors compared to intact dioestrus mice (Figure 34). As with acute ovariectomy, this reduction was largely due to a significant 27% reduction in the number of low affinity,high capacity insulin receptors (Table 18). However, contrary to the increases in $\bar{K}e$ and $\bar{K}f$ noted after acute (2 weeks) ovariectomy, these affinity constants did not change significantly after chronic (10 weeks) ovariectomy (Table 18). It is important to note that the reduction in the number of hepatocyte insulin receptors after ovariectomy of 10 weeks duration was not due to an alteration in hepatocyte size, the diameters being 20.20 ±0.64um and 20.53 ±0.54um for hepatocytes from intact and ovariectomised animals respectively.

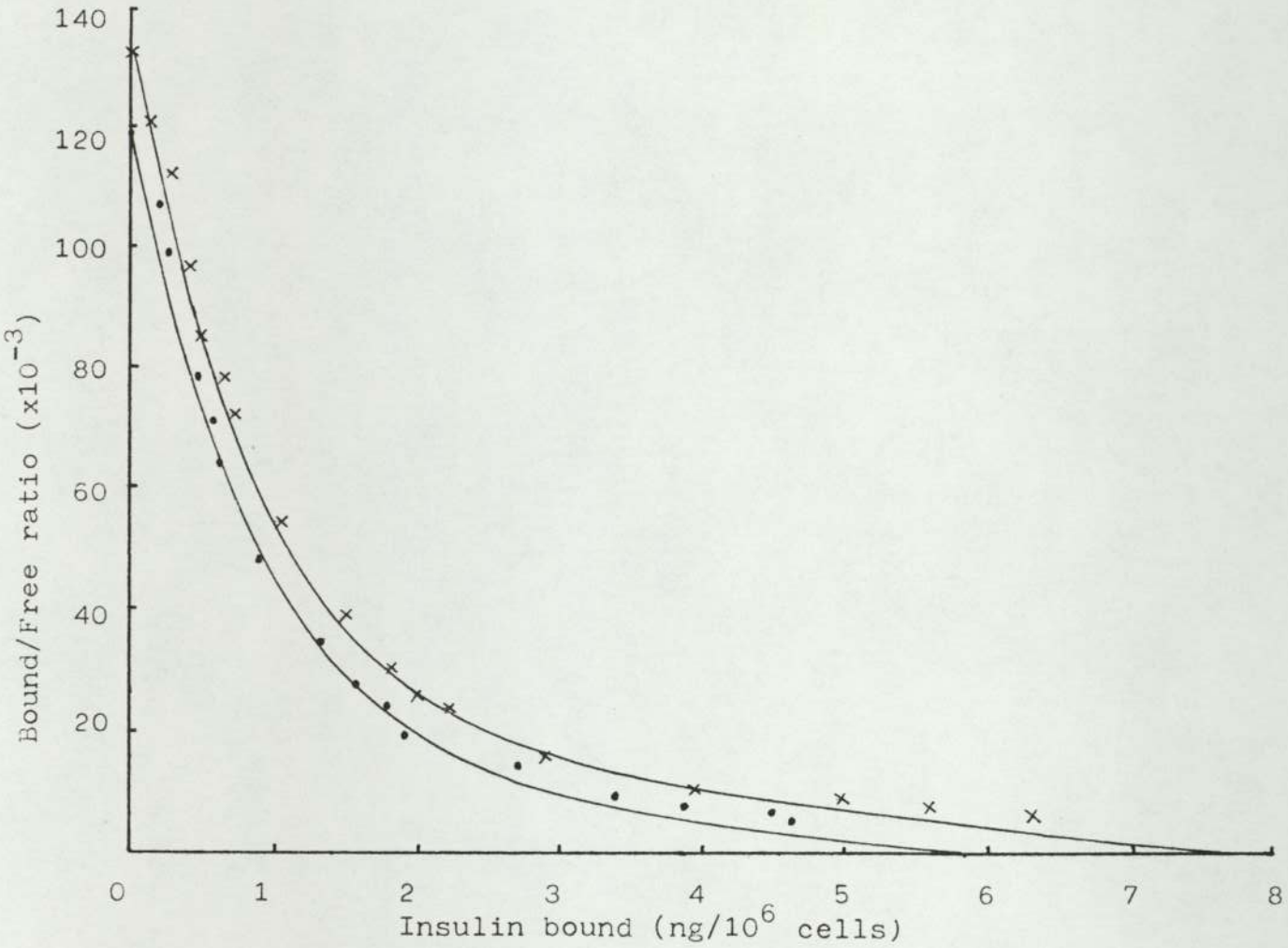

Figure 34. Scatchard plots of insulin binding to isolated hepatocytes from intact (dioestrus) mice(X) and mice ovariectomised for a period of 10 weeks (•). Each point represents the mean of 5 determinations. Data derived from these plots are provided in Table 18.

3.3. The effect of oestradiol and progesterone administration on insulin-receptor binding and insulin action in ovariectomised mice.

The effect of oral sex steroid administration on the parameters selected for study was determined in ovariectomised mice.

Interpretation of the effect on plasma oestradiol concentrations of the administration of oestradiol (5μg/kg/day) either alone or in combination with progesterone (1mg/kg/day) was difficult because of the relative insensitivity of the assay at low concentrations of oestradiol (<60pg/ml, Appendix, pages 224-226). The administration of oestradiol at a higher dose (500μg/kg/day), in an effort to examine the dose dependency of the actions of oestradiol, produced a significantly elevated mean plasma oestradiol concentration (180pg/ml, Table19) but with a wide distribution of values (68-450 pg/ml). Vaginal smears of these mice were typical of mice in the oestrus phase of the oestrous cycle (Appendix, pages 218-219) and consistent with vaginal cornification observed after the administration of high doses of oestradiol (352). Mice administered with oestradiol (5μg/kg/day) and progesterone (1mg/kg/day) either alone or in combination had vaginal smears typical of mice in the dioestrus phase of the cycle.

In general, the plasma glucose and insulin concentrations of freely fed mice, both ovariectomised and hormone treated for 10 weeks, were not significantly different.

	n	OVX	E	E(HD)	E+P	P
Plasma Oestradiol pg/ml	6	<25	33	180*	33	33
Fed plasma glucose mmol/l	18	8.56 ±0.34	9.24 ±0.27	6.92*** ±0.22	8.56 ±0.27	8.85 ±0.21
Fed plasma insulin ng/ml		2.31 ±0.38 (17)	3.03 ±0.59 (13)	2.02 ±0.43 (17)	1.48 ±0.25 (18)	2.07 ±0.50 (14)

TABLE 19.

The effect of 10 week hormone replacement regimes on the plasma oestradiol, glucose and insulin concentrations in mice. * and ***indicate $P<0.05$ and <0.01 respectively when compared to untreated ovariectomised mice. The number of determinations are given in brackets.

	n	OVX	E	E(HD)	E+P	P
Panel A Saline (4.36ml/kg) i.p. K_B	6	3.84 ±1.21	7.00 ±1.21	7.40 ±1.60	2.76 ±1.36	6.77 ±1.36
Panel B Insulin (0.35U/kg) i.p. K_A	6	2.24 ±1.79	4.41 ±1.93	3.46 ±1.44	11.34* ±3.14	6.51 ±4.04
Panel C K_T	6	6.08 ±1.30	11.41 ±1.78	11.04* ±1.44	14.12 ±2.75	13.28* ±3.55

TABLE 20.

The effect of 10 week hormone replacement regimes on the area below (K_B) and above (K_A) plasma glucose curves during the 1 hour period following the intraperitoneal administration of saline and insulin respectively. K_T is the sum of K_B and K_A and represents the total insulin induced plasma glucose disappearance. Units are mmol glucose /hour, * indicates $P<0.05$ when compared to untreated ovariectomised mice.

However, mice treated with the higher dose of oestradiol (500μg/kg/day) had a significantly reduced plasma glucose concentration (8.56 ±0.34 and 6.92 ±0.22 mmol/l for ovariectomised and treated mice respectively).

When considered in isolation, the results of the insulin hypoglycaemia tests on 10 week treated mice suggested that only the combined oestradiol and progesterone replacement regime improved the hypoglycaemic response to intraperitoneally administered insulin (Table 20, Panel B). However, it was noted that mice subjected to the hormone replacement regimes, particularly progesterone, were highly irritable, as arbitarily assessed by the difficulties experienced in handling these mice. It was clearly possible therefore that the hyperglycaemic response, due chiefly to adrenaline release, might vary in mice subjected to different hormone replacement regimes during handling, injection and blood sampling procedures involved with insulin hypoglycaemia tests. Consequently plasma glucose concentrations were also monitored during the 1 hour period following the intraperitoneal injection of a volume of saline equal to that of the insulin injection in the hypoglycaemia tests (4.36mls/kg body weight). Indeed, this study indicated an increase in the hyperglycaemic response to saline injection by all mice that had been hormone treated with the exception of those mice on the combined oestradiol and progesterone regime (Table 20, Panel A). These values masked the elevation in the total insulin induced plasma glucose disappearance (K_T) shown by all hormone treated mice compared to ovariect-

omised mice (Table 20,Panel C). It must be noted, however, that although the K_T value was increased by 80% by all hormone regimes compared to untreated ovariectomised mice, significance of differance was not attained in mice treated with oestradiol (5μg/kg/day) both alone and in combination with progesterone because of large standard errors of the mean.

The quantities of food and fluid consumed by8-10 week hormone treated and untreated 8-10 week ovariectomised mice over a 10 day period were not significantly different (Table 21). The body weights of all mice at the time of sacrifice whether ovariectomised or hormone treated for 2 or 10 weeks were not significantly affected by the treatments (Appendix, Table 32 ,Panels 1 -7). Hence, within each experiment mice were always age and weight matched.

The rate of insulin stimulated ^{14}C-glucose oxidation was significantly increased in isolated soleus muscles from mice treated for 10 weeks with either oestradiol (5μg/kg/day and 500μg/kg/day) or progesterone alone, but not in combination,when compared to untreated ovariectomised mice (Table 22). These increases were significant when the data were expressed both in terms of dpm per mg of muscle protein and dpm per muscle. It might be important that these increases were due largely to increases in the rate of soleus muscle ^{14}C-glucose oxidation in the presence of insulin rather than gross reductions in basal ^{14}C-glucose oxidation (Table 22).

Basal ^{14}C-glycogen synthesis by isolated soleus muscle

	Food consumption		Fluid consumption	
	gm/mouse/ day	gm/kg body wt./ day	gm/mouse/ day	gm/kg body wt./ day
OVX	5.03 ±0.63	141.7	6.05 ±0.67	170.4
E	5.59 ±0.74	145.61	7.01 ±0.57	182.6
E(HD)	5.69 ±0.57	171.4	7.62 ±0.68	229.5
E+P	4.99 ±0.54	139.8	5.21 ±0.36	145.9
P	4.44 ±0.56	131.0	4.98 ±0.36	146.9

TABLE 21.

Food and fluid consumption over a consecutive 10 day period for mice ovariectomised for a period of 8-10 weeks and ovariectomised mice on hormone replacement regimes for a period of 8-10 weeks. Mice were housed in cages of 6 and allowed free access to food and water.

		$^{14}CO_2$ production/2hr incubation.					
		Basal		In the presence of insulin (20ng/ml)		Insulin stimulated	
	n	dpm/mg protein	dpm/muscle	dpm/mg protein	dpm/muscle	dpm/mg protein	dpm/muscle
OVX	6	3082 ±507	5404 ±1059	3467 ±398	5813 ±801	385 ±415	556 ±748
E	6	2378 ±274	4740 ±485	4051 ±308	6865 ±608	1638* ±271	3422* ±773
E(HD)	5	3283 ±282	5181 ±629	5064* ±524	6835 ±875	1781* ±345	2873** ±440
E+P	6	3606.3 ±341	6649 ±804	3726 ±844	7034 ±1490	465 ±759	603 ±1559
P	6	3633 ±492	6725 ±669	4256 ±331	8355* ±445	991* ±148	1931* ±258

TABLE 22.

The effect of 10 week hormone replacement regimes on the rate of $^{14}CO_2$ production from 14C-glucose in the absence and presence of exogenous insulin (20ng/ml) by isolated soleus muscle. * indicates $P<0.05$ when compared to untreated ovariectomised mice.

was reduced by 10 weeks of progesterone administration, either alone or in combination with oestradiol (Table23). The rates of ^{14}C-glycogen synthesis in the presence of insulin and also insulin stimulated production by soleus muscles isolated from hormone treated ovariectomised mice were, however, not significantly different from those of untreated ovariectomised mice (Table23). It is of interest to note that the mean value for insulin stimulated ^{14}C-glycogen synthesis by isolated soleus muscles of oestradiol (both 5 and 500 μg/kg/day) treated mice was elevated compared to that of untreated ovariectomised mice (Table23). In these cases, however, significance of difference was obscured by the presence of comparatively large standard errors of the means.

None of the 10 week hormone replacement regimes employed affected the glycogen content of soleus muscles when compared to the glycogen content of soleus muscles from untreated mice (Table24). However, the liver glycogen content was significantly elevated by all hormone treatments when expressed both in terms of the amount of glycogen per gram wet weight and the total liver glycogen content (Table 25). Of all the 10 week hormone replacement regimes, oestradiol at the higher dose of 500μg/kg/day was associated with the most impressive increase in liver glycogen content (Table 25). Oestradiol alone, at both doses employed, was also associated with a significant increase in liver wet weight (Table 25), probably due to hyperplasia of hepatocytes since no increase in hepatocyte

		^{14}C-glycogen production/2hr incubation					
		Basal		In the presence of insulin (20ng/ml)		Insulin stimulated	
	n	dpm/mg protein	dpm/muscle	dpm/mg protein	dpm/muscle	dpm/mg protein	dpm/muscle
OVX	6	1712 ±518	2645 ±645	7165 ±1530	11572 ±2253	5453 ±1452	8745 ±2178
E	6	1557 ±369	3135 ±782	8884 ±1834	16706 ±2805	7326 ±1745	13500 ±2587
E(HD)	6	4592 ±1533	6884 ±2338	11787 ±3097	18999 ±5399	7195 ±1592	11378 ±2737
E+P	6	4569* ±929	9831* ±2551	8010 ±1506	17393 ±4465	3440 ±628	7409 ±1827
P	6	3736 ±748	7662* ±1764	6709 ±1041	14473 ±3838	2972 ±963	6737 ±2587

TABLE 23.

The effect of 10 week hormone replacement regimes on the rate of ^{14}C-glycogen synthesis from ^{14}C-glucose in the absence and presence of exogenous insulin (20ng/ml) by isolated soleus muscle. * indicates $P<0.05$ when compared to untreated ovariectomised mice.

