REGULATION OF PYRUVATE DEHYDROGENASE ACTIVITY BY INSULIN AND CATECHOLAMINES DURING THE REPRODUCTIVE CYCLE IN THE RAT

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ABBREVIATIONS

The abbreviations used in this thesis are as given in 'Instructions to Authors' (1987) Biochem. J. 241: 3-24 with the following exceptions:

AABS	p-(p-amino-phenylazo)benzene sulphonic acid
AAT	arylamine acetyltransferase
ACC	acetyl-CoA carboxylase
ACTH	adrenocorticotropin
approx.	approximately
BAT	brown adipose tissue
BSA	bovine serum albumin
cAMP	adenosine-3'5'-phosphate
cGMP	guanosine-3'5'-phosphate
CURL	compartment of uncoupling of ligand and receptor
DAG	diacylglycerol
DTT	dithiothreitol
EGF	epidermal growth factor
GTPase	guanine triphosphatase
IP	inositol phosphate
MCV	mean cell volume
N_s N_i N_{ins}	guanine nucleotide regulatory proteins
PDH	pyruvate dehydrogenase
PIP_2	phosphatidylinositol-4,5 bisphosphate
TPA	12-0-tetradecanoylphorbol 13-acetate

SUMMARY

Pyruvate dehydrogenase (PDH) controls the ireversible conversion of pyruvate to acetyl-CoA for subsequent oxidation to CO_2 or for synthesis of fatty acids: the enzyme thus has a critical role in the regulation of glucose metabolism (see Denton & Hughes, 1978). The enzyme occurs in an active, dephosphorylated form and an inactive phosphorylated form, the interconversions being effected by PDH-kinase and phosphatase (see Saggerson, 1985).

During pregnancy and lactation a number of adaptations occur in glucose metabolism in the peripheral tissues to accommodate the demands of the foetus and mammary gland respectively (see Williamson, 1980; Bauman & Elliot, 1983; Vernon & Flint, 1983). In view of the important role of PDH in glucose metabolism the aim of this project was to investigate changes in PDH activity and its control by insulin and catecholamines during pregnancy and lactation in the rat.

Around day 7 of pregnancy a transient surge occurred in the proportion of PDH in the active state in white adipose tissue and skeletal muscle. Then by day 18 of pregnancy both total PDH present and the proportion of the enzyme in the active state in skeletal muscle were reduced. Lactation resulted in a decrease in total PDH in white adipose tissue and in the proportion of the enzyme in the active state in skeletal muscle; by 2 days after litter removal both these actvities had recovered and in addition a considerable rise occurred in the proportion of the enzyme in the active state in white adipose tissue. Little change occurred in hepatic PDH activity

during pregnancy and lactation. In contrast to the other tissues studied both the total PDH present and the proportion of the enzyme in the active state in mammary gland increased substantially between parturition and peak lactation; both then fell on weaning.

It is established that insulin increases the amount of active PDH in white adipose tissue both in vivo and in vitro. However, there is disparity between results of various in vitro studies on the effects of catecholamines on PDH in white adipose tissue with inhibitory, stimulatory and biphasic effects all having been reported (see Saggerson, 1985). In the present study insulin in vivo activated PDH in skeletal muscle, white adipose tissue and liver whereas noradrenaline in vivo stimulated PDH activity in white adipose tissue and liver but not skeletal muscle. This effect of noradrenaline on white adipose tissue was completely blocked by either the α_1-antagonist prazosin or the β-antagonist propranolol. Both insulin and adrenaline activated PDH in isolated adipocytes in vitro and the maximum effect of adrenaline required activation of both α_1 and β-receptors.

During lactation the ability of insulin to activate PDH in white adipose tissue was completely lost whereas it was retained in skeletal muscle and liver. Furthermore the catecholamine-induced stimulation of PDH activity in white adipose tissue was muted both in vivo and in vitro but was increased in liver in vivo. The loss of ability of insulin to activate PDH in white adipose tissue both in vitro and in vivo was probably due to a failure to release an unidentified mediator substance from adipocyte plasma membranes.

The results presented in this thesis show that tissue specific changes occur in PDH activity during pregnancy and lactation in the rat. In addition during lactation changes occur in the control of PDH activity by insulin and catecholamines. These various adaptations should limit the use of glucose and lactate carbon by skeletal muscle and white adipose tissue and thereby facilitate their preferential use by the mammary gland.

This thesis is a study of the control of PDH activity by insulin and catecholamines during the reproductive cycle in the rat. The introduction commences with a review of metabolism in the maternal tissues during pregnancy and lactation and of the hormonal signals to the tissues. The structure and hormonal control of the mammalian PDH complex is then described and the knowledge presently available concerning the mechanism of action of insulin and catecholamines is reviewed. The introduction concentrates on information that is available for the rat.

CHAPTER 1

1. INTRODUCTION

1.1. Pregnancy and lactation in the rat

During pregnancy the mother must supply adequate nutrients to ensure the growth and development of the foetus *in utero* and must also establish a reserve store of energy which can be utilised, around parturition when there is a transient fall in food intake and at times during lactation when the dietary intake is insufficient to meet the demands of milk production (see Bauman & Currie, 1980; Baird *et al.*, 1985). To this end, in the rat, food intake is increased during pregnancy (Cole & Hart, 1983; Otway & Robinson, 1968; Cripps & Williams, 1975) and various alterations occur in the metabolism of the peripheral tissues which result in the progressive storage of fat during the initial two-thirds of pregnancy (see Vernon & Flint, 1984) and which facilitate the transfer of nutrients to the foetus during

the last third of pregnancy. Studies in vivo, using the glucose tolerance test (Leturque et al., 1980), the insulin tolerance test (Knopp et al., 1970b) and the euglycemic clamp technique (Leturque et al., 1984a) show that between day 16 of pregnancy and parturition insulin resistance occurs in the peripheral tissues of the rat. This resistance should reduce the use of circulating glucose by the peripheral tissues and hence increase its availability to the foetus.

During lactation the demand that the mammary gland places upon circulating substrates is extremely high (see Williamson, 1980; Williamson et al., 1984). To help meet this demand various physiological changes occur in the rat such as an increase in cardiac output (Chatwin et al., 1969), in the absorptive capacity of the intestines (Fell et al., 1963; Cripps & Williams, 1975) and in food intake (Fell et al., 1963; Otway & Robinson, 1968; Cripps & Williams, 1975). In addition, reciprocal changes occur in the metabolism of the mammary gland and peripheral tissues which result in the preferential utilization of circulating substrates by the lactating gland (see Williamson, 1980; Vernon & Flint, 1983; 1984; Williamson et al., 1984; Zammit, 1985).

In the following section the nature and function of the metabolic changes which occur in the tissues of the rat during pregnancy and lactation are outlined and the hormonal signals involved are considered.

1.1.1. Changes in serum hormone levels during pregnancy and lactation

Various changes in serum hormone levels occur in the rat during pregnancy and lactation (see Cowie et al., 1980) and although the

list below is by no means exhaustive it includes those most likely to influence changes in maternal metabolism.

Serum insulin concentration peaks at around day 9 of pregnancy (Flint et al., 1983) and then remains elevated until late gestation and falls around parturition, reaching levels found in virgin rats by early lactation and even lower levels by mid-lactation (Knopp et al., 1973; Robinson et al., 1978; Kuhn, 1977; Flint et al., 1979b; Burnol et al., 1983; see Fig. 1.1.1.). Little change appears to occur in serum glucagon levels during pregnancy (Saudek et al., 1975) or lactation (Robinson et al., 1978; Burnol et al., 1983) but, as a result of the variation in insulin levels (Fig. 1.1.1) the insulin:glucagon molar ratio is increased during pregnancy (Saudek et al., 1975) and decreased during lactation (Robinson et al., 1978; Burnol et al., 1983). It is not known whether serum catecholamine levels change during pregnancy and lactation in the rat.

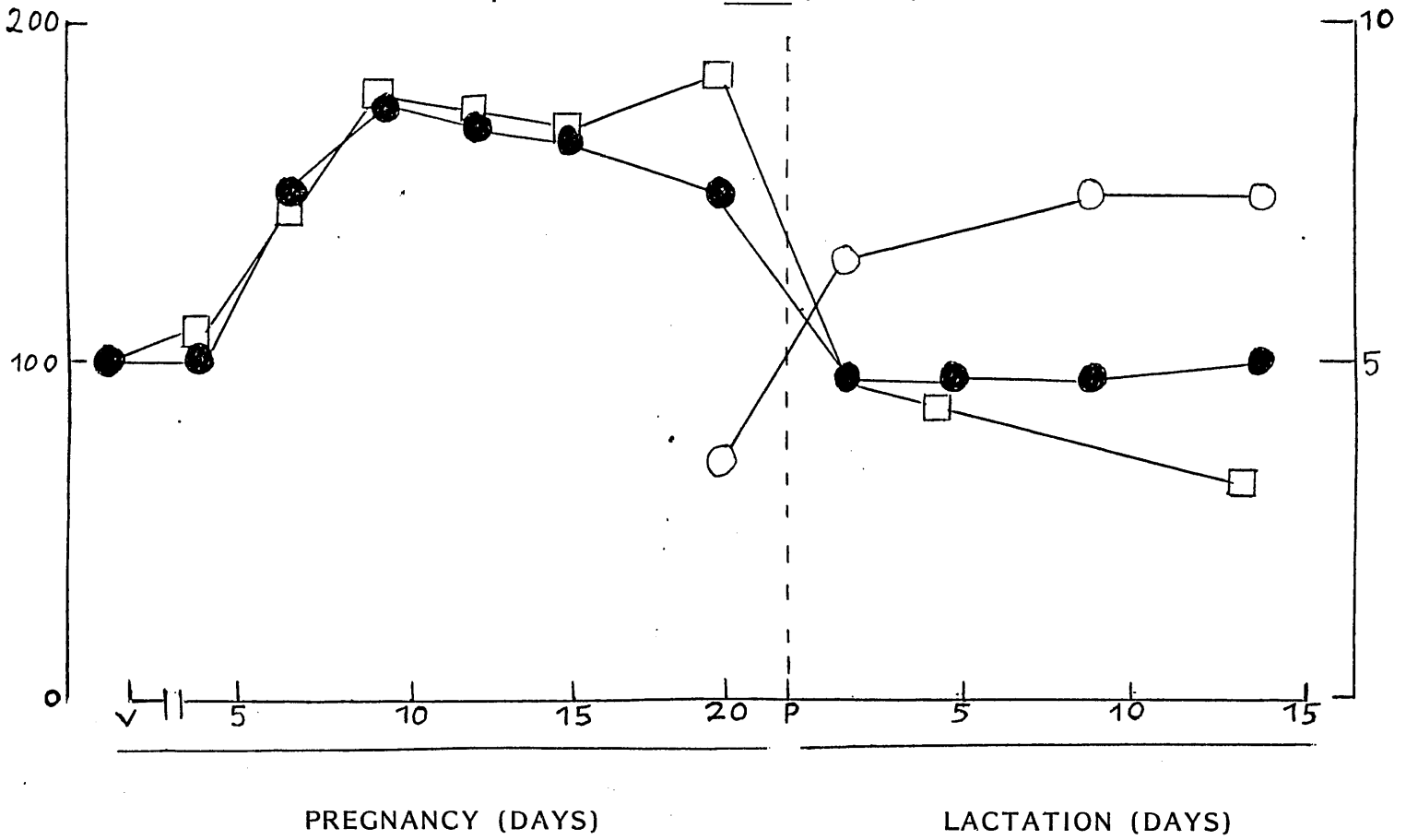
Serum prolactin levels are decreased during pregnancy then rise sharply around parturition and continue to increase during lactation (see Cowie et al., 1980). Two peaks of placental lactogen activity occur during pregnancy, the first occurring around days 11-15 and the second between days 17 and 21 (Shiu et al., 1973; Kelly et al., 1975; 1976). Two distinct forms of placental lactogen have been identified in rat serum: the smaller molecular weight form predominates during days 11-15 and the larger molecular weight form is primarily secreted during the second peak of lactogenic activity (Kelly et al., 1975; Robertson & Friesen, 1981; Robertson et al., 1982).

Progesterone levels in serum are elevated from day 3 of pregnancy, fall sharply before parturition then rise again by day 5

Fig. 1.1.1. Changes in serum insulin concentration and total numbers of insulin receptors on white adipose tissue and mammary gland during pregnancy and lactation

Serum insulin concentration (□), number of insulin receptors in parametrial white adipose tissue (●) and number of receptors in mammary gland (○).

Compiled from Flint et al., 1979b; 1983



of lactation: at all stages of pregnancy and lactation serum progesterone levels are higher than in the virgin rat (see Cowie et al., 1980). Serum oestradiol levels undergo a transient increase, associated with implantation, around day 4 of pregnancy (see Cowie et al., 1980) then peak at parturition and are low throughout lactation (see Cowie et al., 1980). Glucocorticoid levels in serum also peak at parturition but, in contrast to oestradiol, are elevated during lactation (see Cowie et al., 1980).

1.1.2. The effects of pregnancy and lactation on metabolism in the maternal tissues

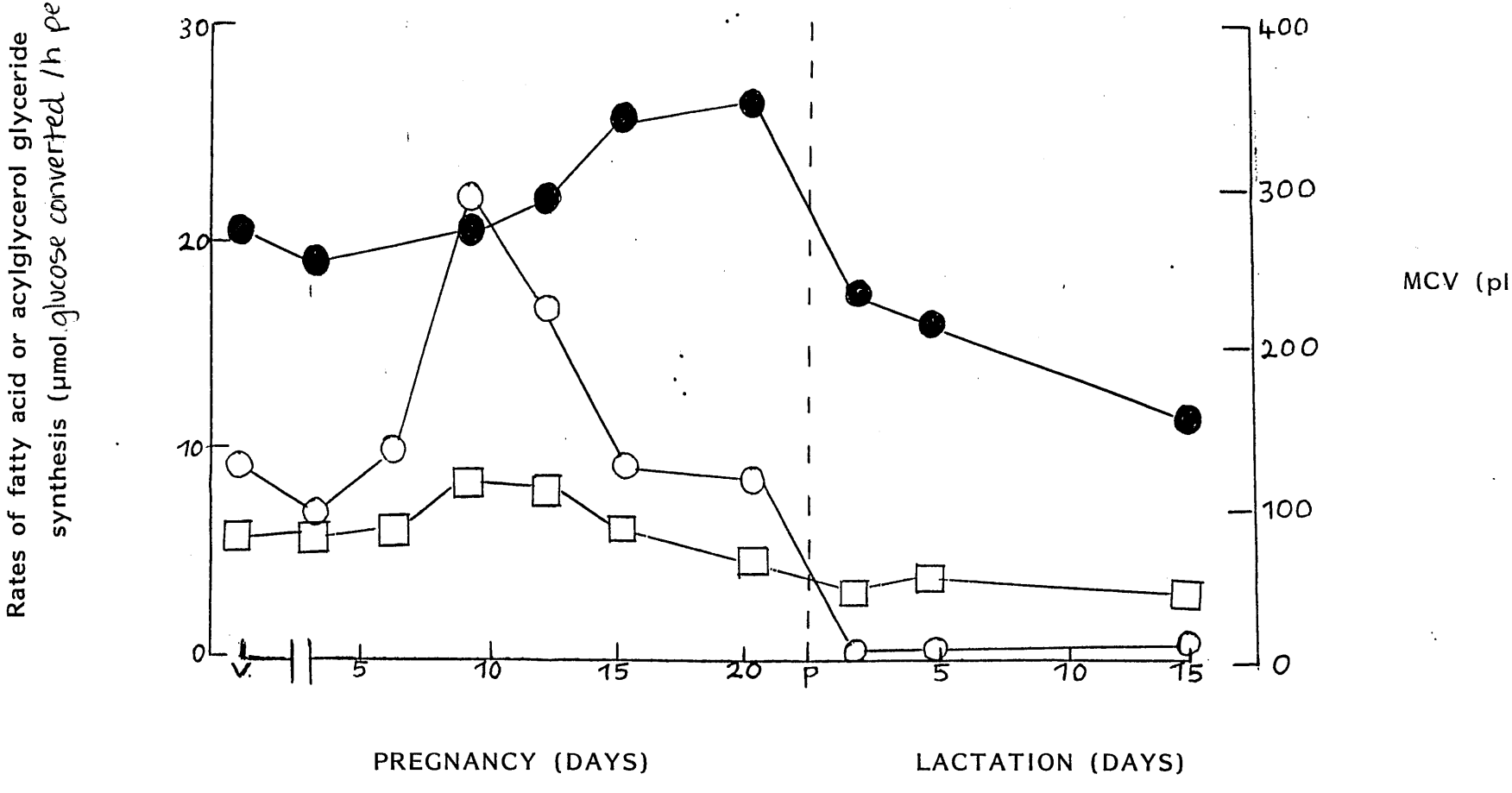
1.1.2.1. White adipose tissue

During pregnancy, when lipid is accumulated in rat adipose tissue, hypertrophy of subcutaneous, parametrial and perirenal adipocytes occurs while the mobilisation of lipid during lactation results in hypotrophy of these fat pads (see Vernon & Flint, 1984 and Fig. 1.1.2).

The rates of fatty acid and acylglycerolglyceride synthesis in rat white adipose tissue increase transiently around day 9 of pregnancy (Knopp et al., 1973; Flint et al., 1983) then return to control levels, prior to a fall in both activities which commences about 2 days before parturition; both rates remain low throughout lactation (Fig. 1.1.2; Smith, 1973b; Robinson et al., 1978; Flint et al., 1979b; Burnol et al., 1983). The fall in the rate of fatty acid synthesis, which commences around parturition (Fig. 1.1.2), is accompanied by a fall in the activity of several key lipogenic enzymes including fatty acid synthetase, ACC and PDH (Farid et al., 1978; Sinnett-Smith et al., 1980, 1982). Most studies show that

Fig. 1.1.2. Changes in the rate of fatty acid (○) and acylglycerol glyceride synthesis (▨) in white adipose tissue and in white adipocyte cell volume (●) during pregnancy and lactation in the rat

Compiled from Flint et al., 1979b; 1983



lipoprotein lipase activity in adipose tissue does not change throughout pregnancy then decreases around parturition and remains low throughout lactation (Otway & Robinson, 1968; Zinder et al., 1974; Flint et al., 1979b, 1983) although Hamosh et al., (1970) did find increased lipoprotein lipase activity during pregnancy. The basal rate of oxidation of glucose to CO_2 is increased during early pregnancy (Smith, 1973b) and suppressed during lactation (Farid et al., 1978; Burnol et al., 1986). The increase in lipogenic activity within white adipose tissue around day 9 of pregnancy should facilitate the storage of lipid in the tissue while the suppression of fatty acid synthesis and lipoprotein lipase activity during lactation will reduce the demand of adipose tissue upon circulating lipogenic precursors and dietary fat required by the mammary gland.

Insulin is the major hormone promoting lipogenesis in adipose tissue (see Saggerson, 1985) and the number of insulin receptors of rat white adipocytes is elevated during pregnancy, which should favour deposition of lipid; around parturition the numbers of receptors returns to levels found in virgin rats and no further change in receptor levels occurs during lactation (Fig. 1.1.1). No change in the affinity of insulin receptors on the fat cell occurs during pregnancy and lactation (Flint et al., 1979b; 1983). The increase in insulin receptor numbers during pregnancy may be attributable to the preceding increase in the serum progesterone concentration (Flint et al., 1983); progesterone implants in virgin rats increase insulin binding to adipocytes (Mendes et al., 1985). However there is a lag phase between the rise in serum progesterone concentration, which occurs by day 3 of pregnancy, and the increase in the number of insulin receptors on adipocytes, which occurs

several days later (Flint et al., 1983). This could be due to a requirement for oestradiol (see Flint et al., 1983) which increases the number of progesterone receptors of adipose tissue (Gray & Wade, 1979); there is a transient rise in serum oestradiol concentration around day 4 of pregnancy (Shaikh & Abraham, 1969). During late pregnancy, when serum progesterone levels decline, the number of insulin receptors of white adipocytes also declines (Flint et al., 1980). This role of progesterone in maintaining insulin receptor numbers on adipocytes is lost during lactation and prolactin may then be responsible for maintaining the receptor levels (Flint et al., 1981). When lactation is interrupted, by removal of suckling pups or by lowering serum prolactin levels via bromocriptine treatment, insulin receptors of white adipose tissue increase; these changes can be prevented by concurrent administration of prolactin, but only as long as the mammary gland is synthesising milk (Flint et al., 1981). This observation, along with the lack of any detectable prolactin binding to adipocytes, suggests that the prolactin effect on adipose tissue is indirect and occurs via an intermediary perhaps produced by the mammary gland (Flint et al., 1981). It has previously been suggested, for different reasons, that the mammary gland may secrete hormones (Maule-Walker & Peaker, 1978).

The elevated serum insulin concentration and insulin binding capacity of adipocytes during pregnancy (Fig. 1.1.1.) is reflected by the surge in lipogenesis which occurs around day 9 (Fig. 1.1.2.). However between days 15 and 20 of gestation, although the serum insulin concentration and insulin receptors of white adipocytes are elevated (FIg. 1.1.1.), the rate of lipogenesis in white adipose tissue is comparable to that in the virgin rat (Fig. 1.1.2.): this

suggests that the tissue is insulin resistant. In vivo the sensitivity of white adipose tissue metabolism to insulin is decreased (Leturque et al., 1986) and this contributes to the overall insulin resistance which occurs at this time (Leturque et al., 1980; 1984a). However studies in vitro have produced conflicting results: some workers have reported that no change occurs in the response or sensitivity of white adipose tissue to insulin during late pregnancy (Knopp et al., 1970a; Leturque et al., 1984b) while others have found a decrease in response only (Toyoda et al., 1985) or in both sensitivity and response to the hormone (Sutter-Dub et al., 1984). The reason for these discrepancies could simply be differences in the age or strain of rats used or differences in the fat depots studied. Alternatively it has been suggested that insulin resistance during late pregnancy is attributable to a systemic factor, such as placental lactogen (see Flint, 1985), and hence the resistant state could be lost when the tissue is removed from the animal.

By early lactation the serum insulin concentration and number of insulin receptors on white adipocytes are comparable to values found in virgin rats (Fig. 1.1.1.) yet lipogenesis in white adipose tissue is suppressed (Smith, 1973b; Flint et al., 1979b; see Fig. 1.1.2.) suggesting that the tissue is resistant to insulin. Some studies have found that the rate of glucose oxidation in white adipose tissue is reduced by mid-lactation in the rat (Farid et al., 1978; Burnol et al., 1986) although Smith (1973b) reported that the rate of glucose oxidation in white adipose tissue of 14 day lactating rats was similar to that in virgin rats. The serum insulin concentration is lower in 14 day lactating rats than in virgin rats (see section 1.1.1.) which could account for the suppression of

glucose oxidation in white adipose tissue. Insulin resistance, as characterised by an elevated serum insulin concentration in response to a glucose load, exists in 24h weaned rats and it has been suggested that this resistance in the tissues involved persists from lactation when it is balanced by the large uptake of glucose by the mammary gland (Burnol et al., 1983). Lipogenesis and glucose oxidation in white adipose tissue are both resistant to stimulation by insulin during lactation (Burnol et al., 1983; Lennox et al., 1985; Burnol et al., 1986); the activity of ACC is extremely low in white adipose tissue of lactating rats (Sinnett-Smith et al., 1980) which will limit any increase in flux through the lipogenic pathway. Certainly if white adipose tissue is resistant to insulin during lactation this will reduce its demand upon circulating substrates and hence increase the availability of these substrates to the mammary gland.