	OVX	E	E(HD)	E+P	P
n	12	12	10	12	10
mg Glycogen/ muscle	0.0182 ±0.0019	0.0194 ±0.0017	0.0144 ±0.0021	0.0220 ±0.0021	0.0188 ±0.0014
mg Glycogen/gm wet weight	2.199 ±0.223	1.891 ±0.189	2.037 ±0.293	2.289 ±0.240	2.076 ±0.105
mg Glycogen/mg muscle protein	0.0135 ±0.0014	0.0109 ±0.0011	0.0126 ±0.0018	0.0119 ±0.0017	0.0121 ±0.0008
Muscle wet weight (mg)	8.41 ±0.34	10.53* ±0.59	7.13* ±0.36	9.51 ±0.46	9.06 ±0.51

TABLE 24.

The effect of 10 week hormone replacement regimes on the wet weight and glycogen content of isolated soleus muscle. * indicates $P<0.05$ when compared with untreated ovariectomised mice.

	OVX	E	E(HD)	E+P	P
n	6	6	5	5	6
Liver wet weight (gm)	1.5034 ±0.0379	1.9472*** ±0.0722	1.8228*** ±0.0313	1.6334 ±0.0932	1.5745 ±0.0701
Total liver glycogen (mg)	8.19 ±1.95	23.65** ±3.96	47.14* ±12.01	19.82** ±2.25	27.97*** ±2.08
mg Glycogen/gm wet weight	5.52 ±1.34	12.21* ±1.97	36.28* ±9.72	12.18* ±1.41	19.37*** ±1.87

TABLE 25.

The effect of 10 week hormone replacement regimes on the wet weight and glycogen content of liver. * $P<0.05$, ** $P<0.02$ and *** $P<0.01$ when compared with untreated ovariectomised mice.

cell size was observed after the 10 week administration of oestradiol or progesterone either alone or in combination (Table 29).

Hormone replacement regimes of 2 weeks duration in ovariectomised mice did not affect the maximum specific ^{125}I-insulin binding capacity of isolated soleus muscles when compared to muscles from untreated ovariectomised animals (Table 26). Progesterone administered either alone or in combination with oestradiol for 10 weeks also had no effect on the maximum specific ^{125}I-insulin binding capacity of isolated soleus muscle. There was some suggestion that the administration of oestradiol at doses of 5 and 500μg/kg/day for a period of 10 weeks was associated with an elevated maximum specific ^{125}I-insulin binding capacity of isolated soleus muscle. However, the only significant increase (46%) was observed after oestradiol (5μg/kg/day) administration when the results were expressed in terms of the maximum specific ^{125}I-insulin binding capacity per muscle (Table 27).

Hormone replacement regimes of both acute (2 weeks) and chronic (10 weeks) duration were associated with more dramatic effects on the insulin receptor status of isolated hepatocytes. The administration of oestradiol (5μg/kg/day) for 2 weeks was associated with a significant 37% increase in the total number of hepatocyte insulin receptors , due primarily to a significant 49% increase in the number of low affinity, high capacity receptors (Table 28). However, in addition, there was a parallel significant reduction

	OVX	E	E(HD)	E+P	P
n	5	6	6	6	6
% of total cpm ^{125}I-insulin/ mg protein	0.173 ±0.032	0.204 ±0.038	0.175 ±0.021	0.180 ±0.037	0.239 ±0.021
% of total cpm ^{125}I-insulin/ muscle	0.278 ±0.050	0.318 ±0.048	0.287 ±0.035	0.267 ±0.038	0.336 ±0.031
Insulin bound (ngx10^{-3})/mg protein	2.477 ±0.960	3.082 ±0.648	2.476 ±0.274	2.614 ±0.581	3.397 ±0.386
Insulin bound (ngx10^{-3})/ muscle	3.995 ±0.687	4.563 ±0.670	4.053 ±0.465	3.867 ±0.607	4.771 ±0.871

TABLE 26.

The effect of 2 week hormone replacement regimes on the maximum specific ^{125}I-insulin binding capacity of isolated soleus muscle from ovariectomised mice

	OVX	E	E(HD)	OVX	E+P	P
n	6	6	5	6	6	6
% of total cpm ^{125}I-insulin /mg protein	0.134 ±0.015	0.170 ±0.014	0.157 ±0.024	0.175 ±0.023	0.171 ±0.017	0.171 ±0.031
% of total cpm ^{125}I-insulin /muscle	0.169 ±0.013	0.246* ±0.027	0.204 ±0.033	0.278 ±0.046	0.333 ±0.038	0.307 ±0.067
Insulin bound (ngx10^{-3})/mg protein	2.017 ±0.223	2.574 ±0.226	2.331 ±0.365	2.600 ±0.323	2.537 ±0.238	2.536 ±0.448
Insulin bound (ngx10^{-3}) /muscle	2.543 ±0.210	3.705* ±0.410	3.024 ±0.503	4.117 ±0.657	4.938 ±0.536	4.539 ±0.974

TABLE 27.

The effect of 10 week hormone replacement regimes on the maximum specific ^{125}I-insulin binding capacity of isolated soleus muscle from ovariectomised mice* indicates $P<0.05$ when compared to untreated ovariectomised mice.

	OVX	E	E+P	P	OVX	E(HD)
n	6	6	6	6	6	6
High affinity receptor number /cell	176,552 ±10,717	180,134 ±15,833	134,701** ±7,066	127,930* ±8,258	130,862 ±5,713	96,907** ±6,037
Low affinity receptor number /cell	523,933 ±41,887	780,002* ±64,271	396,451 ±80,537	442,127 ±40,007	440,590 ±39,809	432,255 ±75,251
Total receptor number/cell	700,485 ±45,396	960,137*** ±57,711	531,153 ±82,716	601,162 ±39,532	571,631 ±38,698	529,160 ±79,552
$\bar{K}_e$ nM^{-1}	0.158 ±0.008	0.116*** ±0.009	0.203 ±0.028	0.200 ±0.023	0.229 ±0.017	0.180 ±0.013
$\bar{K}_f$ nM^{-1}	0.0291 ±0.0015	0.0227* ±0.0019	0.0400 ±0.0065	0.0325 ±0.0081	0.0329 ±0.0048	0.0264 ±0.0035

TABLE 28.

The effect of 2 week hormone replacement regimes on the insulin receptor status of isolated hepatocytes from ovariectomised mice. *P<0.05,**P<0.02 and ***P<0.01 when compared to untreated ovariectomised mice.

in both affinity constants $\bar{K}_e$ and $\bar{K}_f$ by 27% and 22% respectively. This suggested that the insulin binding capacity of hepatocytes from ovariectomised mice and ovariectomised mice treated with oestradiol (5μg/kg/day) would not be significantly different, particulary at insulin concentrations below those required to saturate the receptors. This was indeed established by plotting the two competition curves (not shown) which indicated an unchanged binding capacity for hepatocytes from mice in the two groups at all the insulin concentrations employed. The acute administration of progesterone, both alone and in combination with oestradiol, and also the administration of oestradiol at 500ug/kg/day were all associated with a significant reduction in the number of high affinity, low capacity insulin receptors (Table 28).

The alterations in hepatocyte insulin receptor status observed after chronic (10 weeks) hormone administration did not parallel the alterations observed in the present study after the acute (2 weeks) hormone administration. Thus the administration of either oestradiol (5μg/kg/day) or progesterone for 10 weeks was associated with a significant increase in the values of $\bar{K}_e$ and $\bar{K}_f$ while the two hormones in combination only increased $\bar{K}_f$ significantly (Table 29). The administration of oestradiol at a dose of 500μg/kg/day was, however, associated with a significant 95% increase in the total number of hepatocyte insulin receptors. This increase was due to significant increases in the numbers of both high affinity, low capacity and low affinity, high capacity receptors (Table 29).

	OVX	E	E+P	P	OVX	E(HD)
n	5	8	6	6	5	6
High affinity receptor number /cell	145,120 ±9,549	176,653 ±22,053	152,265 ±20,886	187,245 ±22,381	98,847 ±6,899	138,299* ±13,582
Low affinity receptor number /cell	498,593 ±55,146	493,463 ±69,465	522,523 ±76,423	474,778 ±54,805	342,891 ±37,116	724,908* ±134,263
Total receptor number/cell	630,777 ±61,141	670,114 ±80,339	674,789 ±91,337	662,028 ±69,743	441,739 ±61,227	863,213* ±141,210
$\bar{K}_e$ nM^{-1}	0.118 ±0.012	0.157*** ±0.012	0.117 ±0.006	0.175*** ±0.017	0.153 ±0.015	0.107 ±0.021
$\bar{K}_f$ nM^{-1}	0.0245 ±0.0034	0.0441*** ±0.0040	0.0338* ±0.0018	0.0419* ±0.0036	0.0286 ±0.0037	0.0218 ±0.0041
Hepatocyte cell size (μm)	20.71 ±0.53	19.05 ±0.67	21.25 ±0.50	21.35 ±0.63	20.81 ±0.67	21.01 ±0.50

TABLE 29. The effect of 10 week hormone replacement regimes on the cell size and the insulin receptor status of isolated hepatocytes from ovariectomised mice. *P<0.05,***P<0.01 when compared to untreated ovariectomised mice.

4. Discussion.

The present work has shown that both the maximum ^{125}I-insulin binding capacity of isolated soleus muscle and the insulin receptor status of isolated mouse hepatocytes, were not significantly different when mice at oestrus were compared to mice at the dioestrus stage of the oestrous cycle. Ovariectomy and the oral administration of either oestradiol (5 and 500µg/kg/day) or progesterone (1mg/kg/day) or the two in combination (5µg/kg/day and 1mg/kg/day respectively) for a period of two weeks to ovariectomised mice had no significant effect on the maximum ^{125}I-insulin binding capacity of isolated soleus muscle. However, the maximum specific ^{125}I-insulin binding capacity was significantly reduced by ovariectomy for a period of 10 weeks when the data were expressed in terms of the binding capacity per soleus muscle. This reduction was reversed by the administration of oestradiol (5µg/kg/day) for 10 weeks but not by either progesterone (1mg/kg/day) or the two in combination.

The insulin receptor status of isolated mouse hepatocytes was influenced to a much greater extent than the maximum specific binding of ^{125}I-insulin to isolated mouse soleus muscle by experimentally imposed increases and decreases in the plasma titres of ovarian sex steroids. Alterations in hepatocyte insulin receptor status induced by ovariectomy and oestradiol and progesterone replacement regimes were both time and dose dependent.

Thus, Scatchard analysis of insulin binding data revealed a reduction in the total hepatocyte insulin receptor number after 2 weeks ovariectomy. This reduction in receptor number was accompanied, however, by a concomitant increase in the insulin-receptor binding affinity constants $\bar{K}e$ and $\bar{K}f$. Thus, as shown by competition curves of the binding data, the amount of insulin bound by isolated mouse hepatocytes at all the insulin concentrations employed was not significantly reduced by ovariectomy of 2 weeks duration. This reduction in insulin receptor number and parallel increases in the values of $\bar{K}e$ and $\bar{K}f$ were reversed by the administration of oestradiol at a dose of 5μg/kg/day for a period of 2 weeks .The administration of progesterone (1mg/kg/day) for 2 weeks alone or in combination with oestradiol (5μg/kg/day) and also oestradiol at the higher dose of 500μg/kg/day was associated with a reduction in the number of high affinity, low capacity, insulin receptors in isolated mouse hepatocytes.

Long-term ovariectomy, for a period of 10 weeks, was associated with a reduction in the number of hepatocyte insulin receptors. The reduction in hepatocyte insulin binding capacity was partially or totally rectified by the administration for 10 weeks of all the hormone replacement regimes employed in the present study. Both oestradiol (5μg/kg/day) and progesterone (1mg/kg/day) increased the binding capacity via increases in the affinity constants $\bar{K}e$ and $\bar{K}f$, while the two hormones in combination only increased the value of $\bar{K}f$. Oestradiol at the higher dose of 500μg/kg/

day was associated with the most impressive increase in the hepatocyte insulin binding capacity producing a significant increase in both high affinity, low capacity and low affinity, high capacity insulin receptors.

The improved hypoglycaemic action of insulin, the enhanced rate of isolated soleus muscle glycogen formation from ^{14}C-glucose in the presence of insulin (when expressed per muscle) and the elevated soleus muscle glycogen content in mice in the oestrus phase compared to the dioestrus phase of the oestrous cycle, indicated an improved insulin action in the presence of high physiological concentrations of oestrogens and progestogens. The improved hypoglycaemic action of insulin at oestrus compared to dioestrus was consistent with the improved glucose tolerance at oestrus observed in rats (96). The present work suggested that this improved glucose tolerance observed in rats at oestrus might therefore not be due solely to the enhanced insulin response to the glucose challenge (96) but might also be the result of an improved insulin action in peripheral target tissues.

Previous work had indicated that the glycogen content of the liver also differs according to the stage of the oestrous cycle in rats (97). In that study hepatic glycogen content was elevated at proestrus but greatly reduced at oestrus and moderately elevated at metoestrus and dioestrus. Thus, hepatic glycogen content did not correlate with the circulating concentrations of either oestrogens or progestogens.

However, plasma concentrations of corticosteroids also undergo cyclical alterations throughout the oestrous cycle and indeed a precise positive correlation has been observed between the plasma concentration of corticosterone and hepatic glycogen content throughout the oestrous cycle in rats (97). It is interesting to note that in the present study, the hepatic glycogen content was increased, although not significantly, by 53% in livers from dioestrus mice as compared to livers from oestrus mice (Table 8). This exactly parallels the observations in rats (97) as described above, and suggests therfore that the plasma concentrations of corticosterone may well play a role in the regulation of glucose metabolism, especially hepatic glycogen metabolism, throughout the oestrous cycle in mice. Furthermore, evidence has been presented (353,354) which indicates an unchanged oestrogen receptor distribution between the nucleus and cytoplasm of rat hepatocytes during the oestrous cycle. This indirectly suggests that oestrogen action in the liver was unchanged throughout the oestrous cycle, and supports the hypothesis that the cyclical fluctuations in liver glycogen in rats (97) and possibly in mice, in the present work, were not mediated directly by changes in plasma oestrogen concentrations.

The glycogen content of soleus muscle was, however, significantly elevated in mice at oestrus compared to mice at dioestrus (Table 8). Concomitant with this observation, there was some suggestion that the ability of insulin to promote glycogen synthesis by isolated soleus muscle might

indeed be enhanced at oestrus (Table 7). It must be noted however, that a significant difference was apparent only when the results were expressed in terms of the rate of ^{14}C-glycogen synthesis per muscle in the presence of insulin. These data suggested, therefore, that an enhanced glycogen production and an increased sensitivity of glycogen synthesis to insulin in skeletal muscle may, at least in part, be responsible for the improved hypoglycaemic action of insulin at oestrus compared to dioestrus as observed in the present study.

The maximum specific ^{125}I-insulin binding capacity of isolated soleus muscle was not significantly different at the two selected stages of the oestrous cycle (Table 9). In addition the insulin binding capacity of isolated hepatocytes from mice at oestrus and dioestrus were not significantly different (Table 10). This is consistent with the unaltered insulin binding capacity observed in isolated mammary tissue throughout the oestrous cycle in mice (303). Thus, although the hypoglycaemic action of exogenous insulin, soleus muscle glycogen content and the rate of insulin stimulated ^{14}C-glycogen synthesis in isolated soleus muscle were all increased at oestrus in the presence of high physiological concentrations of oestrogens and progestogens, these increases were not due to an increased insulin binding capacity in either the liver or soleus muscle. These data indicated that the presence of high physiological concentrations of both oestrogens and progestogens were associated with an increased insulin action, at least in

soleus muscle, at the post-receptor level. A role for the liver in mediating the observed increases in the hypoglycaemic action of exogenous insulin at oestrus has yet to be elaborated.

Thus, in the present study, a reduced plasma concentration of endogenous oestrogens and progestogens at dioestrus was associated with an impaired hypoglycaemic action of insulin (Figure 31). Similarly, the hypoglycaemic action of insulin was reduced after ovariectomy (Table 12) which also represented a condition of reduced plasma oestrogen and progestogen concentrations. However, although a reduction in the hypoglycaemic action of insulin was apparent after ovariectomy of 3 weeks duration, the reduction was more fully developed after ovariectomy of 6 and 9 weeks duration.

Since both ovariectomy, for any of the periods of time employed, and the dioestrus phase of the oestrous cycle represent conditions of reduced plasma oestrogen and progestogen titres, this situation might be responsible for the observed reductions in the hypoglycaemic effect of exogenous insulin. However, the results of the present study suggest that the rapid decrease in the hypoglycaemic action of insulin during the dioestrus phase of the oestrous cycle and the time dependent deterioration in the hypoglycaemic action of insulin after ovariectomy might be mediated through different mechanisms.

The reduction in the hypoglycaemic action of insulin

after short-term ovariectomy (3 weeks) suggested a reduction in insulin action in peripheral target tissues. An unaltered insulin binding capacity of both isolated hepatocytes and soleus muscle after 2 weeks ovariectomy suggested that such a reduction in insulin action, induced by ovariectomy, was occurring at the post-receptor levels, at least in these two tissues.

It is of interest to note, however, that ovariectomy of 2 weeks duration was not without effect on hepatocyte insulin receptor status. Thus, although the total binding capacity was not significantly altered by short-term ovariectomy this was due to a complex phenomenon involving a decrease in receptor number with a parallel increase in receptor affinity (Table 18). Such a phenomenon has been noted elsewhere (290,195) and it might be significant that both of these studies were similar to the present study in so far as they both considered the effects of certain ovarian steroids on insulin receptor status. These ovarian steroids included endogenous 17β-oestradiol, progesterone and 17α-hydroxyprogesterone and a combination of a synthetic oestrogen and progestogen. However, the models of insulin receptor status employed were human monocytes and erythrocytes.

An explanation for such parallel but opposite changes in insulin receptor number and affinity has not previously been offered. However, it seems plausible to suggest that an increase in receptor affinity may be the

immediate effect of a reduced receptor concentration via a reduction in site-site interaction. On a physiological basis, an increase in insulin receptor affinity, occurring in parallel with a decrease in receptor number, might serve to maintain cellular sensitivity to insulin following insulin-induced down regulation of its receptors. It is important to stress that the observed behaviour of insulin receptor status did not depend upon the use of mean values, since it was present in each hepatocyte preparation.

In the present work, ovariectomy for a period of 10 weeks had no significant effect upon fed plasma glucose or insulin concentrations (Table 11) confirming the results of similar studies in the literature (78). Mice ovariectomised for a period of 15 weeks have, however, previously been shown to have an increased rate of gluconeogenesis from L-alanine; a decreased glycogen content of liver, uterus and cardiac muscle (98); an impaired glucose tolerance; a reduced plasma insulin response to glucose and arginine; a reduced in vitro insulin secretion from isolated pancreatic islets in response to a glucose challenge and a reduced insulin content of the whole pancreas and pancreatic islets (78). The literature suggested, therefore, that long-term ovariectomy induced a state of glucose intolerance in mice, mediated at least in part by a reduction in insulin secretion. The present work confirmed that long-term ovariectomy (10 week duration) of mice resulted in a reduced hepatic glycogen content (Table 16). In addition ovariectomy for a period of 10 weeks resulted in an impaired insulin

action in peripheral insulin target tissue as assessed by the hypoglycaemic response to exogenous insulin (Table 12). This suggested, therefore, a further mechanism through which long-term ovariectomy might be able to aggravate glucose tolerance in mice.

At the level of specific insulin target tissues the present work revealed that the basal rate of ^{14}C-glucose oxidation by isolated soleus muscle was reduced by ovariectomy of 10 weeks duration (Table 4). This suggested that ovariectomy might also impair glucose oxidation in skeletal muscle independently of insulin action. This reduction in glucose metabolism appeared to be specific to glucose oxidation since the rate of basal ^{14}C-glycogen production from ^{14}C-glucose by isolated soleus muscle was unaffected by ovariectomy. Indeed, both the rate of insulin stimulated ^{14}C-glycogen synthesis (Table 15) and the glycogen content of soleus muscle (Table 16) were significantly increased after ovariectomy for a period of 10 weeks compared to intact dioestrus mice. The reduced hypoglycaemic action of insulin after long-term ovariectomy clearly indicated that these increases did not entirely compensate for other reductions in insulin action in peripheral target tissue.

An increased rate of insulin stimulated ^{14}C-glycogen synthesis and glycogen content of soleus muscle after long-term ovariectomy was also inconsistent with the reduction in the rate of ^{14}C-glycogen synthesis and glycogen content

of skeletal muscle in dioestrus compared to oestrus mice. These opposite responses in muscle glycogen production to conditions of low plasma concentrations of oestrogens and progestogens suggested either time dependent effects of the ovarian steroids or the influence of other factors. Within the present work there was no suggestion that either oestradiol or progesterone ,in the long-term, impaired soleus muscle glycogen synthesis (Table 23-24). This would suggest that the increased soleus muscle glycogen production after ovariectomy was due to factors other than the absence of ovarian steroids. In this respect it might be significant that although glucocorticoids enhance hepatic glycogen deposition by increasing the activity of the glycogen-synthetase-activating enzyme (355), they antagonize insulin mediated glucose uptake into skeletal muscle (356). Furthermore it has been suggested that oestrogens increase the concentrations of circulating glucocorticoids, particarly cortisol (357) through adrenal hyperplasia (358). It is plausible to suggest therefore, that the reduced levels of oestrogens associated with ovariectomy may result in a reduction in glucocorticoid levels with a consequential reduction in glucocorticoid antagonism to insulin mediated muscle glycogen deposition.

The increased glycogen content of soleus muscle after ovariectomy of 10 weeks duration was not in agreement with a previous study which had indicated a decreased skeletal muscle glycogen content in mice after an identical period of ovariectomy (98).

However, it should be pointed out that this earlier study was conducted with 6 hour starved mice, which in terms of the level of glycogen reserves, was inconsistent with the present study. It should also be emphasized that the glycogen metabolism (glycogenesis and glycogenolysis) and the glycogen content of skeletal muscle depend upon the fibre composition of the muscle (334). It was therefore inappropriate to compare either the glycogen content or the rate of glycogen synthesis in soleus muscle, which is composed predominantly of slow oxidative fibres (328), with that in other muscle types such as the biceps femoris which is composed predominantly of fast glycolytic and fast oxidative-glycolytic fibres (328) and which has been used in previous studies (98).

In the present study, ovariectomy of mice for a period of 10 weeks was associated with a reduction in the total number of insulin receptors on isolated hepatocytes (Table 18). This suggested that a reduced insulin binding capacity, and therefore insulin action, was responsible for the reduction in the hepatic glycogen content observed after 10 weeks ovariectomy (Table 16). Furthermore, in view of the important role of the liver in translating the hypoglycaemic action of insulin, the present study suggested that a reduction in insulin action, through a reduced insulin-receptor binding in hepatocytes might be responsible, at least in part, for the reduced hypoglycaemic action of exogenous insulin after long-term ovariectomy.

There was also some suggestion of a reduced maximum specific ^{125}I-insulin binding capacity in isolated soleus muscle of mice ovariectomised for a period of 10 weeks compared to intact mice (Table 17). However, this reduction was significant only when the results were expressed in terms of the ^{125}I-insulin binding capacity per muscle. However, the rate of insulin stimulated ^{14}C-glycogen synthesis by isolated soleus muscle was significantly increased when the results were expressed either in terms of per mg muscle protein or per muscle (Table 15). Taken together, these data suggested that both the specific increase in the rate of insulin stimulated ^{14}C-glycogen synthesis and the reduction in the basal ^{14}C-glucose oxidation by isolated soleus muscle after ovariectomy for a period of 10 weeks, were mediated predominantly at a post-receptor location.

Alterations in the rates of both glucose oxidation and glycogen synthesis at a post-receptor location in muscle, whether dependent or not on insulin action, might take the form of changes in the activities of key gluco-regulatory enzymes. In this respect the enzymes glycogen synthase (EC2.4.1.11) and oxoglutarate dehydrogenase (EC1.2.4.2) are suitable candidates as they critically influence the rates of glycogen synthesis and the tricarboxylic acid cycle respectively (334). Indeed, oestradiol has been reported to increase the maximum activities of both of these enzymes in mouse soleus muscle during <u>in vitro</u> insulin stimulation (116).

The administration of either oestradiol (5µg/kg/day) or progesterone (1mg/kg/day) for a period of 10 weeks or indeed the two in combination was not associated with alterations in the plasma glucose or insulin concentrations of freely fed ovariectomised mice (Table 19). These observations are consistent with data obtained with mice in a previous study which was comparable to the present work (78). These data indicated therefore, that any long-term effects of either oestradiol (5µg/kg/day) or progesterone (1mg/kg/day) or the two in combination observed in the present study, were not mediated through changes in the plasma concentrations of either glucose or insulin.

It should be noted, however, that oestradiol, at the higher dose of 500µg/kg/day, caused a significant reduction in the fed plasma glucose concentration (Table 19). This might have been the result of the dramatic increase in the hepatic insulin binding capacity (Table 29), which suggested that the high dose of oestradiol caused the greatest increase in insulin action in the liver. This hypothesis was further supported by the observation that oestradiol administered at this dose for 10 weeks also caused the most dramatic increase in hepatic glycogen content (Table 25).

The reduced fed blood glucose concentration of ovariectomised mice treated for 10 weeks with oestradiol at 500µg/kg/day in the face of an unaltered plasma insulin concentration suggested that, over all, the hypoglycaemic action

of insulin was improved. This was fully supported by the significantly increased hypoglycaemic action of exogenous insulin (0.35U/kg body weight) after the administration of oestradiol at a dose of 500μg/kg/day for 10 weeks (Table 20). Indeed there was some suggestion that the hypoglycaemic action of insulin was improved by 10 weeks administration of either oestradiol (5μg/kg/day) or progesterone or the two in combination when compared to untreated ovariectomised mice (Table 20).

The liver plays a major role in translating the hypoglycaemic action of insulin. Thus, the significant increases of either the number of hepatocyte insulin receptors or their affinity constants after the administration of all the hormone regimes employed in the present study (Table 29) would suggest that an improved insulin action in the liver, brought about by enhanced insulin binding, might be responsible for the observed improvement in the hypoglycaemic action of insulin after hormone treatment. It should also be noted that a previous study (98) reported a reduction in the rate of in vivo gluconeogenesis from ^{14}C-alanine in mice after the 15 week administration of either oestradiol or progesterone alone or in combination to ovariectomised mice. Thus, a reduced rate of gluconeogenesis, which might in any case have been the result of an increased insulin action (335), might also contribute to the increased hypoglycaemic action of insulin after the 10 week administration of all the hormone replacement regimes employed.

The present work also suggested that the increased liver glycogen content observed after the 10 week administration of all the hormone replacement regimes, was the product of an enhanced insulin action in liver brought about by an increased hepatocyte insulin binding capacity. It should be noted, however, that a reduced glycogenolytic effect of both glucagon and adrenaline in ovariectomised mice treated with either oestradiol or progesterone, or the two in combination (99) would assist the preservation of hepatic glycogen.

Previous work (101) had also indicated an increased glycogen content in rats after the administration of either progesterone or oestradiol and progesterone in combination. Although in that study oestradiol alone did not increase the liver glycogen content, it is significant that control animals were intact rather than ovariectomised animals as used in the present work. This may have obscured oestradiol induced increases in the liver glycogen content. In the present study a significant increase in liver wet weight was also observed after the 10 week administration of both doses of oestradiol (Table 25). As previously noted (page 173), this was probably due to the hyperplasia of hepatocytes and supported earlier studies (101) which suggested that ovarian sex steroids enlarged the liver by increasing the number of hepatocytes.

As previously considered (page 192), oestrogens have been observed to increase the circulating concentrations

of glucocorticoids (357) which enhance hepatic glycogen deposition (355). It is possible, therefore, that increased glucocorticoid titres after oestradiol administration were also, in part, responsible for the observed increase in hepatic glycogen content. There is, however, no evidence to suggest that glucocorticoids are able to increase the insulin binding capacity of hepatocytes. Indeed, the administration of dexamethasone, although a synthetic glucocorticoid, has been observed to reduce the insulin receptor binding capacity of both isolated hepatocytes (359) and liver plasma membranes (360) in a dose dependent manner.

About 50% of the glucose undergoing degradation in the liver enters the pentose-phosphate pathway (355), and oestrogens, including oestradiol, have been observed to stimulate this pathway in rat liver (101,358). In addition oestrogens, specifically stilboestrol, have been observed to increase the synthesis of lipid from ^{14}C-glucose (358). It is possible therefore that both an increased rate of glucose oxidation by the pentose-phosphate pathway and an increased rate of lipid synthesis in the liver, at least after the 10 week administration of oestradiol, might be in part responsible for the observed increase in the hypoglycaemic action of insulin. However, the contribution of such effects, and whether or not they would be dependent on increased insulin action, particularly through an increased hepatocyte insulin binding capacity, remains to be elaborated.