Noradrenaline, adrenaline, glucagon and possible ACTH are the main lipolytic hormones in rats (see Belfrage, 1985). The maximal rate of lipolysis in white adipose tissue (measured in the presence of noradrenaline and theophylline) is unaltered during pregnancy and lactation (Aitchison et al., 1982) but the actual lipolytic rate in vivo will depend on the hormonal environment of the tissue. β-adrenergic agents stimulate lipolysis by increasing intracellular cAMP levels: the activity of the adipocyte phosphodiesterase, which hydrolyses cAMP, is reduced during pregnancy and lactation which should promote lipolysis (Aitchison et al., 1982). The stimulatory effect of noradrenaline itself on lipolysis in isolated adipocytes is increased during pregnancy but surprisingly is reduced during lactation (Aitchison et al., 1982) which is paradoxical in view of

the net mobilisation of lipid which occurs in lactation (Spray, 1950; Beaton et al., 1954; Knopp et al., 1970a; Bershtein & Aleksandrov, 1977; Flint et al., 1979b). This decrease in response to noradrenaline during lactation is due to an increased sensitivity of white adipocytes to adenosine (Vernon et al., 1983) which is an antilipolytic agent produced within adipose tissue resulting in its accumulation during incubations in vitro (Schwabe et al., 1973). The purpose of this increase in sensitivity to adenosine is unknown. It is difficult to quantify the effect of adenosine on adipose tissue lipolysis in vivo as the agent is rapidly metabolised by the blood (Fredholm & Sollevi, 1981) and hence may not accumulate to the same extent as in vitro and also adenosine increases vasodilation and therefore blood flow in adipose tissue (Sollevi & Fredholm, 1981) and hence promotes the removal of plasma non-esterified fatty acids which is an important determinant in the rate of lipolysis (Scow, 1965). However the plasma adenosine concentration is 0.1-0.3μM which should be sufficient to inhibit lipolysis (Arch & Newsholme, 1978). The factors responsible for net lipolysis during lactation probably include low plasma insulin concentration (Kuhn, 1977; Robinson et al., 1978; Flint et al., 1979b; Burnol et al., 1983; see Fig. 1.1.1.), a high glucagon: insulin molar ratio (Robinson et al., 1978; Burnol et al., 1983), an increase in the response of adipose tissue to glucagon (N.A. Robson, R.A. Clegg and V.A. Zammit, unpublished observation) and a reduction in the rate of re-esterification of fatty acids (Smith & Walsh, 1976) the latter possibly being attributable to a reduction in the rate of synthesis of acylglycerolglyceride (Flint et al., 1979b).

1.1.2.2. Brown adipose tissue

Brown adipose tissue (BAT) is the main site of diet-induced and thermoregulatory non-shivering thermogenesis in rats (Foster & Frydman, 1978; Stock & Rothwell, 1985) while noradrenaline is the major hormonal mediator of thermogenesis (Mory et al., 1984). The tissue may play an important part in the regulation of energy balance in rodents (see Rothwell & Stock, 1983; Trayhurn, 1984; Bukowiecki, 1985; Trayhurn & Richard, 1985).

Like white adipose tissue BAT undergoes hypertrophy during pregnancy and the rate of lipogenesis in the tissue is increased (Agius & Williamson, 1980b). In contrast to the situation in the virgin animal, during pregnancy the already high rate of lipogenesis is not stimulated by glucose administered intragastrically (Agius & Williamson, 1980b). The high rate of lipogenesis in BAT during pregnancy does not appear to be paralleled by increased thermogenesis and probably only serves to accumulate a lipid store within the tissue (see Trayhurn & Richard, 1985). High serum progesterone levels during pregnancy (see Cowie et al., 1980) may inhibit the dietary activation of thermogenesis in BAT (see Trayhurn & Richard, 1985).

The amount of interscapular BAT is decreased in the rat during lactation and, as in white adipose tissue, the rate of lipogensis is low (Agius & Williamson, 1980a,b). Thermogenesis in lactating rats is suppressed (Isler et al., 1984), lipogenesis in BAT is little stimulated by the intragastric administration of glucose (Agius & Williamson, 1980a,b) and the ability of insulin to stimulate glucose utilization and lipogenesis in BAT is reduced (Burnol et al., 1983; Ferré et al., 1986). These changes in the metabolism of BAT during

lactation will serve to reduce the uptake of circulating glucose by the tissue and will reduce non-essential energy expenditure.

1.1.2.3. Liver

The liver is a major site of fatty acid synthesis in fed rats (Leveille, 1967) and is responsible for 80% of endogenous glucose production during the postabsorptive period (Owen et al., 1969). Liver weight increases in the rat during pregnancy and lactation and this has been reported to be due solely to hypertrophy (Flint, 1980) or to both hypertrophy and hyperplasia (Herrera et al., 1969). Davidson (1984) found no change in the number of insulin receptors of hepatocytes during pregnancy whereas Flint (1980) reported an increase which was maintained until 2 days prior to parturition after which the numbers of receptors declined to values similar to those in virgin rats.

The rate of fatty acid synthesis in the rat liver is increased during pregnancy then declines around parturition, to levels found in the virgin rat, and subsequently rises by the first day of lactation (Smith, 1973a; Benito & Williamson, 1978; Agius & Williamson, 1980b; Benito et al., 1982). These changes are paralleled by changes in the activities of several lipogenic enzymes (Smith, 1973a) including ACC and fatty acid synthetase (Zammit & Corstorphine, 1982a). The increase in hepatic lipogenesis during pregnancy reflects the elevated serum insulin concentration (Fig. 1.1.1.) while the increased rate of lipogenesis in the liver of lactating rats is somewhat surprising, as the glucagon: insulin molar ratio in the portal vein is elevated (Burnol et al., 1983) which should favour the suppression of lipogenesis. This suggests that the response of the liver to either glucagon or insulin changes during lactation.

The activities of the glycolytic enzymes hexokinase and glucokinase are elevated in the liver during pregnancy and lactation while the activities of key enzymes of gluconeogenesis are either unchanged, as is glucose-6-phosphatase activity, or decreased, as are phosphoenolpyruvate, carboxykinase and pyruvate carboxylase activities and this has led to the suggestion that glycolysis is increased in the liver during pregnancy and lactation (Smith, 1975): in support of this hepatocytes from lactating rats have a higher glycolytic flux and a lower output of glucose as compared to hepatocytes isolated from virgin rats (Whitelaw & Williamson, 1977).

With respect to the suppression of glucose production in vivo the sensitivity of the liver to insulin appears to be decreased in 19 day pregnant rats (Leturque et al., 1980; 1984a) yet hepatic insulin receptors are either higher than (Flint, 1980) or the same (Davidson, 1984) as in the virgin rat. However neither the sensitivity or response of insulin stimulated incorporation of glucose into glycogen is impaired in isolated hepatocytes from pregnant animals (Davidson, 1984): it has been suggested that insulin resistance during late pregnancy is caused by systemic factors, such as placental lactogen (see Flint, 1985) and hence when the tissue is removed from the animal the resistance could be lost. Hepatic gluose production in the post-absorptive state during lactation is elevated, probably due to the high glucagon:insulin molar ratio and the low glucose concentration in the portal vein (Burnol et al., 1983).

1.1.2.4. Skeletal muscle

Comparatively little is known about skeletal muscle metabolism during pregnancy and lactation yet it is one of the major insulin-dependent tissues in the rat, contributing to 35% of glucose

uptake in response to an intravenous glucose load (Curtis-Prior et al., 1969).

The ability of insulin to stimulate glucose utilisation in the perfused rat hind-limb (Rushakoff & Kalkhoff, 1981) and in rat skeletal muscle in vivo (Leturque et al., 1986) is reduced during late pregnancy. However Leturque et al., (1981) failed to detect any insulin resistance in isolated strips of soleus muscle from late pregnant rats: insulin resistance may develop in skeletal muscle during late pregnancy due to a systemic factor and so the resistance could be lost when the tissue is removed from the animal.

It has been suggested that the insulin resistance of the peripheral tissues in 24h weaned rats, as measured by the glucose tolerance test, actually originates during lactation (Burnol et al., 1983). If this is the case it is likely that skeletal muscle contributes to this resistance (Burnol et al., 1983) although this has not been further investigated in the rat. However, studies in the sheep using the euglycemic clamp technique, showed that both the response and sensitivity of hind-limb skeletal muscle to insulin is reduced during lactation (R.G. Vernon et al., unpublished observation).

1.1.2.5 Mammary gland

Around parturition lactogenesis commences in the rat mammary gland and this is reported to be dependent on the rise in serum prolactin and fall in serum progesterone concentrations; there is also a requirement for glucocorticoids (Baldwin & Yang, 1974; Kuhn, 1977; Cowie et al., 1980). Maintenance of lactation in the rat is dependent on prolactin, glucocorticoid and insulin (see Tucker, 1979; Cowie et al., 1980).

Lipogenesis in the mammary gland is elevated during lactation (see Baldwin & Yang, 1974; Vernon & Flint, 1983): in the rat mammary gland the increase in the rate of fatty acid synthesis commences post-partum (Martyn & Hansen, 1980). The elevated rate of fatty acid synthesis in the rat mammary gland during lactation is accompanied by increased activities of several key lipogenic enzymes including ACC (Mackall & Lane, 1977; Munday & Williamson, 1982; McNeillie & Zammit, 1982), fatty acid synthetase (Baldwin & Milligan, 1966) and PDH (Coore & Field, 1974; Kankel & Reinauer , 1976). Removal of pups depresses the rate of mammary gland lipogenesis by more than 90% within 24h (Agius et al., 1979, 1981) while administration of bromocryptine, to reduce serum prolactin levels, has a similar effect (Robinson & Williamson, 1977) and prolactin deficiency has been shown to reduce the activities of PDH (Field & Coore, 1975, 1976) ACC (McNeillie & Zammit, 1982) and fatty acid synthetase (Flint et al., 1981) in the gland. The increase in lipogenesis in the mammary gland during lactation (Robinson et al., 1978; Agius et al., 1979) along with the reciprocal fall in lipogenesis in white adipose tissue (see section 1.1.2.1.) will ensure that lipogenic precursors in the circulation are preferentially used by the lactating gland (see Williamson, 1980).

Lipoprotein lipase activity in the mammary gland rapidly increases at the end of pregnancy to reach high levels at peak lactation (McBride & Korn, 1963; Otway & Robinson, 1968; Hamosh et al., 1970; Zinder et al., 1974) and again prolactin deficiency results in a rapid decrease in this enzyme activity in the gland (McBride & Korn, 1963; Hamosh et al., 1970; Zinder et al., 1974; Flint et al., 1981). Reciprocal changes in the activity of

lipoprotein lipase in mammary gland and white adipose tissue (see section 1.1.2.1.) during lactation will result in triacylglycerol in the circulation being used by the lactating gland rather than by white adipose tissue (see Williamson, 1980).

The mammary gland is an insulin sensitive tissue (see Williamson, 1980) with the lipogenic pathway as a whole (Burnol et al., 1983; Jones & Williamson 1984) as well as glucose transport (Threadgold & Kuhn, 1984) ACC, activity (McNeillie & Zammit, 1982) and PDH activity (Field & Coore, 1976; Baxter & Coore, 1978) all being responsive to insulin. The number of insulin receptors on rat mammary acini increases around parturition and remains elevated during lactation (Fig. 1.1.1.) although it is still much lower than the number found on adipocytes or hepatocytes (Flint, 1982a,b; Flint 1980; Flint et al., 1979b). Progesterone injections prevent the rise in mammary receptor numbers that occurs at parturition whereas bromocriptine (which prevents the surge in serum prolactin levels that occurs at parturition) is without effect (Flint, 1982b). It therefore appears that the rise in insulin receptor numbers of the mammary gland during parturition is dependent on a fall in serum progesterone levels. During lactation in the rat progesterone receptors are absent from the mammary gland possibly due to the low serum oestradiol concentration (Shyamala & McBlain, 1979); hence the role of progesterone in inhibiting insulin binding to mammary cells is lost and prolactin appears to maintain insulin receptor numbers on the mammary cell (Flint, 1982a). Removal of pups from lactating rats or reduction of serum prolactin levels via administration of bromocriptine both result in a fall in the insulin binding capacity of mammary acini and both effects can be prevented by concurrent administration of prolactin (Flint, 1982a).

The rat mammary gland lacks glucagon receptors and hence does not respond to the hormone (Robson et al., 1984). β-adrenergic receptors have been found on rat mammary epithelial cells (Clegg & Mullaney, 1985) but curiously the post-receptor effects of β-agonists are neutralised by the very active cAMP phosphodiesterases of the gland (Clegg & Mullaney, 1985).

1.2. The mammalian pyruvate dehydrogenase complex

The enzyme pyruvate dehydrogenase (PDH) catalyses the conversion of pyruvate to acetyl-CoA for subsequent oxidation via the citric acid cycle or for use as a substrate in the synthesis of fatty acids and sterols. In mammalian tissues no pathway exists to convert acetyl-CoA to pyruvate and hence PDH has an important role in determining the extent of irreversible utilisation of glucose for both energetic and synthetic purposes. It is therefore not surprising that the enzyme is subject to stringent short-term control by metabolic effectors and hormones and to long-term changes in its activity in response to the physiological state of the animal, the latter occurring during starvation, diabetes and lactation (for reviews see Denton & Hughes, 1978; Wieland, 1983; Saggerson, 1985).

1.2.1. The structure of the pyruvate dehydrogenase complex

The PDH complex is located within the mitochondrial inner membrane-matrix space (Schnaitman & Greenawalt, 1968; Addink et al., 1972; Nestorescu et al., 1973) and is composed of multiple copies of three different proteins which catalyse the irreversible oxidative decarboxylation of pyruvate by the following series of reactions:

1. Pyruvate + thiamine pyrophosphate - ENZ_1 $\longrightarrow$ CO_2 + 2α-hydroxyethyl-thiamine pyrophosphate - ENZ_1

2. 2α-hydroxyethyl-thiamine pyrophosphate - ENZ_1 + lipoamide. ENZ2 $\longrightarrow$ thiamine pyrophosphate - ENZ_1 + dihydrolipoamide. S Acetyl - ENZ_2

3. dihydrolipoamide. S Acetyl - ENZ_2 + CoA. SH $\longrightarrow$ Acetyl CoA + dihydrolipoamide. SH. ENZ_2

4. dihydrolipoamide. SH. ENZ_2 + NAD^+ $\xrightarrow{ENZ_3}$ lipoamide.s. ENZ_2 + NADH + H^+

The core of the complex is composed of dihydrolipoate acetyl transferase (ENZ_2) units (Barrera et al., 1972; Kresze et al., 1980a,b) to which are bound the other enzymes, pyruvate decarboxylase (ENZ_1) consisting of non-identical polypeptide chains, α and β, which form α_2 β_2 tetramers (Barrera et al., 1972) and flavoprotein dihydrolipoate dehydrogenase (ENZ_3) which consists of two identical subunits each containing a molecule of FAD (Barrera et al., 1972). The overall molecular weight of the complex is between 7 and 10 million (see Wieland, 1983). Interaction of the three subunits occurs via lipoic acid residues which are covalently attached to lysine side chains on ENZ_2 and which rotate among the catalytic sites of the three component enzymes (see Reed, 1974; Denton et al., 1975).

1.2.2. The regulation of pyruvate dehydrogenase activity

Regulation of PDH activity is probably brought about through alterations in the activity of ENZ_1 since the first step is the most thermodynamically favourable and is irreversible (Walsh et al., 1976). Short-term modulation of the enzyme's activity occurs via two different regulatory mechanisms, namely end-product inhibition (Garland & Randle, 1964; Bremer, 1969; Wieland & Siess, 1970; Tsai et al., 1973) and interconversion between a phosphorylated inactive form and a non-phosphorylated active form (Linn et al., 1969a,b; Wieland & Siess, 1970).

1.2.2.1. End product inhibition of pyruvate dehydrogenase activity

The products of pyruvate oxidation, NADH and acetyl-CoA, are competitive inhibitors of PDH with respect to NAD^+ and CoA (Garland & Randle, 1964; Bremer, 1969; Wieland & Siess, 1970; Tsai et al., 1973). Mitochondrial ratios of [acetyl-CoA]/[CoA] and [NADH]/[NAD^+] are therefore important in controlling the rate of pyruvate oxidation in vivo.

1.2.2.2. Regulation of pyruvate dehydrogenase activity via phosphorylation-dephosphorylation reactions

The PDH complex can exist in two forms, an active dephosphorylated form and an inactive phosphorylated form (fig. 1.2.1.). Phosphorylation and inactivation of the complex is brought about by a $MgATP^{2-}$-dependent PDH-kinase and dephosphorylation is catalysed by a Mg^{++}-dependent PDH-phosphatase (Linn et al., 1969a,b; Wieland & Jagow-Westermann, 1969; Wieland & Siess, 1970; Denton et

al., 1971; Jungas, 1971; Weiss et al., 1971; Coore & Field, 1974; Bailey et al., 1976). Three seryl residues in the α-chains of ENZ-1 are phosphorylated sequentially by the kinase. Site 1 is phosphorylated much faster than sites 2 and 3 and phosphorylation at site 1 suffices to completely inactivate the enzyme (Barrera et al., 1972; Davis et al., 1977; Yeaman et al., 1978; Sugden et al., 1979) while phosphorylation of site 3 has no effect on inactivation of PDH (Reed et al., 1980; Radcliffe et al., 1980). The physiological significance of this multi-site phosphorylation is not fully understood (see Randle et al., 1981).

The kinase is bound tightly to the PDH complex, probably to the ENZ-2 core (Linn et al., 1972; Pettit & Reed, 1982; Stepp et al., 1983) and to the ENZ-1 subunit (Linn et al., 1969a; Kerbey & Randle, 1985) while the phosphatase is only loosely bound to the PDH complex and can be separated from it by high speed centrifugation (Linn et al., 1972; Siess & Wieland, 1972).

The PDH-kinase is inhibited by pyruvate, NAD^+, coenzyme A, ADP and excess Mg^{++} and Ca^{++} and is activated by NADH and acetyl-CoA (Linn et al., 1969a,b; Cooper et al., 1974, 1975; Pettit et al., 1975; Kerbey et al., 1976, 1979; Pratt & Roche, 1979). The phosphatase in Ca^{++}-stimulated and Mg^{++}-dependent (Linn et al., 1969a,b; Randle et al., 1974; Kerby & Randle, 1979). For a summary of the properties of PDH-kinase and PDH-phosphatase see Fig. 1.2.1.

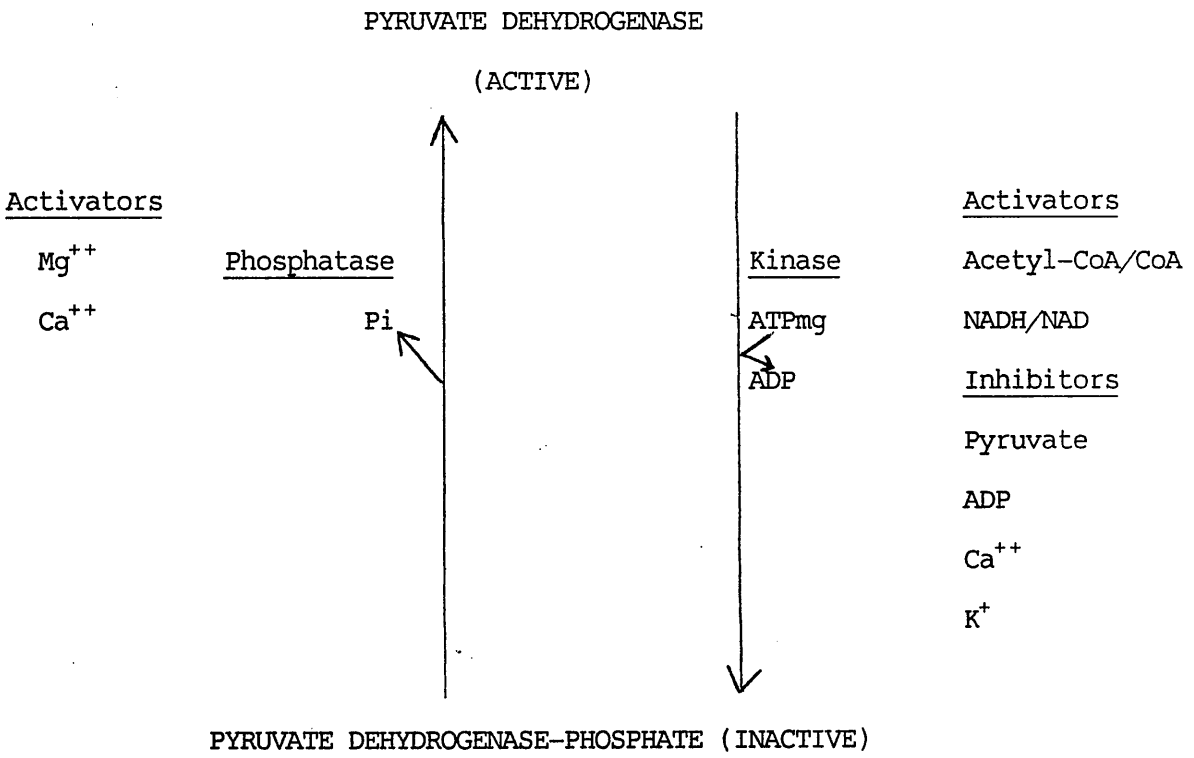
1.2.2.3. In vivo regulation of pyruvate dehydrogenase activity via phosphorylation-dephosphorylation reactions

In vivo it is largely the mitochondrial concentrations of effectors that regulate the PDH-kinase. Increasing concentration

Figure 1.2.1.

SUMMARY OF THE PROPERTIES OF PYRUVATE DEHYDROGENASE PHOSPHATASE AND PYRUVATE DEHYDROGENASE KINASE

Taken from Denton and Halestrap (1979)



ratios of ATP/ADP, NADH/NAD^+ and acetyl-CoA/CoA activate the kinase while pyruvate and Ca^{++} decrease kinase activity (Cooper et al., 1974; Hansford, 1976; Kerbey et al., 1977; Hansford & Cohen, 1978). The reactivation of PDH by the phosphatase requires Mg^{++} and Ca^{++} (Severson et al., 1974).

Fatty acids inhibit PDH activity in white adipose tissue, liver, heart and kidney (Wieland et al., 1971b, 1972; Guder & Wieland, 1974; Kerby et al., 1976; Sooranna & Saggerson, 1976a, 1979b) probably as a result of their oxidation within mitochondria which will increase the mitochondrial NADH: NAD^+ and acetyl-CoA: CoA concentration ratios and hence activate PDH-kinase (Batenburg & Olson, 1976; Kerby et al., 1976, 1977; Hansford, 1977; Saggerson, 1985). Conditions in vivo which are associated with enhanced oxidation of lipid fuels, for example diabetes and starvation, therefore result in inactivation of the PDH complex (Wieland et al., 1971a,b).

1.2.3. Short-term hormonal regulation of pyruvate dehydrogenase activity

1.2.3.1. Control of pyruvate dehydrogenase activity by insulin

It is clear that insulin acts directly upon rat white and brown adipose tissue to increase the proportion of PDH present in the active, dephosphorylated state without affecting the total amount of PDH present in the tissues (Jungas, 1970; Stansbie et al., 1976a; Denton et al., 1977; Paetzke-Brunner et al., 1979): this effect is an important component of the mechanism whereby insulin stimulates fatty acid synthesis. The nature of short term regulation of PDH activty by insulin in other tissues of the rat is less well established.

1.2.3.1.1. Adipose tissue

Exposure to insulin produces a 2-3 fold increase in the proportion of PDH in the active state, without affecting the total amount of PDH present, in white adipose tissue pieces and isolated white adipocytes (Jungas, 1970; Coore et al., 1971; Weiss et al., 1971; Martin et al., 1972; Sica & Cuatrecasas, 1973; Wieland et al., 1973; Weiss et al., 1974) and also in white adipose tissue in vivo (Stansbie et al., 1976a). Insulin has similar effects on PDH activity in brown adipose tissue both in vitro (Paetzke-Brunner et al., 1979) and in vivo (Denton et al., 1977).

Several agents which mimic insulin action on white adipose tissue also increase the proportion of PDH in the active state for example prostaglandin E_1, nicotinate (Taylor et al., 1973) H_2O_2 (May & de Häen, 1979) concanavalin A (Mukherjee et al., 1980) and oxytocin (Mukherjee & Mukherjee, 1982). As oxytocin does not stimulate glucose transport (Mukherjee & Mukherjee, 1982) and the effects of insulin on PDH activity can often be observed when no carbohydrate substrate is present (Taylor et al., 1973; Weiss et al., 1974) the stimulation of PDH activity can occur independently of an increase in glucose transport.

Insulin increases the V_{max} of PDH but has no effect on the K_m for pyruvate (Coore et al., 1971; Sica & Cuatrecasas, 1973) which is consistent with an increase in the proportion of enzyme in the active form. Denton & Hughes (1978) reported that during incubation of adipocytes with ^{32}P-inorganic phosphate insulin diminished the ^{32}P-labelling of the α-subunit of the pyruvate decarboxylase core in the PDH complex. Insulin therefore stimulates PDH activity by

increasing the amount of enzyme present in the tissue in the active dephosphorylated form which suggests that insulin either induces an increase in the activity of the PDH-phosphatase or a decrease in the PDH-kinase activity.