The present work indicated that increases in the

insulin sensitivity of specific pathways of glucose metabolism in skeletal muscle might also contribute towards the increased hypoglycaemic action of insulin observed after 10 week hormone replacement regimes. Thus, although only significant after the administration of oestradiol at the higher dose of 500ug/kg/day, there was some suggestion of an increased rate of glucose oxidation by isolated soleus muscle in the presence of insulin (20ng/ml) after the 10 week administration of all hormone replacement regimes (Table 22). Indeed, an increased sensitivity of glucose oxidation to insulin in isolated soleus muscle was demonstrated by the observation of a significantly elevated rate of insulin stimulated glucose oxidation after the administration of either oestradiol (5 and 500ug/kg/day) or progesterone (1mg/kg/day). Administration of the two hormones in combination at these doses did not, however, result in an increased rate of insulin stimulated glucose oxidation These data suggested therefore that there was some antagonism between oestradiol and progesterone as regards their ability to increase sensitivity of glucose oxidationby isolated soleus muscle. This observation was supported by earlier work which had reported an antagonism by progesterone of oestradiol induced increases in the rate of *in vivo* gluconeogenesis from ^{14}C-alanine (98), the hypoglycaemic action of insulin (99) and the glycogen content of both myometrial tissue (109,123) and liver (102). The mechanism underlying this phenomenon is unclear. However, it is plausible to suggest

that a reduction in the number of cellular oestrogen receptors by progesterone, (observed in human uterine muscle (361)), with a subsequent reduction in oestrogen action could be responsible for such progesterone antagonism to oestrogen action.

This aspect of the present work may highlight the point that the administration of either oestradiol or progesterone alone, while revealing individual effects on the parameters under study,may not be physiological since these effects may depend upon interactions between the two hormones. A further illustration of the action of one ovarian hormone depending on the concentration of the other can be provided by the observation that the increase in rat carcass fat by progesterone requires oestrogen priming (362). This potentiation in the action of progesterone might be explained in terms of an oestrogen induced increase in progesterone receptor number with the concomitant increase in the action of progesterone. Such an oestrogen induced increase in the number of progesterone receptors has been observed in chick oviduct (363) and in mammary tissue of lactating rat (364).

Thus, the action of either oestradiol or progesterone appears to depend not only on the molar concentrations of the two hormones, but also on the ability of the target tissues to respond to them, and this in turn may also be subject to regulation at a number of levels (361).

It is of interest to note that the 10 week administration of progesterone either alone or in combination with oestradiol to ovariectomised mice resulted in a significant reduction in the basal rate of ^{14}C-glycogen synthesis by isolated soleus muscle (Table 23). This suggested that progesterone is capable of reducing glycogen synthesis in skeletal muscle independently of an effect on the action of insulin.

Although there was a suggestion of an increased rate of insulin stimulated ^{14}C-glycogen synthesis by isolated soleus muscle after the 10 week administration of oestradiol at doses of both 5μg/kg/day and 500μg/kg/day, these increases were not significant (Table 23). Indeed, apart from the reduced rate of basal ^{14}C-glycogen synthesis outlined above, the rate of ^{14}C-glycogen synthesis by isolated soleus muscle either in the absence or presence of insulin was not significantly changed after 10 weeks administration of any of the hormone replacement regimes employed. However, as discussed above, insulin stimulated ^{14}C-glucose oxidation in isolated soleus muscle was significantly increased by both concentrations of oestradiol and by oestradiol in combination with progesterone (Table 22). This specific increase in the sensitivity of glucose oxidation to insulin and the fact that the rate of basal ^{14}C-glucose oxidation by isolated soleus muscle, unlike the rate of basal ^{14}C-glycogen synthesis, was not significantly reduced by progesterone suggested that oestradiol and progesterone induced increases in soleus muscle glucose

metabolism predominantly at a post-receptor location. Indeed, this was further supported by the observation that there was no significant change in the maximum ^{125}I-insulin binding capacity per mg protein in isolated soleus muscle from 10 week hormone treated mice when compared to soleus muscles from untreated ovariectomised mice (Table 27).

The present study suggested, therefore, that while oestradiol and progesterone increased insulin action in the liver, at least in part, at the level of the insulin receptor, the observed increases in glucose metabolism in isolated soleus muscle were mediated predominantly at the post-receptor level. Thus, the insulin receptor status of isolated hepatocytes was more sensitive than the maximum ^{125}I-insulin binding capacity of isolated soleus muscles to long-term changes in the plasma concentrations of the ovarian steroids. It must be emphasized that, this difference in sensitivity was not simply a product of the oral route of administration of exogenous oestradiol and progesterone. This was apparent as ovariectomy alone for 2 and 10 weeks also induced a greater response in the insulin receptors of hepatocytes compared to those of soleus muscle (pages 156-165).

Further evidence that the insulin binding capacity of hepatocytes is more sensitive than that of muscle to either increased or decreased plasma concentrations of the ovarian sex steroids has been obtained from experiments

investigating the time dependency of their effects on insulin binding to these two tissues.

Thus, the 2 week administration of all the hormone replacement regimes employed had no significant effect on the maximum specific ^{125}I-insulin binding capacity of isolated soleus muscles when they were compared to soleus muscles from untreated ovariectomised mice (Table 26). However, these hormone replacement regimes had profound effects on the insulin receptor status of isolated hepatocytes. Indeed, although the administration of oestradiol at a dose of 5μg/kg/day for 2 weeks had no significant effect on the total insulin binding capacity of isolated hepatocytes at the insulin concentrations employed, this was due to a phenomenon that involved an increase in receptor number and a parallel decrease in receptor affinity (Table 28). The effects of ovariectomy of 2 weeks duration on hepatocyte insulin receptor status (page 189) was therefore reversed. The 2 week administration of progesterone both alone and in combination with oestradiol and also oestradiol alone at a dose of 500μg/kg/day to ovariectomised mice was, however, associated with a significant reduction in the insulin binding capacity of hepatocytes (page 181).

The work outlined above also revealed that ovariectomy of 2 weeks duration and 2 week hormone replacement regimes, produced effects on hepatocyte insulin receptor status which were inconsistent with the increased insulin receptor binding of isolated hepatocytes produced by 10 week treat-

ments (page181). Thus a clear time dependency could be demonstrated for the actions of oestradiol and progesterone on hepatocyte insulin receptor status.

As previously discussed (page 196) it was significant that the stimulatory effects produced by the 10 week administration of the ovarian sex steroids on the insulin receptor binding capacity of isolated hepatocytes were consistent with the improved hypoglycaemic action of insulin observed after these treatments. However, the reduction in the number of high affinity, low capacity hepatocyte insulin receptors after the two week administration of progesterone alone or in combination with oestradiol and after the high dose of oestradiol (Table 28), suggested a short-term aggravation of insulin action. Although not the subject of this thesis, concomitant studies in our laboratory have revealed a marked deterioration in the hypoglycaemic action of insulin after the short-term treatment of ovariectomised mice with either progesterone alone or in combination with oestradiol, while oestradiol (5µg/kg/day) alone had little or no effect (Dr.C.J.Bailey, personal communication). It must be emphasized, however, that the potential hyperglycaemic effects of handling and injecting the mice have not been accounted for in that study. In addition an earlier investigation that formed part of the present work indicated a marked deterioration in the hypoglycaemic action of insulin after 2 weeks administration of the high dose of oestradiol, (500µg/kg/day), (K_A=0.899 ±2.149 and 6.736 ±0.924 mmol glucose/hour

for oestradiol treated and ovariectomised mice respectively). These reductions in the hypoglycaemic action of insulin outlined above coincided with the observed reductions in the insulin binding capacity of isolated hepatocytes (see above). These data further support a role for the ovarian sex steroids in regulating the hypoglycaemic action of insulin, particularly through regulation of hepatocyte insulin receptor status.

From the results discussed over the previous few pages it is apparent that progesterone, both alone and in combination with oestradiol, and oestradiol alone at the higher dose of 500µg/kg/day, resulted in a biphasic alteration in hepatocyte insulin receptor binding capacity. That is, a short-term decrease and long-term increase in hepatocyte insulin binding capacity have been observed. The parallel changes in insulin action that were suggested provide a possible mechanism for the observed initial oestrogen induced aggravation of diabetes produced by subtotal pancreatectomy in rats which occurred before the long-term ameliorative effects (69), (page 10). Furthermore, this aspect of the present work suggested a possible reason for the discrepancies within the literature regarding the impact of oestrogens and progestogens on carbohydrate metabolism (pages 1 - 20).

It is of interest to note that increased plasma concentrations of oestrogens have been observed to increase the number of oestrogen receptors in human uterine tissue

(361). The biphasic alterations in hepatocyte insulin receptor status caused by oestradiol administration, might therefore, reflect an increased ability of tissues, especially the liver, to respond to oestradiol after exposure to the steroid over a long period of time.

The observed time dependency of the effects of oestradiol and progesterone on the insulin binding capacity of hepatocytes casts some doubt on the existence of a direct relationship between insulin receptor status and the concentration of oestradiol or progesterone at one point in time. This particularly applies when the ovarian steroids undergo relatively short-term fluctuations in their plasma concentrations. However, direct relationships have been suggested in several studies that have compared the insulin receptor status of erythrocytes and monocytes throughout the menstrual cycle in women (289-291). Furthermore, these studies used models of insulin receptor status such as the erythrocyte which, unlike the liver, are neither classical insulin nor sex steroid target tissues, and which have insulin receptors which respond relatively slowly to factors affecting their status. This information may go some way towards explaining the short-term reduction in insulin receptor binding capacity by oestradiol and progesterone observed in these studies which are in direct contrast to the long-term increases observed in the present work. It cannot be excluded, however, that the observed reduction in erythrocyte and monocyte insulin receptor

binding capacity in the presence of elevated plasma concentrations of oestradiol and progesterone (289-291) might be equivalent to their short-term effects on hepatocyte insulin receptor binding capacity observed in the present work. This might suggest that the mediation of reduced insulin binding during the luteal phase of the menstrual cycle might be brought about by insulin-counteracting hormones, for example, glucocorticoids, rather than either oestradiol or progesterone per se.

The consumption of food and fluid was unaltered by either 10 week ovariectomy or 10 week hormone replacement regimes (Table 21), and this result is consistent with earlier work (78). This observation suggested that the effects of ovariectomy along with the effects of oestradiol and progesterone administration were not, at least in the long-term, a result of an altered food or fluid consumption. The possibility of alterations in gastrointestinal absorption cannot, however, be excluded. It should be emphasized that reduction in the plasma concentrations of both ovarian oestrogens and progestogens after ovariectomy has been reported to increase food consumption in rats (314). Similar observations have been made during progesterone replacement regimes in rats (102), while oestradiol has been shown to decrease food consumption (102,314). Although still requiring confirmation, recent work from our laboratory has suggested that the alterations in food consumption shown in rats may well occur on a short-term (2-3 weeks) basis in mice (Dr.C.J.

Bailey, personal communication). This suggests that the ovarian steroids, particularly oestradiol and progesterone, produce a transient change in food consumption in mice supporting the observation made earlier in rats (314). It was possible therefore, that the reduction in hepatocyte insulin binding observed after the administration of progesterone for 2 weeks might be the result of an elevated insulin concentration caused by an increased food consumption. However, such a hypothesis would also suggest that a reduced food consumption after short-term oestradiol administration would result in an increased insulin binding capacity. This was clearly not the case as the 2 week administration of oestradiol at 500μg/kg/day resulted in a significant decrease in hepatocyte insulin binding capacity (Table 28). Furthermore, the initial aggravation by oestradiol of diabetes induced in rats by subtotal pancreatectomy (69) was shown not to be due to alterations in food consumption (56,67). This suggested therefore, that the biphasic response in hepatocyte insulin receptor binding capacity (and the possible biphasic changes in the hypoglycaemic action of insulin - see above) induced by oestradiol and progesterone were true reflections of hormone action. It is possible, however, that the observed short-term reduction in hepatocyte insulin receptor binding capacity was mediated, at least in part, by increased levels of circulating insulin-counteracting hormones such as glucoagon, catecholamines, growth hormone, cortisol and thyroid hormones.

Oestrogens have indeed been reported to increase the levels of many plasma components including, cortisol, angiotensinogen, several clotting factors (II,VII,IX and X), growth hormone and the plasma globulins (especially carrier proteins of the sex steroids, thyroxine, growth hormone, cortisol, iron and copper) (365). The precise biological significance of these changes in plasma components has, however, yet to be defined. As growth hormone and cortisol are regarded as insulin-counteracting hormones (47), it is possible that an increased action of one or both of these could be responsible for the short-term reduction in hepatocyte insulin receptor binding capacity (and possibly the hypoglycaemic action of insulin) observed after the 2 week administration of oestradiol at 500 μg/kg/day. It was considered unlikely, however, that changes in any of the above would be responsible for either the increased rate of ^{14}C-glucose oxidation by isolated soleus muscle, the increased liver wet weight and glycogen contents, the increased hypoglycaemic action of insulin or the increased hepatocyte insulin receptor binding capacity observed in the present study after the 10 week administration of either dose of oestradiol. In addition to the increased concentration of the plasma components reported above, oestrogens have been shown to promote the production and release of hypothalamic luteinizing hormone-releasing hormone (HLH-RH). This hormone in turn stimulates the secretion of the pituitary gonadotrophins, luteinizing hormone (LH) and follicle stimulating hormone (FSH). A combined treatment of LH and FSH has

been shown to improve glucose tolerance when administered to ovariectomised rats (96). However, the pharmacological doses employed in that study did not reveal a physiological role for the gonadotrophins in the regulation of glucose homeostasis.

At the present time there is no overwhelming evidence to indicate that the long-term effects of oestradiol and progesterone on liver and muscle observed in the present study are mediated entirely by secondary effects of the steroids. This implies that both the liver and skeletal muscle are capable of responding directly to both oestradiol and progesterone.

The currently accepted mechanism of sex steroid action involves the free diffusion of the steroid through the outer plasma membrane of the cell and its binding to a cytoplasmic receptor, which in turn is translocated to the nucleus to interact with chromatin where gene transcription or translation may be altered (88). This mechanism of action therefore requires the presence of specific sex steroid receptors. Specific ovarian steroid receptors have yet to be identified in either mouse liver or soleus muscle. However, as the liver of the mouse is the major organ for the metabolism of steroids, including sex steroids, it does not seem unreasonable to suggest that receptors within hepatocytes specific for oestrogens and progestogens might exist. Indeed, oestrogen receptors have been identified in the liver of several mammalian species including

the rat (354). Oestrogen receptors have also been suggested to exist in rat skeletal muscle (366) although this observation has not been substantiated (367). There is no evidence in the literature to confirm or deny the presence of specific progesterone receptors in either mouse liver or skeletal muscle, although such receptors have been identified in a human hepatic neoplasm (368). It is of interest to note that both oestrogens and progesterone have been reported to interact, with a certain degree of specificity, with androgen receptors (367,369). This would suggest at least one mechanism through which oestrogens and progestogens could exert their metabolic effects, particularly in skeletal muscle, in which androgen receptors have been well characterized (367).

It is apparent that the mechanisms suggested for both the post-receptor mediation of insulin action (Section1.4) and the regulation of insulin receptor status (Section1.8) contain scope for their regulation by oestradiol and progesterone. However, the precise cellular mechanisms through which either oestradiol or progesterone, or possibly their metabolites, influence both hepatocyte insulin receptor status and insulin action at the post-receptor level in soleus muscle remain to be elaborated.

From the present work it may be suggested that in the long-term, endogenous and exogenous natural ovarian sex steroids were responsible for the increased hepatocyte insulin receptor binding capacity. This appeared to play

a fundamental role in the regulation of insulin action in the liver. However, besides converting the binding of insulin into a biological response, the insulin receptor also plays a vital role in the further metabolism of the insulin molecule. Indeed, after binding to its receptor and the subsequent internalization of the insulin-receptor complex, insulin is degraded within the cells of insulin target tissues (pages 59 -66). Since the insulin concentration in the hepatic portal vein is at least three times that found in the arterial circulation (370) and the liver has a high insulin degradative capacity (275), it is probable that the liver plays a major role in determining the amount of insulin reaching the peripheral circulation. Indeed, the high insulin concentration of the hepatic portal vein and the high insulin degradative capacity of the liver, are probably the reason for the high number of insulin receptors reported on hepatocytes compared to other insulin target tissues (182).

It is important, therefore, to consider alterations in the insulin binding capacity of the liver not only in terms of the consequent effects on insulin action, but also in terms of the ability of the liver to degrade insulin. This,in turn, would affect the amount of insulin entering the peripheral circulation.

The administration of either oestradiol or progesterone, or the two in combination to ovariectomised mice has been reported to increase the amount of insulin released

from isolated pancreatic islets in response to a glucose challenge (78). In the present work the 2 week administration of progesterone, both alone and in combination with oestradiol and also oestradiol alone at a dose of 500μg/kg/day reduced the number of high affinity, low capacity insulin receptors. It is possible, therefore that this reduction in hepatocyte insulin receptor number was the product of a down regulatory effect of an increased insulin release. It was apparent, however, in the present study that the long-term administration of all the hormone replacement regimes was not associated with an increased plasma insulin concentration. This observation, although consistent with earlier reports in the literature (78), was not in accord with the increased plasma insulin concentration expected from the reported stimulatory effects of oestradiol and progesterone on the pancreas (75,76,78-80,90,94). Although there is no evidence in the literature, this observation would suggest that the rate of insulin degradation by the liver might be increased by oestradiol and progesterone. Bearing in mind the vital role of the insulin receptor in the initiation of insulin degradation (273-275), this might in turn suggest that an increased insulin receptor binding capacity would result in an increased rate of insulin degradation. Thus, the increased hepatocyte insulin binding capacity observed after the long-term administration of oestradiol and progesterone might conceivably represent an adaptive response to prevent peripheral hyperinsulinaemia. Similarly the reduced hepatocyte insulin binding capacity observed after

ovariectomy for a period of 10 weeks might be an adaptive response to prevent peripheral hypoinsulinaemia in the face of the reduced insulin secretion observed after ovariectomy (78).

This hypothesis would necessitate hepatocytes overcoming the down and up regulatory effects of elevated and reduced insulin concentrations respectively on their insulin receptors. A number of studies during pregnancy and lactation have indeed failed to show a down regulatory effect of insulin in either rat liver, rat adipose tissue (309) or human erythrocytes (291).

4.1. Concluding remarks.

The present study has indicated that high plasma concentrations of the ovarian steroids in mice at oestrus, and after the 10 weeks administration of either oestradiol or progesterone, or the two in combination were associated with an improved hypoglycaemic action of insulin compared to mice at dioestrus and after ovariectomy. The short-term cyclical fluctuations in the hypoglycaemic action of insulin during the oestrous cycle, were mediated predominantly by changes in insulin action at the post-receptor level, at least in liver and soleus muscle.

Ovariectomy for a period of 10 weeks resulted in a reduced hypoglycaemic action of insulin, mediated, at least in part, via a post-receptor reduction in the ability of insulin to promote glucose oxidation in skeletal

muscle. More importantly, however, the reduced hypoglycaemic action of insulin might have been due to a reduced insulin action in the liver. This was suggested by the reduced insulin binding capacity of hepatocytes and further supported by the observed reduction in liver glycogen content.

The reduced hypoglycaemic action of insulin, reduced rate of glucose oxidation by soleus muscle and reduced liver glycogen content and hepatocyte insulin binding capacity after 10 weeks ovariectomy were either partially or totally rectified by the 10 week administration of either oestradiol (5µg/kg/day) or progesterone (1mg/kg/day) or the two in combination. The most dramatic increase in these parameters was observed after the 10 week administration of oestradiol at the higher dose of 500µg/kg/day. These observations confirmed that oestrogens and progestogens might act on peripheral insulin target tissues to increase insulin action.

The present work also indicated that although neither 2 week ovariectomy nor the 2 week oral administration of oestradiol (5µg/kg/day) to ovariectomised mice, had any significant effect on the insulin binding capacity of isolated hepatocytes, the latter was significantly reduced by the 2 week oral administration of oestradiol at the higher dose of 500µg/kg/day or by progesterone (1mg/kg/day) alone or in combination with oestradiol (5µg/kg/day). The maximum specific ^{125}I-insulin binding capacity of soleus

muscle was not significantly altered by these treatments. These data suggest a short-term antagonism of insulin action, at least in the liver by progesterone (1mg/kg/day) and the higher dose of oestradiol (500µg/kg/day).

Thus, the ovarian steroids oestradiol and progesterone appear to be capable of two contrasting, divergent effects. On one hand they may act on the liver and skeletal muscle, in the long-term to increase the ability of insulin to lower blood glucose and ameliorate carbohydrate intolerance. On the other hand they might in the short-term, produce a metabolic setting of carbohydrate intolerance by reducing the ability of insulin to lower blood glucose. This may be produced by either their own inherent properties, or via insulin antagonists, possibly of pituitary or adrenal origin. Thus, the outcome, particularly on hepatocyte insulin receptor status, of either the removal of the major source of endogenous ovarian sex steroid production through ovariectomy, or the oral administration of oestradiol and progesterone has been shown to depend on both the dose and duration of treatment.

It is of interest to note, however, that in the present work the plasma glucose and insulin concentrations of freely fed mice were not significantly different at dioestrus and oestrus.Neither were they different in 10 week ovariectomised mice nor ovariectomised mice treated for 10 weeks with either oestradiol (5ug/kg/day) or progesterone (1mg/kg/day) or the two in combination. This

would suggest that neither the increased insulin action at oestrus compared to dioestrus nor the long-term increase in insulin action by oestradiol and progesterone play a major role in the regulation of glucose homeostasis under conditions of normal dietary intake and normal endocrine pancreatic reserve. However, in conditions in which there may be a tendency for glucose intolerance, through, for example, a compromised pancreatic B cell function or peripheral antagonism to insulin action, the influence of such ovarian steroids on carbohydrate metabolism might be of greater significance.

Although extrapolation to the clinical situation should, of course be undertaken with extreme caution, the short-term effects of oestradiol and progesterone, especially the high dose of oestradiol, suggests caution in the administration of these steroids for contraceptive or therapeutic purposes to women predisposed to glucose intolerance. The present study does, however, draw attention to the long-term beneficial effects of the natural ovarian hormones, especially oestradiol and progesterone, in respect of an improvement in insulin action.

5. Appendix.

5.1.1. Sex hormones and vaginal cytology in female mice.

Sex hormone titres vary throughout the oestrous cycle of both rats and mice.

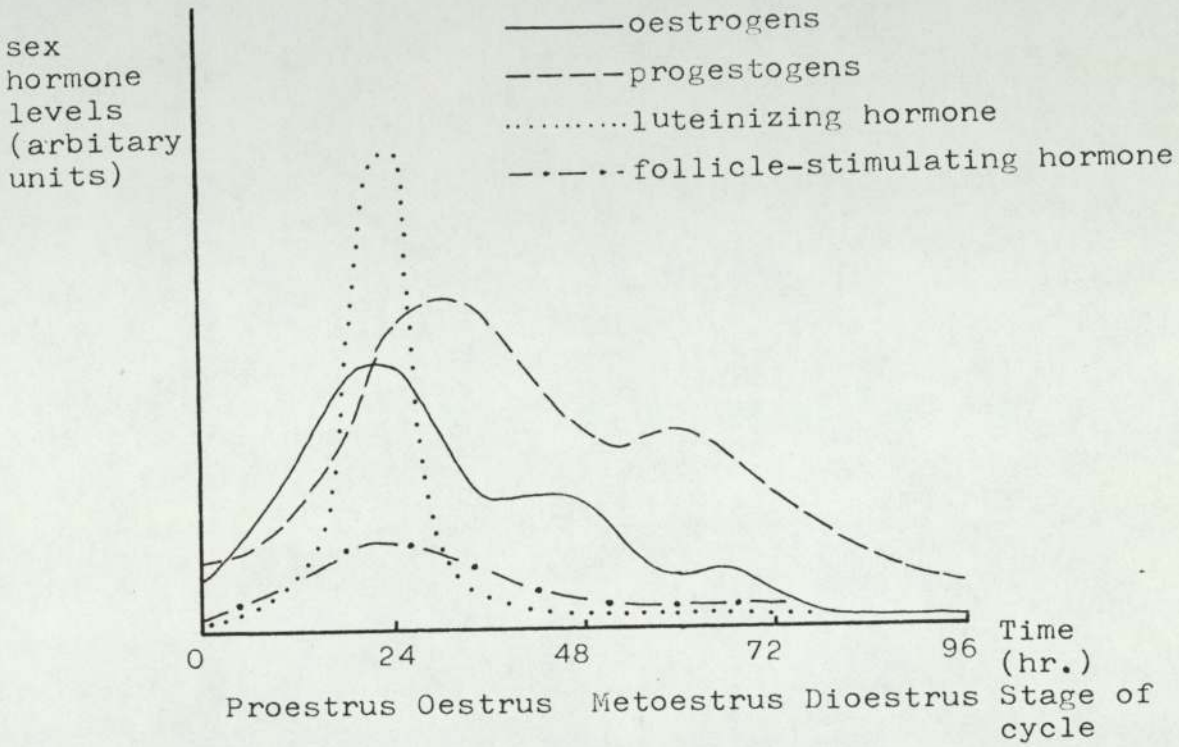

Figure 35. Sex hormone titres throughout the oestrous cycle in rats and mice together with the designated stages.

The levels of circulating sex steroids (oestrogens- chiefly 17-β-oestradiol, and progesterone) are principally responsible for the changes observed in the vaginal cytology throughout the oestrous cycle. Both classes of sex steroid promote the proliferation and growth of the vaginal epithelial cells at proestrus, resulting in a vaginal

coating of oval nucleated epithelial cells. Oestrus is characterized by an excess of large cornified epithelial cells. However, after this time, if fertilization has not occured, sex steroid titres plumet and leucocytes invade to ingest and thereby resorb the outer layers of the vaginal epithelium. Metoestrus is thus characterized by the presence of numerous leucocytes and dioestrus by the presence of leucocytes but in much reduced numbers.

5.1.2. Vaginal smears.

A smooth, clean glass rod (1-1.5mm diameter) moistened with saline was carefully inserted into the vagina, twisted and withdrawn. The tip of the rod was then brought into contact with a drop of saline on a microscope slide. After twisting the rod to facilitate transfer of cells into the saline drop, the slide was observed under a microscope (magnification x200). The stage of the oestrous cycle was then determined by identification of the cell types present in the smear (see above).

5.2. The radioimmunoassay of insulin.

Insulin concentrations were determined in serum samples by the double antibody technique of Hales and Randle (371).

A fixed quantity of insulin binding reagent (guineapig anti-insulin serum and rabbit ant-guineapig-globulin serum) was incubated with a constant amount of ^{125}I-insulin and aliquots of insulin standards or samples of unknown insulin concentrations. At the binding reaction equilibrium point, labelled and unlabelled insulins were bound specifically to the antibody in proportion to their molar ratios. Insulin binding reagent was then precipitated bv centrifugation, thus separating the bound and free insulin. The ^{125}I-activity associated with the bound fraction was then determined. The amount of unlabelled insulin in a sample is inversely related to the amount of labelled insulin bound and was determined by the construction of a standard curve.

Procedure.

Samples and insulin standards (0-10ng/ml) were pipetted into plastic LP3 tubes (Luckhams Ltd.) and made up to 50µl if required with diluent buffer (0.04M sodium phosphate, 0.03M EDTA, 0.1% sodium azide, 0.5% BSAV,pH7.4) Insulin standards were determined in triplicate although plasma insulin concentrations were generally the estimate of one sample because of the small blood volumes obtained from mice by tail bleeding. The insulin standard used was porcine monocomponent insulin (Lot No.S8311160, Novo Laboratories).

Insulin binding reagent (Wellcome Laboratories) was reconstituted to the suggested concentration (8ml of distilled water per vial) and 50μl aliquots added to all tubes. After gentle vortex mixing the tubes were left for a chase incubation of 6 hours at 4°C which produces better assay results than the direct incubation of all final reactants.

Following the initial incubation 50μl of ^{125}I-insulin (~10,000cpm, specific activity 50μCi /μg) were added to each tube including three replicate tubes containing 100μl of diluent buffer (blanks) for determination of non-specific ^{125}I-insulin binding. Aliquots were also added to empty tubes for replicate determinations of the total ^{125}I-activity (cpm). Labelled insulin was prepared by the choramine-T iodination method to an average specific activity of 247.6uCi/ug (page 91). The specific activity was reduced by the addition of an appropiate amount of unlabelled insulin. It is important not to add a significantly greater amount of labelled insulin as the resulting saturation of insulin binding sites severely reduces the assay sensitivity.