The stimulation of PDH activity persists through the isolation and subsequent incubation of mitochondria from insulin treated brown and white adipose tissue (Denton et al., 1984); this has permitted the study of intramitochondrial changes which occur on exposure of the tissue to insulin. After exposure of white or brown adipose tissue to insulin no significant change was found in the intramitochondrial concentrations of the kinase regulators ATP, ADP, NADH, NAD^+, acetyl-CoA or CoA (Denton et al., 1984). In mitochondria prepared from epididymal adipose tissue after insulin treatment the rate of phosphorylation of PDH was found to be increased indicating an increase in PDH-kinase activity (Hughes & Denton, 1976). If insulin were to stimulate adipose tissue PDH activity via PDH-kinase a decrease in kinase activity would be expected. This has led to the proposal that insulin increases the amount of PDH in the active dephosphorylated form via an increase in phosphatase activity and that this in turn results in a stimulation of PDH-kinase due to an accumulation of its substrate (Hughes & Denton, 1976).

Changes in intramitochondrial levels of the phosphatase regulators Mg^{++} and Ca^{++} are not thought to be responsible for the insulin stimulation of PDH-phosphatase activity (Denton et al., 1984; Cheng & Larner, 1985; McCormack & Denton, 1985) although the possibility that an altered distribution of Ca^{++} and Mg^{++} within mitochondria is involved can not be ruled out. It has been reported

that insulin causes a persistent increase in PDH-phosphatase activity in crude extracts from fat pads (Sica & Cuatrecasas, 1973; Mukherjee & Jungas, 1975) but this has not been confirmed by others (Marshall et al., 1984; Severson et al., 1974; Stansbie et al., 1976b). Very recently Denton and co-workers have presented evidence to show that insulin treatment of white adipose tissue results in an increase in the affinity of mitochondrial PDH-phosphatase for Mg^{++} (Thomas et al., 1986; Thomas & Denton, 1986). However the nature of the signal that is generated by insulin binding to receptors on its target cell's plasma membrane and which is transmitted to the PDH complex in the mitochondria remains to be found.

1.2.3.1.2. Liver

Conflicting evidence exists with respect to the effect of insulin on hepatic PDH activity. Wieland et al. (1972) reported that injection of rats with a high dose of insulin increased the proportion of PDH in the active state in the liver but Stansbie et al., (1976a) found no change in hepatic PDH activity after manipulation of serum insulin levels with glucose and anti-insulin serum even though changes in PDH activity in epididymal white adipose tissue occurred in the rats studied. Some workers have reported that insulin has no effect on PDH activity in perfused liver (Patzelt et al., 1973; Mukherjee & Jungas, 1975) in isolated hepatocytes (Oviasu & Whitton, 1984) or in primary hepatocyte cultures (Wieland, 1983) while other workers have observed a stimulation of PDH activity during incubation of perfused liver with insulin (Topping et al., 1977; Assimacopoulos-Jeannet et al., 1977) or after exposure of isolated hepatocytes to pharmacological concentrations of insulin

(Vaartjes et al., 1981). It appears that the increase in PDH activity produced by maximally effective concentrations of insulin is much less in hepatocytes (Assimacopoulos-Jeannet et al., 1982) than in adipocytes (see section 1.2.3.1.1.) which may explain the confusion that has arisen concerning the effect of insulin on hepatic PDH. It therefore seems that the control of PDH activity by insulin is less important in liver than in white adipose tissue.

1.2.3.1.3. Skeletal muscle

The effect of short-term exposure to insulin on skeletal muscle PDH activity has not been investigated, possibly due to the lack of a suitable in vitro system to work on. However it has been shown that starvation for 48h and streptozotocin induced diabetes both result in a decrease in the proportion of PDH in the active state in skeletal muscle but have no effect on the total amount of PDH present in the tissue (Hennig et al., 1975; Hagg et al., 1976). This suggests that insulin has a role in controlling skeletal muscle PDH activity.

1.2.3.1.4. Mammary gland

Starvation of rats for 24h and withdrawal of insulin from the circulation for 3h both result in a decrease in PDH activity in the mammary gland due to a fall in the proportion of the enzyme in the active state while little change occurs in total PDH in the gland (Field & Coore, 1976; Kankel & Reinauer, 1976; Baxter & Coore, 1978): a decrease in PDH-phosphatase activity (Baxter & Coore, 1978) and an increase in PDH-kinase activity (Baxter & Coore, 1978; Baxter et al., 1979) accompany this fall in PDH activity in the mammary gland. The production of lactate and pyruvate by acini in vitro is increased

when the cells are isolated from the mammary gland of rats after starvation or withdrawal of insulin from the circulation. This increase in lactate and pyruvate production is not accompanied by an increase in glucose uptake and therefore suggests that the low PDH activity in the mammary gland of starved and diabetic rats persists during the isolation of acini (Robinson & Williamson, 1977; Munday & Williamson, 1981; Williamson et al., 1983). Although insulin administered in vivo reverses the effect of starvation and insulin deficiency on mammary PDH, PDH-phosphatase and PDH-kinase activities (Field & Coore, 1976; Baxter & Coore, 1978; Baxter & Coore, 1979; Baxter et al., 1979; Munday & Williamson, 1981) studies on the mechanism of this action of insulin have been hindered by the lack of a suitable in vitro system (Goheer & Coore, 1977). Therefore the direct effect of short-term exposure to insulin on mammary gland PDH has not been established.

1.2.3.2. Control of pyruvate dehydrogenase activity by catecholamines

Catecholamines increase the proportion of PDH in the active state in rat liver (Assimacopoulos-Jeannet et al., 1983; Oviasu & Whitton, 1984), heart muscle (Hiraoka et al., 1980; McCormack & Denton, 1981) and, it has been shown recently, in brown adipose tissue (Gibbins et al., 1985). This effect on liver PDH occurs via the stimulation of α_1-adrenergic receptors and is secondary to a rise in intramitochondrial Ca^{++} levels (Assimacopoulos-Jeannet et al., 1983; Oviasu & Whitton, 1984; McCormack, 1985b; McCormack & Denton, 1985) while the effect in heart muscle is attributed to the stimulation of muscle contraction via β-adrenergic receptors

resulting in an increase in cytoplasmic Ca^{++} levels which is relayed into the mitochondria (Hiraoka et al., 1980; McCormack & Denton, 1981; McCormack & Denton, 1985). The stimulation of brown adipose tissue PDH activity by noradrenaline has recently been demonstrated in vivo (Gibbins et al., 1985) but the mechanism of this stimulation of PDH is unknown.

In contrast the effect of catecholamines on PDH activity of white adipose tissue is not well established. Some early studies reported that catecholamines stimulated PDH activity in isolated fat pads (Jungas, 1971; Sica & Cuatrecasas, 1973; Taylor et al., 1973; Weiss et al., 1974) but these effects could have been due to inappropriate incubation conditions such as a lack of albumin or carbohydrate substrate or the use of very high concentrations of hormone. Later studies showed that concentrations of adrenaline above 0.1μM reduced insulin stimulated PDH activity in incubated fat pads (Coore et al., 1971; Weiss et al., 1974; Smith & Saggerson, 1978) and inhibited PDH activity in isolated adipocytes in the presence and absence of insulin (Coore et al., 1971; Sooranna & Saggerson, 1976b; Smith & Saggerson, 1978). The concentration of adrenaline used in these studies also stimulate lipolysis and the inhibitory effect on PDH activity may have been secondary to the accumulation of fatty acids in the incubations which is known to suppress PDH activity (Sooranna & Saggerson, 1976a, 1979b). Studies in vitro, using low concentrations of adrenaline (3-100nM), which do not stimulate lipolysis, have shown that the hormone increases PDH activity in isolated adipocytes (Smith & Saggerson, 1978; Cheng & Larner, 1985). It therefore appears that adrenaline may have a

stimulatory effect on white adipose tissue PDH activity which is separate from its effect on lipolysis. As adrenaline inhibits fatty acid synthesis in white adipose tissue (Katz et al., 1966; Saggerson & Greenbaum, 1970; Smith & Saggerson, 1978) the increase in flux through PDH would serve to provide acetyl-CoA units for the citric acid cycle which is stimulated by the hormone (Katz et al., 1966; Saggerson & Greenbaum, 1970).

1.2.3.3. Other examples of short-term regulation of pyruvate dehydrogenase activity

Glucagon, vasopressin and angiotensin increase the proportion of PDH in the active state in rat liver via an increase in intramitochondrial Ca^{++} levels (Hems et al., 1978; Assimacopoulos-Jeannet et al., 1983; Oviasu & Whitton, 1984; McCormack, 1985b; Assimacopoulos-Jeannet et al., 1986). Similarly glucagon and other inotropic agents increase PDH activity in rat heart: the stimulation of contraction results in an increase in cytoplasmic Ca^{++} levels which is relayed into the mitochondria (Illingworth & Mullings, 1976; Hiraoka et al., 1980; McCormack & Denton, 1981; McCormack & Denton, 1985).

The amount of PDH in the active state in skeletal muscle increases several fold during exercise (Hennig et al., 1975; Hagg et al., 1976). This may be due to a rise in mitochondrial Ca^{++} levels or to a fall in the ATP/ADP concentration ratio but these possibilities have not been further investigated (see Randle et al., 1981).

1.2.4. Long-term alteration in the PDH system

Alloxan-diabetes and starvation for 48h both result in a decrease in the proportion of PDH in the active state, but have little effect on the total amount of PDH present, in a number of tissues in the rat including liver, kidney, white adipose tissue and skeletal muscle (Wieland et al., 1971a, 1973; Kerby et al., 1976, 1977; Stansbie et al., 1976b) and during lactation in the mammary gland (Baxter et al., 1979). In fat pads from diabetic or starved rats the decrease in PDH activity persists during the incubation of the tissue with insulin (Stansbie et al., 1976b): therefore the low PDH activity is not due simply to reduced plasma insulin levels but involves a persistent alteration in the regulation of the PDH complex.

The sensitivity of PDH activity to stimulation by pyruvate is reduced in adipose tissue, heart, brain and mammary gland from starved or diabetic rats (Hutson & Randle, 1978; Kerbey et al., 1976, 1977; Baxter et al., 1979; Lyn & Coore, 1985). This suggests that either the phosphorylation of PDH by the kinase is activated or that dephosphorylation by the phosphatase is inhibited. Evidence suggests that in the heart dephosphorylation of the PDH-complex by PDH-phosphatase is inhibited, by some unknown mechanism, during starvation and diabetes (Randle et al., 1981) and that there is also an increase in an uncharacterised protein activator of PDH kinase as well as in the amount of PDH kinase present in the tissue (Kerbey & Randle, 1982; McCormack et al., 1982; Kerbey et al., 1984). During starvation or alloxan-diabetes in the rat there is an increase in the concentration of free fatty acids and in the ratio of glucagon to

insulin in the circulation and recently it has been shown that glucagon and n-octanoate increase the activity of PDH-kinase in cultured hepatocytes (Fatania et al., 1986). Therefore glucagon and free fatty acids may mediate at least some of the effects of starvation and diabetes on PDH-kinase activity.

In contrast to alloxan-diabetes and starvation, which produce a decrease in the proportion of PDH in the active state but have little effect on total PDH activity, feeding rats a high-fat diet for more than 5-6 days is reported to produce a fall in total PDH activity in white adipose tissue and have little effect on the proportion of the enzyme in the active state (Stansbie et al., 1976b; Begum et al., 1982a). However the PDH activity in these studies was not expressed on a per cell basis and an increase in adipocyte volume during fat feeding may account for the apparent fall in total PDH activity.

1.3. Insulin

The peptide hormone insulin has both rapid short term effects, mediated by changes in the activities of metabolic enzymes and membrane transporters, and long term effects, involving changes in the rate of synthesis and breakdown of proteins, on its target tissues. The following discussion will concentrate on the short term effects and in particular on the possible molecular mechanisms by which these effects occur.