All tubes were then incubated for 16-18 hours at 4°C after which time 1ml aliquots of diluent buffer were added to all but the total ^{125}I-activity tubes. The tubes were then centrifuged at 2,000 g for 20 minutes. The centrifuge was allowed to 'run-down' with the brake off to prevent disturbing the small volume (not visible) of precipitate which consisted of antibody-bound insulin. Supernatants

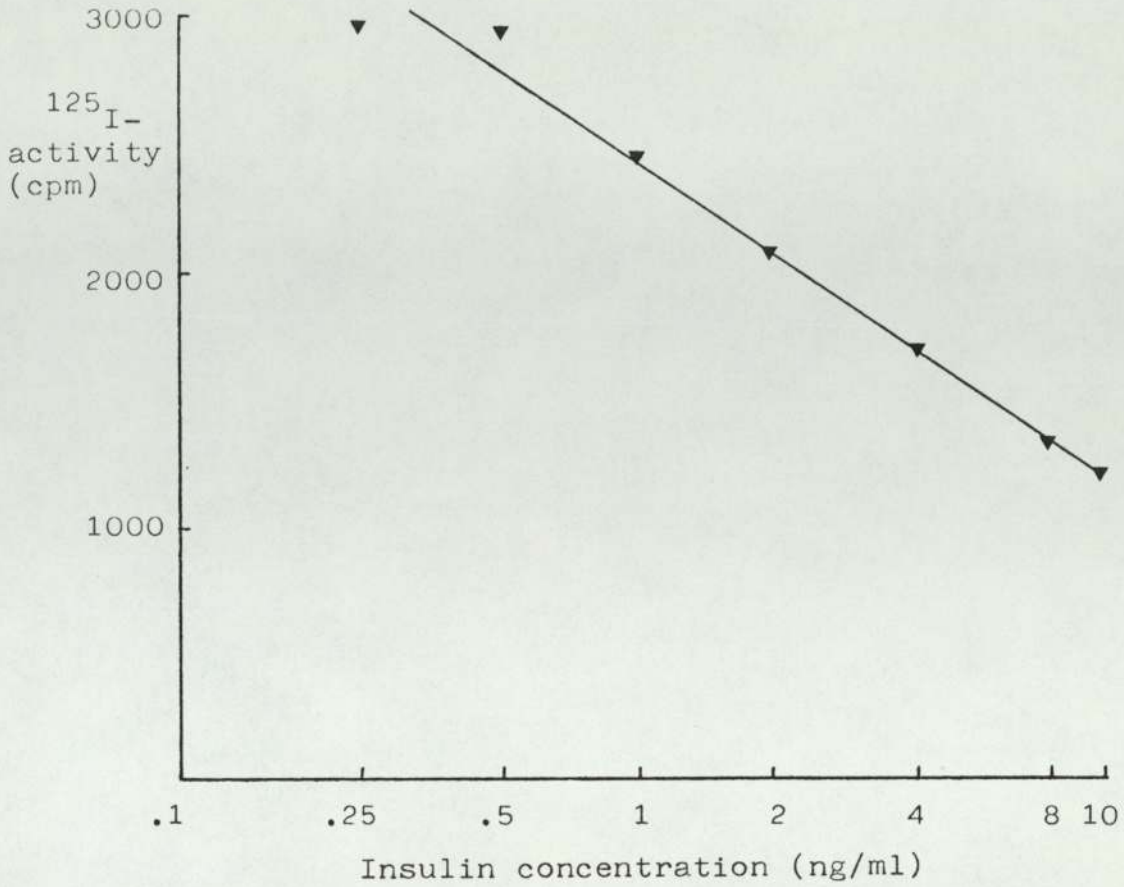

Figure 36. A typical standard curve produced for the double antibody radioimmunoassay of insulin. Correlation coefficient (Pearson's r)=0.991, P<0.002. Average SEM ±31cpm, n for each point =3.

were carefully decanted off and all but the total ^{125}I-activity tubes were then left at an angle of 30° for 1 hour. The last drop of supernatant was then removed from the mouth of each tube with absorbant paper.

The ^{125}I-activity was determined for 1 minute on a Gamma counter (ICN, Tracer Lab) with a counting efficiency of 57% and the values corrected for background ^{125}I-activity. Standard curves were constructed of $\log_{10}$[insulin] vs. mean cpm but did not include the value for zero insulin (Figure 36). The best straight lines were fitted by the method of least squares, allowing determination of sample insulin concentrations by equation ($y=mx + c$) or direct graph reading.

Assays were considered to be satisfactory if there was good agreement between replicates, the blank value was less than 5% of the total counts, the zero insulin standard was approximately 25% of the total count and the correlation coefficient (Pearson's r) of the standard curve was equivalent to $P<0.002$. Assay sensitivity was 0.18ng /ml.

A computer program was designed ('INS' pages 235-237) to facilitate the rapid analysis of results.

5.3. The radioimmunoassay of oestradiol.

Plasma oestradiol concentrations were determined with the aid of an oestradiol radioimmunoassay kit (Serono Diagnostics). The assay was based on the same principal as the insulin radioimmunoassay (see above). Thus the assay relied on the competition of ^{125}I-17 β-oestradiol and cold 17 β-oestradiol for oestradiol binding sites, followed by the separation of bound and free hormone fractions and the determination of the ^{125}I-activity associated with the bound fraction.

Procedure.

The assays were carried out without modification to the suggested procedure and the protocol has been summarised in Table 30 . All tubes except samples were assayed in duplicate. The high cost of the kits, the relatively small sampling capacity and the small blood volumes from mice obtained by tail bleeding prevented replicate sample determinations.

Replicate counts were meaned, background and non-specific binding subtracted and the percentage of maximum tracer binding (Bo) calculated for each standard and sample (%relative binding). Sigmoidal standard curves were constructed by plotting % relative binding versus $\log_{10}$[oestradiol] (Figure 37). Plasma oestradiol concentrations were determined by comparing the percent of ^{125}I-activity bound relative to the zero oestradiol standard with those of the standards by means of the standard curve.

Tubes / Reagents	Total Counts	NSB	B_o	Standard	Sample
Distilled H_2O		100			
Zero 17 B-oestradiol standard		50	50		
17 B-Oestradiol standards (25-50-100-200-500-1000-2000 pg/ml)				50	
Sample					50
^{125}I-17 B-Oestradiol	100	100	100	100	100
17B-oestradiol antiserum			100	100	100

Volumes are expressed in µl. Tubes were mixed and incubated at room temperature for 4 hours.

Rabbit gamma globulin to second antibody		100	100	100	100
PEG solution (cold, at 4°C.) (polyethylene glycol)		1000	1000	1000	1000

Tubes were mixed thoroughly.

TABLE 30

Procedure scheme for the radioimmunoassay of oestradiol. All tubes were centrifuged (except total count tubes) at 2000 g for 15 minutes. Supernatants were then aspirated and ^{125}I-activity associated with each tube determined.

Assays were considered satisfactory provided there was good correlation between standard replicates, non-specif binding was low (<5% of total cpm), specific binding was in the range of 55-65% of total cpm and the value of the kit test sample was within the given range.

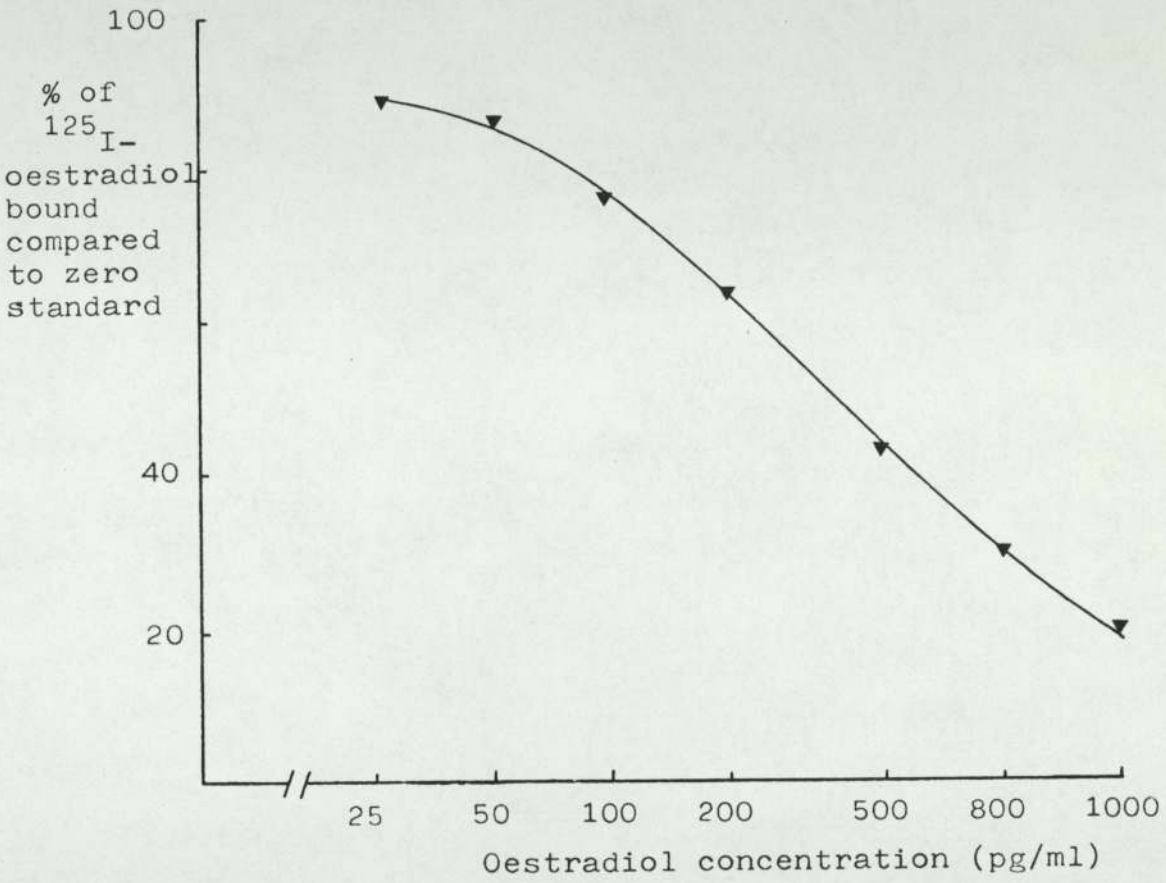

Figure 37. A typical standard curve produced for the radioimmunoassay of oestradiol. Average SEM ±0.53%, n for each point =2.

5.4. Protein determination.

Protein determinations were based on the method of Lowry, Rosebrough, Farr and Randall (338). Protein and Cu^{++} are first reacted in alkali. After this initial reaction, phosphomolybdic and phosphotungstic acids are reduced to molybdenum blue and tungsten blue respectively by both the Cu^{++}-protein complex (~75%) and the tyrosine and tryptophan residues of the protein (~25%). The blue colouration produced has an absorbance maximum at 750nm.

Procedure.

10-25μls aliquots of samples (muscle digests) were placed in glass tubes and made up to 1ml with distilled water. 5ml of an alkaline copper reagent were then added and the tube contents vortex mixed. The working alkaline tartrate reagent consisted of 90% alkaline tartrate solution (2% Na_2CO_3, 0.05% sodium or potassium tartrate in 0.1M NaOH) and 10% of 0.1gm /100ml $CuSO_4 5H_2O$ solution. All reagents were prepared on the day of use.

After standing for 15 minutes at room temperature, 0.5 ml of 1N Folin-Ciocalteau reagent was added to each tube followed by immediate vortex mixing. Tubes were left to stand for a further 30 minutes at room temperature and their absorbance read at 750nm. A standard curve was constructed (Figure38) using BSA (fraction V) at 0-0.4mg /ml. All readings were made against a reagent blank (1ml H_2O, 5ml alkaline copper reagent and 0.5ml phenol reagent) and sample protein concentrations were determined by comparison

of absorbance values to those of the standards. Standard curves were fitted by the method of least squares (correlation coefficient- Pearson's r generally $>0.98, P<0.02$).

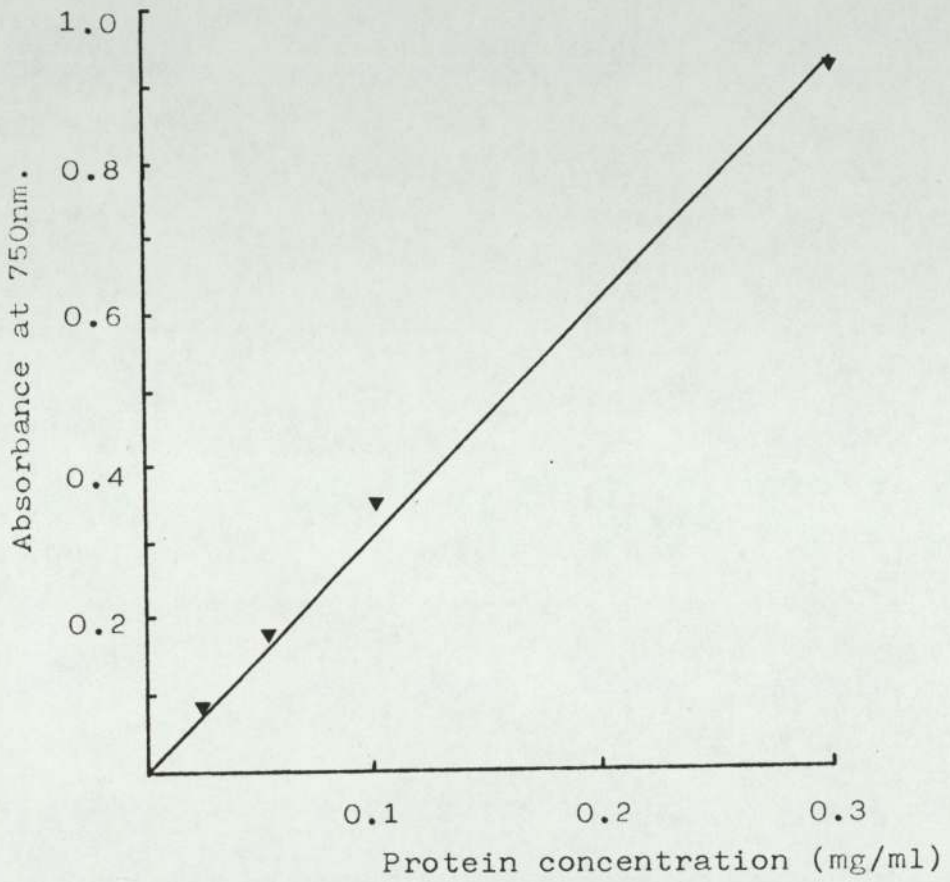

Figure 38. A typical standard curve for protein determination by the method of Lowry, Rosebrough, Farr and Randall (338). Correlation coefficient (Pearson's r)=0.996, P<0.002.

5.5. Glycogen determination.

Glycogen determinations were based on the method of Lo, Russell and Taylor (341).

Procedure.

The liver or muscle digests were placed on ice and 1ml of 95% ethanol was added to each digest to precipitate glycogen and the tubes retained in ice for 30 minutes. Following centrifugation at 840 g, 5°C for 30 minutes, supernatants were carefully aspirated and the tubes left inverted for a further 30 minutes. Distilled water (1ml for muscles, 3ml for liver) was then added to redissolve the glycogen and the appropriate volume transferred for use in the glycogen assay.

1ml of 5% (wt:vol) phenol solution was added to the samples (generally 1-3ml of 100% sample) followed rapidly (within 10-20 seconds) by 5ml of 98% H_2SO_4. These reactions were carried out in pyrex tubes of large capacity (20-30 ml) as the reaction was strongly exothermic and the mixture often boiled. Tubes were allowed to stand for 10 minutes, vortex mixed and placed in a water bath at 25°C for 20 minutes. The brown/red colour produced was then measured at the absorbance maximum of 490nm.

Standards were prepared using rabbit glycogen (0-0.01 mg/ml) in volumes equivalent to those used for the samples. All tubes were read against a reagent blank (H_2O, 1ml 5% phenol, 5ml 98% H_2SO_4) and standard curves (Figure 39) were fitted by

the method of least squares (correlation coefficient - Pearson's r generally >0.98, P<0.02).

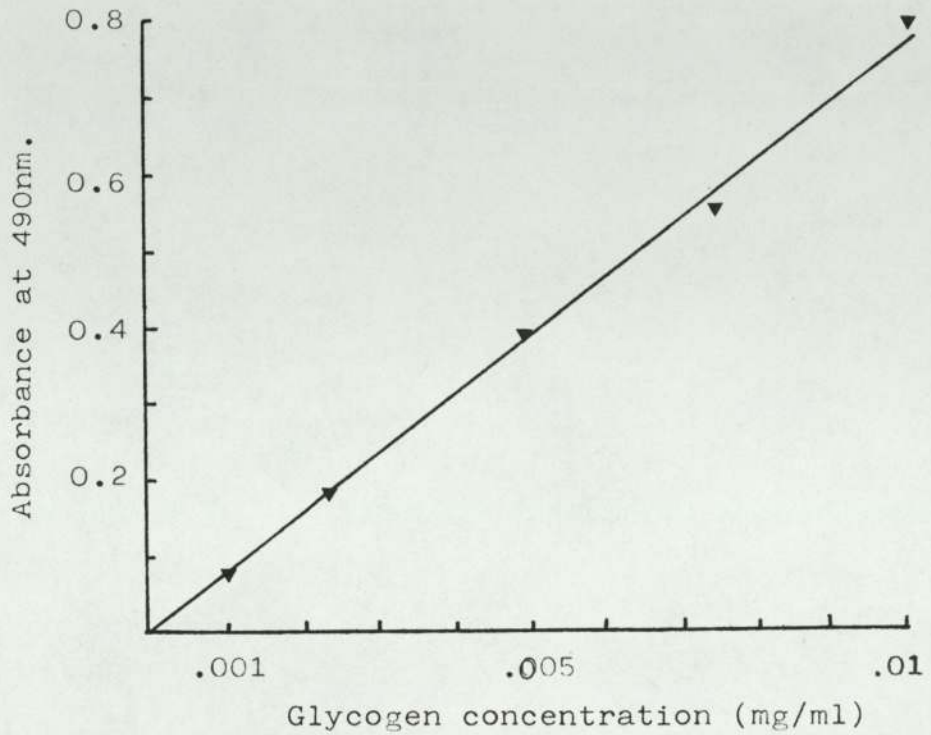

Figure 39. A typical standard curve for glycogen determination by the phenol-sulphuric acid method (341). Correlation coefficient (Pearson's r)=0.997, P<0.002.

5.6. Computer programs.

Data from insulin radioimmunoassays and hepatocyte insulin binding assays were analysed routinely by computer. Table 31 gives details of the programs employed, followed by listings of each program.

Program name	Program function	Program language	Computer
INS.	Analysis of insulin radioimmunoassay data	BASIC	Harris
RECEPTORSK	Scatchard analysis of hepatocyte insulin-receptor binding data	ALGOL-68R	ICL 1904s
RECEGRAFICA	Graph (Scatchard) plot) production from data compiled by RECEPTORSK	ALGOL-60	ICL1904s

TABLE 31.

Details of computer programs used throughout the present work.

5.6.1. RECEPTORSK.

```
Listing of : SBS 7006.RECEPTORSK(11/)   Produced on 9 Aug
82 at 15.26.59
G8.65G AT ASTON  IN ':SBS7006.M418WED' on 17Nov82 at
10-53.41 using U15
DOCUMENT    RECEPTORSK

BINDING
'BEGIN'
'REAL' CELLCT, TOTALCT, TRAH, FREE;
[1:18] 'REAL' COUNT;   [1:3]'REAL' C, M;   [1:17]'REAL'  HCONC;
'INT' K:=1, N;  'STRING' S;

'PROC' CALC = ('INT' B) 'REAL':
       'BEGIN' 'REAL' R:=0;    'INT' DATA;
       'TO' B 'DO''BEGIN' READ(DATA); R:=R + DATA 'END';
       R:=(R*100)/(B*N);
       R       'END';

'PROC' PRINTVAL1 = ('REAL' C) 'VOID':
       'BEGIN' OUTF(STANDOUT, S<5.2>2XS, C)  'END';

'PROC' PRINTVAL2 = ('REAL' C) 'VOID':
       'BEGIN' OUTF(STANDOUT, S<2.5>2XS, C)  'END';

'PROC' REGRESSION = ('INT' FIRST, LAST) 'VOID':
       'BEGIN' 'REAL' SX:=0.0, SXQ:=0.0, SXY:=0.0, SY:=0.0,
       SYQ:=0.0, XB:=0.0;
       FREE:=0.0;  N:=N+1;
'FRO   'FOR' I 'FROM' FIRST 'TO' LAST 'DO''BEGIN'
            SX:=SX + HCONC[I];  SXQ:=SXQ + HCONC[I]+2;
            SY:=SY + COUNT[I];  SXY:=SXY + HCONC[I]* COUNT[I];
            SYQ:=SYQ + COUNT[I]+2; FREE:=FREE + 1.0  'END';
       XB:=SX/FREE;  SY:=SY/FREE;
       M[N]:= (SXY*(SX*SY)) / (SXQ*(SX*XB));
       PRINT((NEWLINE,NEWLINE,"POINTS ", FIRST,"" -",LAST,
            ":              SLOPE M ="));    PRINTVAL2(M[N]);
       C[N]:=SY-(M[N]*XB);  PRINT("  Y-INTERCEPT C =");
       PRINTVAL2(C[N]);  PRINT((NEWLINE, "       CORRELATION
       COEFFICIENT R ="));
       PRINTVAL2((SXY/FREE - XB*SY) / SQRT((SXQ/FREE -XB+2)*
       (SYQ/FREE - SY+2))); FREE:=XB-9SY/M[N]); 'IF'[M]N  <0.0
       'THEN' PRINT("    X-INTERCEPT ="); 'IF' FREE  <100.0
       'THEN' PRINTVAL2(FREE) 'ELSE' PRINTVAL1(FREE) 'FI'
       'FI'    'END';

SIGN'OFNUMBERSTYLE:=29;  INT'OF NUMBERSTYLE:=2; SPACES'OF'
NUMBERSTYLE:=0;
START:   READ((S, N)); 'IF' N   0 'THEN K:=N;  'GOTO' DEPOT
'FI';
'FOR' I 'TO' 16 'DO' COUNT[I]:=CALC(3);
```

```
'FOR' I 'FROM' 17 'TO' 18 'DO' COUNT[1]:=CALC(2);
'C'1-16 ARE TRIPLICATE STANDARDS, 17-18 ARE DUPLICATE 1000
AND N.S. BINDING 'C'
TOTAL CT:=CALC(1);  READ((CELLCT, TRAH)); CELLCT:=CELLCT * 0.8;
PRINT((K, NEWLINE, S, NEWLINE, NEWLINE,"        TOTAL COUNT =
"));
PRINTVAL1(TOTALCT);
PRINT(("COUNTS/MINUTE", NEWLINE, CELL COUNT(4/5 OF VALUE
INPUT) =");
PRINTVAL2(CELLCT);
PRINT(("MILLION CELLS/ML", NEWLINE,"      TRACER HORMONE ="));
PRINTVAL2(TRAH);
PRINT(("NG/ML", NEWLINE,"         NON-SPECIFIC BINDING ="));
PRINTVAL1(COUNT[18]);
PRINT(("COUNTS/MINUTE",  NEWLINE, NEWLINE,
" MEAN    HORMONE   APPROX.   FRACTION   AMOUNT  AMOUNT  B/F",
    NEWLINE,
"COUNT    CONC.   CTS.BOUND   BOUND     BOUND     FREE  RATIO",
    NEWLINE,
"(COUNT/MIN)  (NG/ML)    (CPM/M.CELLS)   (NG/ML)  (NG/ML)",
    NEWLINE));
'FOR' I 'FROM' 0 'TO' 2 'DO''FOR' J 'TO 5'DO' HCONC[I*5+J+2]:
     2*J*(10 I);
HCONC[1]:=0;  HCONC[2]:=1;
'FOR' I 'TO' 17 'DO''BEGIN'
     PRINT(NEWLINE);  PRINTVAL1(COUNT[I] );  'C' MEAN COUNT 'C'
     HCONC[I]: HCONC[I] + TRAH;  COUNT[I]:=(COUNT[I] -
        COUNT[18]) / CELLCT;
     PRINTVAL1(HCONC[I]);  PRINTVAL1(COUNT[I]);
        'C' HORMONE CONC, APPROX.COUNTS BOUND 'C'
     COUNT[I]:=COUNT[I]/TOTALCT;  PRINTVAL2(COUNT[I]); 'C'
        FRAC BOUND 'C'
     COUNT[I]:=COUNT[I]  * HCONC[I]; FREE: HCONC[I] - COUNT[I];
     PRINTVAL2(COUNT[I]);  PRINTVAL1(FREE); 'C' BOUND ( X),
       FREE 'C'
     HCONC[I]:=COUNT[I];  COUNT[I]:=COUNT[I]/FREE;
       PRINTVAL2(COUNT[I])
     'C' BOUND/FREE RATIO (=Y) 'C'  'END';
PRINT((NEWLINE, NEWLINE, "NOTE: APPROX.COUNTS BOUND CALCULATED
     ON4/5 OF VALUE OF CELL COUNT AS INPUT"));  N:=0;
REGRESSION(1, 7);  REGRESSION(12, 17);
PRINT((NEWLINE, NEWLINE, "*"));
'FOR' I 'TO' 17 'DO''BEGIN'
     PRINT(NEWLINE);  PRINTVAL2(HCONC[I]); PRINTVAL2(COUNT I )
     'END'
'FOR' I 'TO' 3 'DO''BEGIN'
     PRINT(NEWLINE);  PRINTVAL2(M[I]);  PRINTVAL2(C[I])  'END';
PRINT((SPACE, S, "        @@@", NEWLINE)); READ(NEWLINE);
K:=K+1;  'IF' K<17 'THEN''GOTO' START 'FI';
DEPOT:  PRINT(K)     'END'
'FINISH'
```

5.6.2. RECEGRAFICA.

```
'BEGIN' 'INTEGER''PROCEDURE' INTS (MAX); 'REAL' MAX;
      'BEGIN' 'INTEGERS' NI, S; I:=0;
      STAR:    'IF' MAX < 3.0 'THEN''BEGIN' MAX:=MAX*10;
      I:=I+1;
            'GOTO' STAR     'END';
      'IF' MAX > 30.0 'THEN''BEGIN' MAX:=MAX/10; I:=-1  'END';
      'IF' MAX > 15 'THEN' S:=5 'ELSE''IF' MAX > 10 'THEN'
      S:=3 'ELSE''IF' MAX > 5 'THEN' S:=2 'ELSE S:=1;
      MAX:=MAX/S;
      NI:=ENTIER(MAX); 'IF' (MAX - NI) > 0.0 'THEN'
      NI:=NI+1;     MAX:=NI*S / (10+I);   INTS:=NI
      'END' OF INTS;

'PROCEDURE' READTRAP(P); 'PROCEDURE' P; 'EXTERNAL';
'PROCEDURE' T; 'BEGIN' WRITETEXT ('(''('2C')'DATA%ERROR%
FOUND')');
   'GOTO' DEPOT   'END';
'INTEGER''ARRAY' W[1:20]; 'REAL''ARRAY' X, Y [1:17], M,
C[1:3];
'INTEGER' I, K;    'REAL' XM, YM;

OPENGINOGP; SOFCHA; DEVPAP (2500.0, 340.0, 1); READTRAP(T);
START: K:=READ; 'IF' K<0 'THEN''GOTO' DEPOT;
'IF' K=17 'THEN''BEGIN' WRITETEXT('('CAPACITY%OF%GRAPHPLOTTER%
     REACHED%-%NO%MORE%DATA%PROCESSED')'); 'GOTO'DEPOT 'END';
COPYTEXT('('*')'); NEWLINE(4); XM:=YM:=0;
'FOR' I:=1 'STEP' 1 'UNTIL' 17 'DO''BEGIN' X[I]:=READ;
     Y[I]:=READ;
     'IF' X[I] > XM 'THEN' XM:=X[I];
     'IF' Y[I] > YM 'THEN' YM:=Y[I]   'END';
'FOR' I:=1 'STEP' 1 'UNTIL' 3 'DO''BEGIN' M[I]:=READ;
     C[I]:=READ          'END';
INSTRARR('('@@@')',W);
LINTO2 (0.0, 160.0); MOVTO2 (297.0, 160.0); LINTO2
     (297.0, 0.0);
SHIFT2 (35.0, 15.0);   CHASIZ (2.5, 3.5);
AXIPOS (0, 0.0, 0.0, 240.0, 1); AXISCA (3, INTS(XM), 0.0, XM, 1);
AXIPOS (0. 0.0, 0.0, 125.0, 2); AXISCA (3, INTS(YM), 0.0, YM, 2);
AXIDRA (1, 1, 1);   AXIDRA (-1, -1, 2);
MOVTO2 (100.0, -15.0);     CHASTR ('('AMOUNT%BOUND%(NG)*.')');
MOVTO2 (-15.0, 45.0);      CHAANG (90.0);
CHASTR ('('BOUND-FREE%RATIO*.')');
GRASYM (X, Y, 17, 3, 0);
GRAMOV (0.0, C[1]);     GRALIN (X[7], M[1]-X[7] + C[I]);
GRAMOV (X[8], M[2]-X[8] + C[2]);
GRALIN (X[11], M[2]*X[11] + C[2]);
GRAMOV (X[12], M[3]*X[12] + C[3]); M[1]:=M[3]-XM + C[3]
'IF' M[1] < 0 'THEN' GRALIN (-C[3]/M[3], 0) 'ELSE'
     GRALIN (XM, M[1]);
MOVTO2 (60.0, 135.0); CHASIZ (3.0, 5.0); CHAANG (0);
CHAARR (W, 20, 4);
'IF' K/2 - K'/'2 = 0 'THEN' SHIFT2 (265.0, -180.0) 'ELSE'
     SHIFT2 (-35.0, 150.0);
MOVTO2 (0.0, 0.0);       'GOTO' START;
DEPOT:     DEVEND       'END'
```