1.3.1. Insulin secretion

Insulin is released into the circulation from the β-cells in the pancreatic islets of Langerhans (for reviews see Miller, 1981;

Fain, 1984). The main physiological stimulus for insulin release is glucose although various other agents, such as amino acids and glucagon, also have a role in regulating the secretion of the hormone (Cerasi et al., 1972; Lambert, 1976; Matschinsky et al., 1979; Ashcroft, 1980; Hedeskov, 1980; Joost & Beckmann, 1980; Malaisse et al., 1981; Wollheim & Sharp, 1981). Increases in the intracellular concentration of cAMP or Ca^{++} are known to act as signals for insulin release (Ashcroft, 1980; Malaisse et al., 1981; Wollheim & Sharp, 1981) although it is not certain whether cAMP acts directly on insulin release or indirectly through increasing intracellular Ca^{++} (see Fain, 1984). The mechanism by which glucose stimulates insulin release is not clear but it is likely that the effect is mediated by elevated intracellular Ca^{++} (see Fain, 1984).

Catecholamines can stimulate insulin release from the β-cells in the pancreas through β-adrenergic receptors (Loubatieres et al., 1971; Kaneto et al., 1975; Lundquist & Ericson, 1978; Ahrén & Lundquist, 1981) but this effect is usually swamped by the inhibitory α_2 effect (Nakadate et al., 1980; Roy et al., 1981; Wollheim, 1981; Ismail et al., 1983). Therefore both adrenaline and noradrenaline inhibit insulin release from pancreatic β-cells both in vitro and in vivo (Mialhe, 1965; Kris et al., 1966; Porte et al., 1966; Malaisse et al., 1967; Morgan & Montague, 1985; Madon et al., 1985). Although activation of α_2-adrenergic receptors is known to reduce intracellular cAMP concentrations (see section 1.4.1.2.) it has been suggested that the α_2-inhibitory effect on insulin secretion occurs via a novel mechanism which is independent of alterations in islet cell cAMP levels (Wollheim & Sharp, 1981; Morgan & Montague, 1985).

1.3.2. The control of metabolism by insulin

The major target organs for insulin in the non-lactating animal are liver, adipose tissue and muscle and in general the hormone stimulates anabolic processes, such as carbohydrate and lipid synthesis, and suppresses catabolic processes (for reviews see Randle et al., 1966; Fritz, 1972; Fain, 1974; Pilkis & Park, 1974).

Insulin stimulates glucose uptake into muscle and fat cells (Levine et al., 1949; Randle & Morgan, 1962; Crofford & Renold, 1965) by increasing the V_{max} of the plasma membrane glucose carrier activity (See Czech, 1980). Much of the glucose removed from the blood by muscle is converted into glycogen; insulin stimulates the activity of skeletal and cardiac muscle glycogen synthase (See Larner et al., 1978; Miller, 1978). In adipose tissue the glucose is mostly used in the synthesis of the fatty acid and the glycerol moieties of triacylglycerol and to this end insulin increases the activity of several lipogenic enzymes including PDH (see section 1.2.3.1.1.) and ACC (Halestrap & Denton, 1973; Lee et al., 1973; Stansbie et al., 1976a; McCormack & Denton, 1977). Insulin effects on the liver include the inhibition of glucose release and gluconeogenesis and the stimulation of glycogen, cholesterol and triacylglycerol synthesis. Enzymes associated with anabolic pathways in the liver such as glycogen synthase (van de Werve et al., 1977a,b; Witters & Avruch, 1978), ACC (Stansbie et al., 1976a; Geelen et al., 1978; Witters et al., 1979) and hydroxymethyl glutaryl-CoA reductase (Ingebritsen & Gibson, 1979, 1980) are stimulated by insulin and phosphorylase kinase and glycogen phosphorylase, which catalyse glycogen breakdown, are both inhibited by insulin (Van de Werve et al., 1977a,b; Witters & Avruch, 1978).

1.3.3. The effect of insulin on phosphorylation/dephosphorylation reactions

Insulin affects the phosporylation state of a number of proteins in its target cells: PDH (see Wieland, 1983) and glycogen synthase (Villar-Palasi & Larner, 1960) are both activated by insulin via dephosphorylation while the hormone stimulates the phosphorylation of ACC (Brownsey & Denton, 1982) ATP-citrate lyase (Pierce et al., 1981, 1982) ribosomal S_6 protein (Belsham et al., 1980; Smith et al., 1979, 1980) the β-subunit of the insulin receptor (Haring et al., 1982; Kasuga et al., 1982a,b) and two unidentified proteins one a 22k cytoplasmic protein and the other a 61k plasma membrane protein (Belsham et al., 1980, 1982; Blackshear et al., 1982). The phosphorylation of ACC results in an increase in its activity (Brownsey & Denton, 1982) while no change in catalytic activity of ATP-citrate lyase accompanies phosphorylation. It could be that the increase in the phosphorylation of ribosomal S_6 protein plays a role in the long term effects of insulin on protein synthesis (Perisic & Traugh, 1983) and it has been suggested that the phosphorylation of the insulin receptor is an integral step in the insulin transducing system (Gammeltoft & Van Obberghen, 1986). In addition insulin decreases the phosphorylation and activity of triacylglycerol lipase (Khoo et al., 1973; Steinberg et al., 1975; Belfrage et al., 1980; Nilsson et al., 1980) phosphorylase (Witters & Avruch, 1978; Steinberg et al., 1975) and phosphorylase kinase (Van de Werve et al., 1977a,b) but only after their phosphorylation has been increased by hormones which elevate intracellular cAMP levels.

1.3.4. The structure of the insulin receptor

The insulin receptor has been purified by affinity chromatography (Cuatrecasas, 1972; Jacobs et al., 1977; Harrison & Itin, 1980) and has been studied by affinity labelling techniques (Yip et al., 1978; Pilch & Czech, 1979). The receptor is a glycoprotein with the carbohydrate portion located on the outer surface of the cell (Cuatrecasas, 1971; Cuatrecasas & Illiano, 1971; Rosenthal & Fain, 1971; Cuatrecasas, 1973; Jacobs et al., 1980; Cherqui et al., 1981). Four disulphide linked subunits with the configuration β-α-α-β make up the receptor: the β-subunit has a molecular weight of 90k and the α-subunit is estimated to be 135k (fig. 1.3.1.).

The α-subunit contains the insulin binding site (Massague & Czech, 1980; Massague et al., 1980; Yip et al., 1980) and the receptor molecule is multivalent with regards to binding of insulin (Harrison et al., 1979; Schlessinger et al., 1980). Generally scatchard plots for insulin binding are concave (De Meyts et al., 1973, 1976); this has been attributed to negative cooperative interactions occurring between binding sites although this hypothesis has met with some scepticism as such plots could also be attributed to the curve from heterogenic binding sites with differing affinities for insulin (see Rodbard, 1979).

1.3.5. Internalisation of the insulin-receptor complex

Insulin is taken up into its target cells via receptor mediated endocytosis (Terris & Steiner, 1975; Terris & Steiner, 1976; Goldfine et al., 1978; Kahn et al., 1978; Schlessinger et al., 1978; Carpentier et al., 1979): degradation of insulin by its target cells

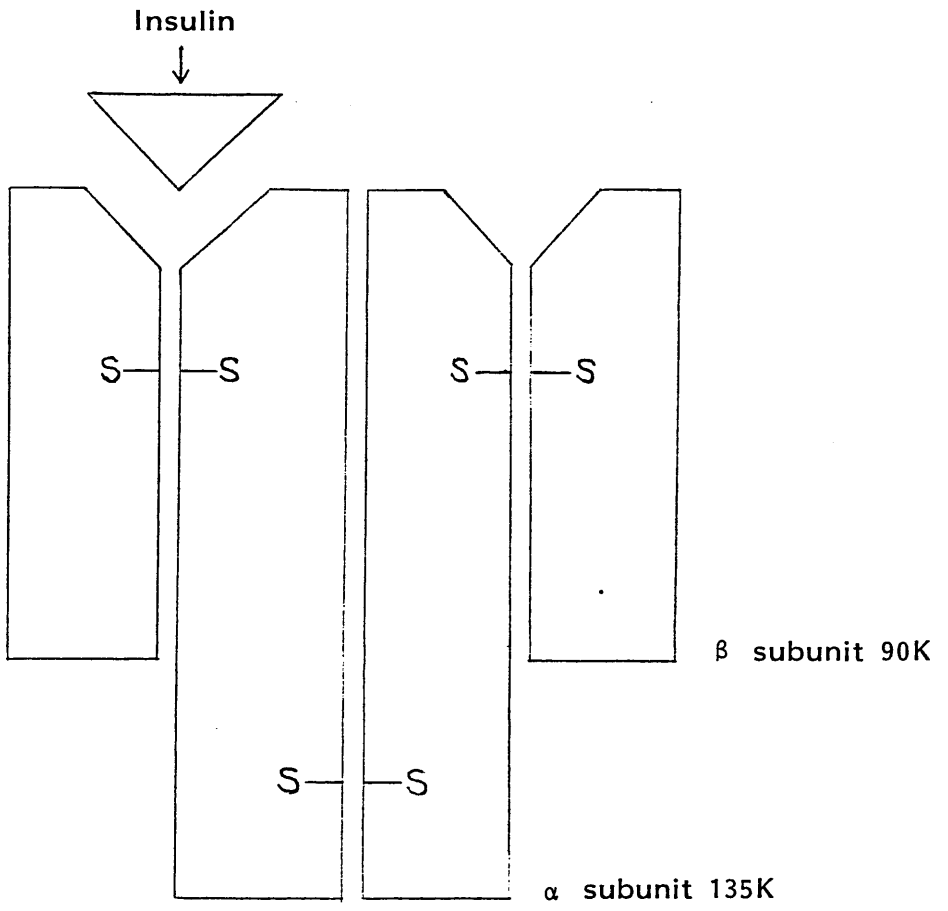


Fig. 1.3.1. Subunit structure of the insulin receptor

(adapted from Jacobs and Cuatrecasas, 1981)

is a receptor mediated process and follows internalisation (Terris & Steiner, 1975; Baldwin et al., 1980; Caro & Amatruda, 1981).

Insulin binding to cells leads to a reduction in the number of receptors on the cell surface (Roth et al., 1975; Blackard et al., 1978; Livingston et al., 1978; Peterson et al., 1978; Bar et al., 1979; Mott et al., 1979; Marshall & Olefsky, 1980; Krupp & Lane, 1981) - a phenomenon known as 'down' regulation (Gavin et al., 1974). 'Down' regulation is attributed to the intracellular degradation of receptors (Blackard et al., 1981; Van Obberghen et al., 1981; Ronnett et al., 1983) and the lysosomes have been identified as the site of degradation of both insulin and its receptor (Goldstein & Livingston, 1981; Gleiman & Sonne, 1978; Marshall & Olefsky, 1979; Hammons & Jarett, 1980; Hoffman et al., 1980; Marshall & Olefsky, 1980). However, the rate of entry of insulin into the cell exceeds the rate at which the reduction of receptor numbers on the plasma membrane occurs indicating that some receptors are recycled back to the cell surface (Terris & Steiner, 1980) and the use of photoaffinity analogues of insulin to follow the fate of the internalised hormone-receptor complex confirms that the recycling of receptors occurs (Fehlmann et al., 1982a,b; Carpentier et al., 1986).

The insulin receptor complex appears to undergo internalisation via a process similar to that identified for receptors for asialoglycoproteins (Wiegel & Oka, 1981), α_2-macroglobulin (Wiegel & Oka, 1981) low density lipoproteins (Brown & Goldstein, 1976; Brown et al., 1983) and EGF (Schechter et al., 1978; Schlessinger et al., 1978). After the binding of hormone

insulin receptors congregate at coated pits, which are invaginated areas of the plasma membrane coated in the protein clathrin (Maxfield et al., 1978; Fan et al., 1982; Brown et al., 1983). Coated pits pinch off or bud to form endosome vesicles which transport the hormone-receptor complex to the golgi region (Wall et al., 1980; Willingham & Pastan, 1980) where dissociation of the hormone and receptor occurs in acidic endosomes (Wall et al., 1980; Willingham & Pastan, 1980; Brown et al., 1983; Helenius et al., 1983). Specialised endosomes termed CURL (Compartment of Uncoupling of Receptor and Ligand) have been identified and the hormone receptor complex is thought to enter these structures then, because of the low pH within CURL, the hormone dissociates into the luminal space and the receptors migrate to the more membrane-rich area (Geuze et al., 1983a,b, 1984). It is presumed that following this segregation receptor-rich membrane vesicles bud from CURL and return to the plasma membrane (Geuze et al., 1984). Meanwhile the hormone and some receptors are degraded in the lysosomes (Goldstein & Livingston, 1981; Carpentier et al., 1985). Therefore receptors can either be recycled back to the plasma membrane or degraded (Rozengurt et al., 1981; Carpentier et al., 1986). Not all of the insulin internalised is degraded and the relative amount extruded back into the extracellular medium depends on the cell type (Sonne & Glieman, 1980; Marshall, 1985). It has also recently been reported that some receptors are recycled back to the plasma membrane with insulin intact (Ezaki et al., 1986).

1.3.6. The molecular mechanism of insulin action

Although the effects of insulin on cellular metabolism are well established (see section 1.3.2.) the molecular mechanism of insulin action is not understood. Following the binding of insulin to plasma membrane receptors on its target cells a protein kinase activity contained within the receptor is stimulated (see section 1.3.6.6.) and the hormone-receptor complex is internalised (see section 1.3.5.): it has been suggested that these processes are involved in the insulin transduction system. In addition a number of agents have been proposed to act as intracellular second messengers for insulin including Ca^{++} (see section 1.3.6.2.), H_2O_2 (see section 1.3.6.5.) and an unidentified protein (N_{ins}) may also be involved in mediating insulin action (see section 1.3.6.3.). In the following section some possible mechanisms of insulin action are discussed.

1.3.6.1. The role of internalisation and degradation of the insulin receptor complex in the action of insulin

It has been proposed that internalisation of the insulin receptor complex is necessary to elicit at least some of the hormone's actions (Goldfine, 1981). Internalisation may be necessary to transport insulin to intracellular receptors while degradation of the hormone may be essential to its action as a fragment of insulin may carry the biological activity or alternatively internalisation and perhaps degradation of the receptor could be an essential step in insulin action and may serve to deliver the tyrosine kinase, on the β-subunit of the receptor, to intracellular sites of action (see Goldfine, 1981; Goldstein & Livingston, 1981; Houslay, 1985). Some

evidence does exist to support these hypotheses. Indeed insulin receptors have been identified on the cell nucleus (Goldfine et al., 1981) and on other intracellular sites such as golgi vesicles and endoplasmic reticulum (Posner et al., 1980; Goldfine, 1981; Kahn et al., 1981; Debanne et al., 1982; Desbuquois et al., 1982; Kahn et al., 1982; Posner et al., 1982; Sonne & Simpson, 1984) and fragments of insulin have been synthesised which do not interact with the receptor but do possess insulin like activity (Weitzel et al., 1971; Fujino et al., 1977). Also the time course for insulin stimulation of glucose transport is identical to that for internalisaton of insulin (Karnieli et al., 1981; Sonne & Simpson, 1984) and both processes are energy dependent (Kono et al., 1977; Chandramouli et al., 1977) suggesting that the internalisation of insulin and stimulation of glucose transport by the hormone may be connected. The stimulation of glucose transport in adipocytes by insulin is a two step process (Karnieli et al., 1981; Cushman et al., 1983; Simpson et al., 1983) the first being the recruitment of glucose carriers from intracellular sites to the plasma membrane and the second step being the activation of the carriers. The translocation of glucose carriers to the plasma membrane of adipocytes is induced by Tris buffers (Cushman et al., 1983) which also inhibit the recycling of the insulin receptor (Olefsky et al., 1982) and therefore increase the number of insulin receptors that are present internally. The overall rate of glucose transport is not stimulated in Tris buffer but this may be due to a requirement for the activation of the transporters once inserted into the plasma membrane. It is therefore possible that internalisation of the

insulin-receptor complex induces the redistribution of cellular glucose transporters which is a necessary step in the insulin-stimulation of glucose transport. However evidence exists to contradict this as it has been reported that the blocking of internalisation or degradation of the insulin-receptor complex does not interfere with the stimulation of glucose transport (Hammons & Jarett, 1979; Marshall & Olefsky, 1980; Hyslop et al., 1985; Jeffreys et al., 1985) and it is difficult to explain how antibodies to the insulin receptor (Kahn et al., 1977; 1978) and plant lectins (Czech & Lynn, 1973) mimic insulin action if either the internalisation or degradation of insulin or its receptor are a necessary prequisite for its action.

1.3.6.2. The role of calcium in mediating the actions of insulin

It has been proposed that the binding of insulin to its receptor on the cell surface triggers an increase in cytoplasmic Ca^{++} levels, which is relayed into the mitochondria, and that Ca^{++} mediates the action of insulin on membrane transport and on enzymes of glycogenesis, lipogenesis, lipolysis and glycogenolysis (Bihler, 1972; Clausen et al., 1974; Clausen, 1975; Kissebah et al., 1975). However, studies investigating the role of Ca^{++} in cellular insulin action have produced varied and contradictory results.

Exposure of adipocytes to insulin has been reported to result in an increase in intracellular Ca^{++} levels (Hope-Gill et al., 1975; Kissebath et al., 1975; Clausen & Martin, 1977) while in L6 muscle cells insulin reportedly had no effect on the intracellular Ca^{++} concentration under conditions which resulted in stimulation of glucose transport (Klip & Logan, 1983, 1984). Several studies have

reported that extracellular Ca^{++} is required for the stimulation of hexose transport by insulin in perfused heart atria (Bihler & Sawh, 1980; Bihler et al., 1980b) and in adipocytes (Taylor et al., 1979) although contrary reports claim that the insulin stimulation of glucose transport is independent of extracellular Ca^{++} in isolated rat soleus muscle (Gould & Chandry, 1970; Yu et al., 1980) in L6 muscle cell cultures (Klip et al., 1983, 1984) and in isolated cardiocytes (Haworth et al., 1982; Eckel et al., 1983). These conflicting reports may be at least partly due to a lack of suitable techniques for measuring intracellular Ca^{++} levels (see Klip, 1984). However if insulin does induce increases in intracellular Ca^{++} levels in target cells and this increase is independent of extracellular Ca^{++} then the hormone must trigger the release of Ca^{++} from intracellular stores such as the endoplasmic reticulum or plasma membrane.

Indirect approaches have also been adopted to attempt to correlate changes in Ca^{++} levels with the actions of insulin. Inducing an increase in the cytoplasmic Ca^{++} concentration, using the ionophore A23187, has been found to either increase hexose transport in primary myoblast cultures of chicken skeletal muscle (Schudt et al., 1976) in rat hemidiaphragm and resting atria (Bihler et al, 1980a) and in rat adipocytes (Taylor et al., 1979) and thymocytes (Reeves, 1977) or to have no effect in L6 muscle cell cultures (Klip et al., 1983, 1984) in isolated cardiocytes (Eckel et al., 1983), or in rat soleus muscle (Grinstein & Erlij, 1976). In addition intracellular perfusion of barnacle muscle fibres with Ca^{++} solutions did not increase hexose uptake (Baker & Carruthers, 1983) and

lowering of intracellular Ca^{++} levels using A23187 in Ca^{++} free media or with the chelator quin-2 did not interfere with the insulin stimulation of hexose uptake in rat soleus muscle (Hall et al., 1982) isolated heart cells (Haworth et al., 1982; Eckel et al., 1983) or in L6 muscle cells (Klip et al., 1983, 1984).

In conclusion the role of Ca^{++} in insulin action remains controversial due to the many conflicting experimental reports which exist. Perhaps the strongest argument against Ca^{++} acting as a mediator of insulin action is the lack of correlation between the effects of Ca^{++} and insulin on some metabolic pathways. For example Ca^{++} stimulates phosphorylase in muscle (Ozawa et al., 1967) and adipocytes (Khoo, 1976) while insulin has an inhibitory effect (Jungas, 1966; Torres et al., 1968) and raising the intracellular Ca^{++} concentration stimulates lipolysis in adipose tissue (Park et al., 1972; Efendic et al., 1970; Exton et al., 1972; Werner et al., 1972; Schimmel, 1976) yet insulin exerts an antilipolytic effect (Londos et al., 1985). In addition it has recently been shown that the stimulation of mitochondrial PDH activity by insulin is not dependent upon Ca^{++} (Cheng & Larner, 1985; McCormack & Denton, 1985). Certainly it therefore seems that Ca^{++} is not the mediator for all of the effects of insulin on intracellular metabolism and it remains to be conclusively proved that is is necessary to elicit any of the responses to insulin. It has been shown that the insulin receptor contains a calmodulin-binding domain (Graves et al., 1985) and that Ca^{++} increases the affinity of the receptor for insulin (Williams et al., 1984): Ca^{++} may therefore have a role in modulating the insulin binding capacity of the cell.

1.3.6.3. Is insulin action mediated by a putative guanine nucleotide binding protein?

Two distinct guanine nucleotide regulatory binding proteins N_s and N_i have been identified and purified in the liver (see Houslay, 1985, 1986). The binding of glucagon to its hepatic receptors results in the activation of N_s which subsequently leads to the stimulation of adenylate cyclase activity. For a description of the structure and function of N_s and N_i see section (1.4.1.1.).

Insulin antagonises the effects of glucagon in the liver by lowering intracellular cAMP levels (see Houslay, 1986); this is achieved by the activation of a plasma membrane and a dense vesicle phosphodiesterase (Heyworth et al., 1983b) and by the inhibition of adenylate cyclase (Heyworth & Houslay, 1983b). It has been proposed that the stimulation of the plasma membrane phosphodiesterase and the inhibition of adenylate cyclase by insulin is mediated by a putative guanine nucleotide binding protein which is coupled to the insulin receptor and has been labelled $N_{ins.}$ while the dense vesicle phosphodiesterase is activated by a different and as yet unknown route (see Houslay et al., 1984; Houslay, 1985, 1986).

Insulin stimulates the phosphorylation of the plasma membrane phosphodiesterase by a plasma membrane bound cAMP-dependent protein kinase (Marchmont & Houslay, 1980, 1981; Heyworth et al., 1983b): cholera toxin also elicits the activation of the plasma membrane phosphodiesterase and insulin then causes no further increase in its activity hence both agents achieve their effect through a common route (Heyworth et al., 1983b). This effect of cholera toxin is not mediated by N_s as cAMP does not increase the activity of the plasma

membrane phosphodiesterase (Heyworth et al., 1983b). The stimulation of the phosphodiesterase is also not mediated by N_i as pertussis toxin has no effect on its activation by insulin (Heyworth et al., 1986). These observations support the hypothesis that another guanine nucleotide binding protein $N_{ins.}$ exists.

Glucagon blocks the activation of the hepatic plasma membrane phosphodiesterase by insulin (Heyworth et al., 1983b) in a similar manner to that whereby glucagon induces desensitisation of adenylate cyclase to hormonal stimulation (Heyworth & Houslay, 1983a): in both instances the effect of glucagon is independent of cAMP, occurs at a concentration of the hormone which is an order of magnitude lower than that required to stimulate adenylate cyclase and is relatively slow to occur (Heyworth & Houslay, 1983a; Heyworth et al., 1983b). Results suggest that the desensitisation of adenylate cyclase by glucagon occurs via the modification of the functioning of N_s and, as desensitisation can be blocked by pertussis toxin, this may be mediated by the activation of Ni (Heyworth et al., 1984a). It therefore seems likely that the glucagon induced inhibition of the insulin effect also occurs through a guanine nucleotide binding protein (see Houslay, 1985, 1986) and this view is further supported by the observations that adenosine (Wallace et al., 1984), the phorbol ester 12-0-tetradecanoylphorbol-13-acetate (TPA) (Heyworth et al., 1984b, 1985b) pertussis toxin (Heyworth et al., 1984a) and the Ca^{++} ionophore A23178 (Houslay, 1986) all prevent glucagon induced desensitisation of adenylate cyclase and inhibition of insulin's ability to activate the plasma membrane phosphodiesterase.