5.6.3. INS.

```
105 REM      INSULIN ASSAY
110 REM
115 REM
120 FILES*
125 DIM M$[6],T$[6],U$[6],S$[12],F$[6],H$[64]
130 REM DATA statements ; "species","units",no. of standards,
standard concns.
135 DATA "Mouse","ng/ml",5,.5,1,2.5,10
140 DATA "Human","uU/ml",4,12.5,25,50,100
145 PRINT "Do you want printout (Y or N) ";
150 INPUT Y$
155 IF Y$#"Y" TNEN 235
160 PRINT LIN(1);"Ascii file name for printout";
165 INPUT F$
170 ASSIGN F$,1,V1
175 IF V1=0 THEN 205
180 PRINT LIN(1);"File does not exist !"
185 PRINT "Create it with command : FILE-******,DS,N"
190 PRINT "where ****** is filename , N is length in blocks"
195 PRINT "and RUN again"
200 STOP
205 PRINT LIN(1);"Experiment title etc. for printout ";
210 INPUT Hs
215 PRINT #1;H$
220 PRINT
225 REM ************************ Input standard cpm's
230 REM ************************ and compute best line
235 PRINT "STANDARDS  :  Mouse or Human or Both ( M or H or B)";
240 INPUT M$
245 IF M$="H" THEN 280
250 IF M$="M" THEN 260
255 IF M$ "B" THEN 235
260 GOSUB 305
265 LET A1=A
270 LET B1=B
275 IF M$="M" THEN 485
280 RESTORE 140
285 GOSUB 305
290 LET A2=A
295 LET B2=B
300 GOTO 485
305 READ T$,U$
310 PRINT LIN(1);T$
315 PRINT "Standards"," cpm ?"
320 PRINT U$
325 IF Y$#"Y" THEN 345
330 PRINT #1;LIN(1);Ts
335 PRINT #1; "Standards"," cpm"
340 PRINT #1;U$
345 LET X=G=Y=S=W=N=0
350 READ K
355 FOR J=1 TO K
360 READ I
```

```
365 PRINT I," ";
370 INPUT C
375 IF Y$##"Y" THEN 385
380 PRINT  1; USING "4D.D8X,6D";I,C
385 LET I=LOG(I)/LOG(10)
390 LET X=X+C
395 LET Q=Q+C ** 2
400 LET Y=Y+I
405 LET S=S+I ** 2
410 LET W=W+I*C
415 LET N=N+1
420 NEXT J
425 LET B=(W-X*Y/N)/(G-X ** 2/N)
430 LET A=(Y-B*X)/N
435 LET Z=(W-X*Y/N)/SQR((Q-X ** 2/N)*(S-Y ** 2/N))
440 PRINT LIN(1);"Computed best line : Intercept (cpm = 0)  ="A
445 PRINT TAB(21);"Slope                ="B
450 PRINT TAB(21);"Correlation coefficient = "Z
455 IF Y$##"Y" THEN 475
460 PRINT #1;LIN(1);"Computed best line : Intercept (cpm=0) ="A
465 PRINT #1;TAB(21);"Slope                    ="B
470 PRINT #1;TAB(21);"Correlation coefficient ="Z
475 RETURN
480 REM ******************** Calculate sample concentrations
485 PRINT LIN(1);"Input sample name , or 'END' to stop program"
490 PRINT "                    or 'DIL' to change dilution
    factor"
495 IF Y$##"Y" THEN 505
500 PRINT "                    or 'SUBH' to insert subheading
for printout"
505 PRINT "followed by cpm"
510 PRINT LIN(1);"'*' indicates result outside linear region
    of curve"
515 PRINT "    and will be incorrectly calculated by this
    program"
520 REM   ************   Limits of linearity set in 735,740,
    780,785
525 IF M$="B" THEN 600
530 PRINT LIN(1);" "," ","   Insulin concn."
535 PRINT " "," ","   ref."Ts" standard"
540 IF Y$##"Y" THEN 555
545 PRINT #1;LIN(1);TAB(36);"Insulin concentration"
550 PRINT #1;TAB(24);"Dilution      ref."Ts" standard"
555 IF M$="H" THEN 580
560 PRINT "Sample","   cpm","  ng/ml","  uU/ml"
565 IF Y$##"Y" THEN 640
570 PRINT #1;"Sample","cpm      factor      ng/ml      uU/ml"
575 GOTO 635
580 PRINT "Sample","   cpm","  uU/ml"
585 IF Y$##"Y" THEN 640
590 PRINT #1;"Sample","   cpm      factor      uU/ml"
595 GOTO 635
600 PRINT LIN(1);TAB(33);"Insulin concentration    Insulin
    concn."
605 PRINT TAB(33);"ref. Mouse standard     ref. Human st."
610 PRINT "Sample","   cpm","  ng/ml","  uU/ml","  uU/ml"
615 IF Y$##"Y" THEN 640
620 PRINT #1;LIN(1);TAB(36);"Insulin concentration   Insulin
    concentration"
```

```
625 PRINT #1;TAB(24);"Dilution     ref. Mouse standard
    ref. Human standard"
630 PRINT #1;"Sample","  cpm     factor      ng/ml
    uU/ml            uU/ml"
635 PRINT  1
640 PRINT
645 PRINT "Dilution factor ",
650 INPUT F
655 PRINT
660 LET P$="*"
665 LET R$="*"
670 INPUT S$
675 IF Y$ "Y" THEN 700
680 IF S$ "SUBH" THEN 700
685 INPUT H$
690 PRINT #1;LIN(1);H$
695 GOTO 670
700 IF S$="END" THEN 840
705 IF S$="DIL" THEN 645
710 PRINT " "," ";
715 INPUT P
720 PRINT S$,P," ";
725 IF M$="H" THEN 775
730 LET D1=INT(EXP((A1+B1*P)*LOG(10))*10+ 5)/10
735 IF D1< 5 THEN 750
740 IF D1>12.8 THEN 750
745 LET P$=" "
750 IF M$="B" THEN 775
755 PRINT  USING "A,4D.D6X,A,5D.D";P$,F*D1,P$,25*F*D1
760 IF Y$#"Y" THEN 770
765 PRINT #1; USING "15A,5DX4,5DX5,A,5D.D5X,A,6D.D";S ,P,F,
    P$,F*D1,P$,25*F*D1
770 IF M$="M" THEN 660
775 LET D2=INT(EXP((A2+B2*P)*LOG(10))+ 5
780 IF D2<12.5 THEN 795
785 IF D2>100 THEN 795
790 LET R$=" "
795 IF M$="B" THEN 820
800 PRINT  USING "A,5D";R$,F*D2
805 IF Y$#"Y" THEN 660
810 PRINT #1; USING "15A,5D4X,A,7D";S$,P,F,R$,F*D2
815 GOTO 660
820 PRINT USING "A,4D.D6X,A,5D.D10X,A,5D";P$,F*D1,P$,25*F*D1,
    R$,F*D2
825 IF Y$#"Y" THEN 660
830 PRINT #1; USING "15A,5D4X,5D5X,A,5D,D5X,A,6D,D9X,A,7D";
    S$,P,F,P$,F*D1,P$,25*F*D1,R$,F*D2
835 GOTO 660
840 IF Y$#"Y" THEN 850
845 PRINT #1; END
850 END
```

5.7. Appendix to results.

Panel 1

Experimental group	OVX	E	E(HD)	E+P	P	O	D	Intact (O+D)
n	6	6	6	6	6	6	6	12
Body weight (gm)	42.43 ±1.62	47.07 ±2.84	49.27 ±2.44	44.12 ±2.15	43.68 ±2.83	40.59 ±2.47	39.91 ±1.04	40.25 ±0.73

Panel 2

Experimental group	OVX	E	E(HD)	E+P	P	O	D	Intact (O+D)
n	5	6	5	5	6	6	6	12
Body weight (gm)	35.59 ±1.02	36.48 ±1.28	31.70 ±0.69	41.80 ±2.43	40.26 ±1.74	37.84 ±1.68	38.22 ±1.39	38.07 ±1.08

Panel 3

Experimental group	O	D
n	9	11
Body weight (gm)	44.92 ±1.93	44.21 ±1.64

Panel 4

Experimental group	OVX	E	E(HD)	E+P	P	O	D	Intact (O+D)
n	5	6	6	6	6	6	6	12
Body weight (gm)	38.44 ±0.60	37.88 ±0.60	36.83 ±1.29	37.58 ±1.04	35.80 ±1.05	43.36 ±1.66	42.14 ±2.35	42.75 ±1.45

TABLE32.continued page 239.

Panel 5

Experimental group	OVX	Intact (D)	E	E(HD)	OVX	E+P	P
n	6	7	6	5	6	6	6
Body weight (gm)	32.82 ±1.02	32.21 ±1.01	32.80 ±1.38	31.85 ±0.51	43.50 ±2.36	46.07 ±2.49	40.15 ±2.05

Panel 6

Experimental group	OVX	E	E+P	P	OVX	E(HD)	OVX	Intact (D)
n	6	6	6	7	6	5	6	6
Body weight (gm)	33.57 ±1.67	33.53 ±1.24	36.83 ±1.86	32.54 ±0.82	42.02 ±1.21	42.77 ±1.29	33.23 ±0.83	32.69 ±1.28

Panel 7

Experimental group	OVX	E	E+P	P	Intact (D)	OVX	E(HD)
n	5	8	6	6	5	5	6
Body weight (gm)	48.41 ±3.43	40.84 ±1.82	45.42 ±2.13	44.25 ±2.82	43.40 ±1.68	36.51 ±1.81	33.97 ±1.02

TABLE 32. (continued)

For legend see pages 240 and 241.

Legend for Table 32.

The body weights (gm) of experimental mice. The key below gives details of the studies performed with each set of experimental mice. Tests for significance of difference (unpaired t tests) were carried out comparing each experimental group with ovariectomised mice. Experimental groups of intact mice were composed of the mice from both the oestrus and dioestrus stages of the oestrous cycle unless otherwise stated. No significant differences were found between oestrus and dioestrus mice. Values are the means ±SEM's.

Panel 1.

The hypoglycaemic action of insulin and the glycogen content of soleus muscles in mice that had been ovariectomised and hormone treated for 10 weeks.

Panel 2.

^{14}C-glycogenesis and $^{14}CO_2$ production from ^{14}C-glucose by isolated soleus muscles, and liver wet weights and hepatic glycogen contents of mice that had been ovariectomised and hormone treated for 10 weeks.

Panel 3.

The influence of the oestrous cycle on hepatocyte insulin receptor status.

Panel 4.

Specific binding of ^{125}I-insulin to isolated soleus

muscles from mice that had been ovariectomised and hormone treated for 2 weeks. Also the hypoglycaemic action of insulin in mice that had been ovariectomised and treated with oestradiol 500μg/kg/day for 2 weeks.

Panel 5.

Specific binding of ^{125}I-insulin to isolated soleus muscles from mice that had been ovariectomised and hormone treated for 10 weeks.

Panel 6.

Hepatocyte insulin receptor status of mice that had been ovariectomised and hormone treated for 2 weeks.

Panel 7.

Hepatocyte insulin receptor status of mice that had been ovariectomised and hormone treated for 10 weeks duration.

Panel 1

Experimental group	OVX	E	E(HD)	E+P	P	O	D	Intact (O+D)
n	12	12	10	10	12	12	12	24
Muscle protein content (mg)	1.68 ±0.05	1.98* ±0.11	1.543 ±0.05	2.01 ±0.13	2.00 ±0.10	1.97 ±0.15	1.71 ±0.90	1.84 ±0.09

Panel 2

Experimental group	OVX	E	E(HD)	E+P	P	O	D	Intact (O+D)
n	12	12	10	10	10	6	6	12
Muscle protein content (mg)	1.38 ±0.07	1.82** ±0.10	1.15* ±0.07	2.04*** ±0.15	1.57 ±0.07	1.76 ±0.10	1.80 ±0.07	1.78** ±0.09

Panel 3

Experimental group	OVX	E	E(HD)	E+P	P	O	D	Intact (O+D)
n	10	12	12	12	12	12	12	24
Muscle protein content (mg)	1.70 ±0.08	1.58 ±0.05	1.63 ±0.04	1.62 ±0.09	1.48* ±0.06	1.79 ±0.09	1.94 ±0.10	1.85 ±0.07

Panel 4

Experimental group	OVX	E	E(HD)	Intact (D)	OVX	P	E+P
n	12	12	10	14	12	12	12
Muscle protein content (mg)	1.34 ±0.05	1.42 ±0.06	1.31 ±0.05	1.42 ±0.06	1.54 ±0.06	1.64 ±0.09	1.90*** ±0.02

TABLE 33. For legend see pages 243 and 244.

Legend for Table 33 .

The protein content (mg) of soleus muscles from experimental mice. The key below gives details of the studies performed with each set of experimental mice. The values shown represent the mean protein content (mg) of both the right and left hind limb soleus muscles of all mice within that group. Paired t values were determined for each group, right versus left muscle protein content and no significant differences were found. * $P<0.05$, ** $P<0.02$, and *** $P<0.01$ when compared with the appropriate ovariectomised control mice. The protein contents of soleus muscles from intact mice are the average protein contents of soleus muscles from mice in the dioestrus and oestrus stages of the oestrous cycle unless otherwise stated. No significant differences were found between the soleus muscle protein contents of diostrus and oestrus mice. Values are means ± SEM's.

Panel 1

^{14}C-glycogenesis and $^{14}CO_2$ production from ^{14}C-glucose by isolated soleus muscles from mice in the dioestrus and oestrus stages of the oestrous cycle and from mice that had been ovariectomised and hormone treated for 10 weeks.

Panel 2

Glycogen content of soleus muscles from mice in the dioestrus and oestrus stages of the oestrous cycle and from mice that had been ovariectomised and hormone treated for 10 weeks.

Panel 3

Specific binding of ^{125}I-insulin to isolated soleus muscles from mice in the dioestrus and oestrus stages of the oestrous cycle and from mice that had been ovariectomised and hormone treated for 2 weeks.

Panel 4

Specific binding of ^{125}I-insulin to isolated soleus muscles from mice that had been ovariectomised and hormone treated for 10 weeks.

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