In contrast to the lack of effect of pertussis toxin on the insulin induced stimulation of the plasma membrane phosphodiesterasee the inhibition of adenylate cyclase by insulin is completely blocked by the toxin (Heyworth et al., 1986) as it is by cholera toxin (Heyworth & Houslay, 1983b). This supports the view that insulin elicits its actions on cAMP metabolism by interacting with the guanine nucleotide binding protein system but could imply that the activation of the plasma membrane phosphodiesterase and the inhibition of adenylate cyclase is not mediated by the same protein. Houslay (1986) has however suggested that pertussis toxin may block the insulin induced inhibition of adenylate cyclase by an indirect action. Hence excess N_i-α subunits could provide a high affinity sink for β and α subunits from $N_{ins.}$ and this may obliterate the inhibition of adenylate cyclase if it were mediated by the β-subunit of $N_{ins.}$

A 25KDa protein has been identified in rat liver plasma membranes as possibly being the putative $N_{ins.}$ (Heyworth et al., 1985a). The ribosylation of this protein by cholera toxin is inhibited by insulin, which may be expected if insulin causes the activation and dissociation of the protein. The ability of insulin to inhibit ribosylation by cholera toxin is reduced in cells pre-treated with glucagon (i.e. cells which are desensitised to insulin). In addition the ability of cholera toxin to ribosylate the 25KDa species is reduced by the phorbol ester TPA and by pertussis toxin and both of these agents also inhibit the activation of the plasma membrane phosphodiesterase by cholera toxin (Heyworth et al., 1985b).

To conclude strong evidence exists to suggest that insulin exerts its effects upon hepatic cAMP metabolism by interacting with the guanine nucleotide regulatory binding protein system. This may involve the existence of a distinct guanine nucleotide binding protein, $N_{ins.}$, although further work is required to show this conclusively and fully characterise the protein.

It has been postulated that insulin may exert many of its effects on cellular metabolism by modifying the activity of membrane bound protein kinases via the activation of $N_{ins.}$ (Houslay, 1986) and indeed evidence is emerging to support this view. The insulin induced stimulation of a cAMP-independent protein kinase in sarcolemma membranes is enhanced by GTP (Walaas et al., 1981), glucose transport in barnacle muscle is stimulated by non-hydrolysable analogues of GTP (Baker & Carruthers, 1983) and the ability of insulin to inhibit lipolysis in adipocytes is abolished by pertussis toxin (Goren et al., 1985). Glucagon and isoprenaline can inhibit insulin stimulated glucose transport (Green 1983a,b; Kashiwagi et al., 1983) though a cAMP-independent mechanism (Cooper, 1982) which is reminiscent of the glucagon desensitisation of the plasma membrane phosphodiesterase and of adenylate cyclase (Wallace et al., 1984): similarly to hepatic desensitisation the inhibition of glucose transport is blocked by adenosine (Green, 1983a,b). If the action of insulin is mediated by $N_{ins.}$ this could explain the amplification of the signal generated by the binding of insulin to its receptor and the mechanism whereby insulin modifies the activity of many membrane associated functions. $N_{ins.}$ could also provide a site for the antagonism of insulin action by catecholamines

and other hormones. However it seems unlikely that insulin elicits all of its effects on metabolism in this way: in particular it is unlikely that enzymes in intracellular organelles, such as the dense vesicle phosphodiesterase (see Houslay, 1985, 1986) and mitochondrial PDH (see section 1.2.3.1.) are affected by the plasma membrane guanine nucleotide regulatory system.

1.3.6.4. Is insulin action dependent upon changes in intracellular cyclic nucleotide levels?

Insulin decreases the cAMP concentration in liver and adipose tissue only after it has been elevated by hormonal stimulation (Butcher et al., 1966, 1968; Siddle et al., 1973; Pilkis et al., 1975; Blackmore et al., 1979; Heyworth et al., 1983b). No effect of insulin has been detected on cAMP levels in muscle (Pilkis & Park, 1974; Tarui et al., 1976; Walkenbach et al., 1978; Severson, 1979) or on basal levels in adipocytes or hepatocytes yet many of the metabolic actions of insulin occur under these conditions (see Czech, 1977; Fain, 1977). Clearly most of the biological effects of insulin occur independently of cAMP levels. However, the ability of insulin to decrease hormonally stimulated cAMP levels probably accounts for the insulin antagonism of the effects of glucagon on hepatic gluconeogenesis and glycogen breakdown (see Denton et al., 1981) and at least partly for the antilipolytic effects of insulin on white adipose tissue (Kono & Barham, 1973; Siddle & Hales, 1974; Steinberg et al., 1975; Wong & Loten, 1981; Londos et al., 1985).

Insulin also induces an increase in the cGMP concentration in white adipose tissue and liver (Illiano et al., 1973; Vydelingum et al., 1975; Fain & Butcher, 1976; Pointer et al., 1976). However it

is unlikely that this nucleotide mediates the biological actions of insulin as other agents, such as carbachol and noradrenaline, which do not have the same biological effects as insulin also increase cGMP levels in fat cells (Fain & Butcher, 1976; Pointer et al., 1976) and hepatocytes (Pointer et al., 1976) while the insulin induced rise in cGMP is suppressed in adipocytes incubated in Ca^{++} free medium yet the metabolic effects of insulin are unaltered (Fain & Butcher, 1976).

1.3.6.5. Does hydrogen peroxide act as a mediator for insulin?

Hydrogen peroxide (H_2O_2) mimics many of the effects of insulin on adipose tissue metabolism including stimulation of glucose transport, glucose oxidation, glycogen synthesis and lipogenesis and inhibition of lipolysis (May & De Haën, 1979; Ciraldi & Olefksy, 1982). In addition the insulin like effects of the polyamines spermine and spermidine are attributed to the formation of H_2O_2 as a result of their oxidation (Lockwood etal., 1971; Lockwood & East, 1974; Livingston et al., 1977). This has led to the suggestion that H_2O_2 is a mediator of insulin action. However there is no conclusive evidence that insulin increases H_2O_2 in adipocytes. Mukherjee & Lynn (1977) reported an increase in H_2O_2 production in fat cells after exposure to insulin but this could not be confirmed by others (May & De Haën, 1979; Wieland, 1983). It has been claimed that exposure of adipocyte membranes to insulin stimulates NADPH oxidase which results in the production of H_2O_2 (Mukherjee & Lynn, 1977) but again other workers have not observed this (Livingston et al., 1977; May & De Haën, 1979; Wieland, 1983). Insulin stimulates formate oxidation in adipocytes (Mukherjee et al., 1978; May & De Haën, 1979;

Paetzke-Brunner et al., 1980) which is claimed to initiate the formation of H_2O_2 (May & De Haën, 1979; Wieland, 1983) but this is by no means conclusive (see Denton et al., 1981; Wieland, 1983).

Similarly to insulin H_2O_2 increases the proportion of the enzyme pyruvate dehydrogenase in the active dephosphorylated form in adipocytes (May & De Haën, 1979; Mukherjee et al., 1980; Paetzke-Brunner et al., 1980; Wieland & Paetzke-Brunner, 1981; Begum et al., 1982b) and in hepatocytes (Wieland, 1983). It has been reported that the exposure of adipocyte mitochondria to H_2O_2 results in the activation of PDH activity (Paetzke-Brunner, et al., 1980) but others have been unable to obtain any effects which are not due simply to a decrease in the mitochondrial ATP/ADP ratio (Denton et al., 1981; Begum et al., 1982b).

1.3.6.6. The insulin receptor expresses protein kinase activity

Recently it was demonstrated that the insulin receptor possesses insulin sensitive protein kinase activity (Avruch et al., 1982; Kasuga et al., 1982a; Petruzzelli et al., 1982; Van Obberghen & Kowalski, 1982). This activity is located exclusively on the β-subunit (Roth & Cassell, 1983; Van Obberghen et al., 1983) which has its autophosphorylation stimulated by the binding of insulin to the receptor (Kasuga et al., 1982b; Roth et al., 1982; Kasuga et al., 1983a,b; Roth et al., 1983b; Nemenoff et al., 1984; Petruzzelli et al., 1984) resulting in a further stimulation of the kinase activity (Rosen et al., 1983; Yu & Czech, 1984). In intact cells insulin stimulates the phosphorylation of the β-subunit on tyrosine and serine residues (Haring et al., 1982; Kasuga et al., 1982a,c; Van Obberghen & Kowalski, 1982; Gazzano et al., 1983; Zick et al., 1983a;

Pang et al., 1985; White et al., 1985); in constrast insulin stimulates the phosphorylation of only tyrosine residues in highly purified receptor preparations (Avruch et al., 1982; Kasuga et al., 1982b; Roth et al., 1982; Petruzzelli et al., 1982; Fujita-Yamaguchi et al., 1983; Gazzano et al., 1983; Kasuga et al., 1983a; Roth & Cassell, 1983; Shia & Pilch, 1983). It has been suggested that while tyrosine kinase activity is inherent within the insulin receptor a serine kinase is non-covalently associated with it and therefore purification of the receptor results in the loss of the serine kinase activity (Gazzano et al., 1983; Gammeltoft & Van Obbeghen, 1986).

The insulin receptor expresses protein kinase activity in all tissues and cells studied so far (Haring et al., 1982; Kasuga et al., 1982a,b,c; Petruzzelli et al., 1982; Grigorescu et al., 1983; Burant et al., 1984; Gammeltoft et al., 1984; Grunberger et al., 1984a; Van Obberghen et al., 1985). Although the kinase can phosphorylate exogenous substrates such as histones and casein (Gazzano et al., 1983; Grunberger et al., 1983; Kasuga et al., 1983b; Stadtmauer & Rosen, 1983; Nemenoff et al., 1984; Pike et al., 1984) the β-subunit of the receptor is the best substrate known (see Gammeltoft & Van Obberghen, 1986).

After the discovery of the insulin receptor kinase attempts to implicate it as a mediator of the metabolic effects of insulin have been disappointing. Dephosphorylation of the insulin receptor results in deactivation of the kinase, as would be expected if it were to have a regulatory function (Zick et al., 1983b; Yu & Czech, 1984), and indeed phosphatase activity co-purifies with but is not an integral part of the receptor (Haring et al., 1984a;

Kowalski et al., 1983). Half-maximal stimulatory effects of insulin on the kinase occur at 300-600μunit/ml (Haring et al., 1982; Petruzzelli et al., 1982; Grigorescu et al., 1983; Kasuga et al., 1983a; Rosen et al., 1983; Roth et al., 1983; Zick et al., 1983b; Haring et al., 1984a; Rees-Jones et al., 1984) while many metabolic effects of insulin occur at about 2-fold lower concentrations (Heyworth et al., 1983b; Sonne & Simpson, 1984; Weiss et al., 1974). Phosphorylation of the insulin receptor is increased by agents which have insulin like effects such as lectins (Roth et al., 1983a) and some polyclonal antisera to the receptor (Petruzzelli et al., 1982; Zick et al., 1984) however other antibodies which have insulin like effects lack the ability to stimulate the kinase (Simpson & Hedo, 1984; Zick et al., 1984). This suggests that the stimulation of the receptor kinase is not an essential step in mediating the effects of insulin upon cellular metabolism. Although many exogenous substrates for the insulin receptor kinase have been identified only one physiological substrate is known: a cellular glycoprotein, with a M_r of about 110000-120000, which has been purified from rat liver and brown adipose tissue although its exact identity and function are unknown (Sadoul et al., 1985; Rees-Jones & Taylor, 1985). All kinases involved in the control of intermediary metabolism are serine or threonine specific (Denton et al., 1981; Cohen, 1982): the insulin receptor tyrosine protein kinase may induce an increase in activity of the receptor-associated serine kinase and this could account for many of the cellular responses to insulin (see Gammeltoft & Van Obberghen, 1986). Perhaps the strongest evidence in favour of the receptor kinase being involved in insulin action is the

impairment of its activity in various insulin resistant states such as type A insulin resistance (Grunberger et al., 1984a,b; Grigorescu et al., 1984), obesity in mice (Le Marchand-Brustel et al., 1985) in catecholamine-treated rat adipocytes (Häring et al., 1986) and in streptozotocin diabetic rats (Kadowaki et al., 1984).

The receptors for several polypeptide growth factors possess tyrosine kinase activity including epidermal growth factor (Cohen et al., 1980), platelet derived growth factor (Ek et al., 1982), transforming growth factor (Reynolds et al., 1981) and insulin like growth factor I (Jacobs et al., 1983; Rubin et al., 1983) implying that receptor kinase activity may be involved in promoting cellular growth responses. Certainly the sensitivity of the kinase activity to insulin, although much less than that of metabolic responses, correlates with the sensitivity of the longer term effects of the hormone (King & Kahn, 1981). In support of a role for the insulin receptor kinase in mediating the growth promoting effects of insulin injection of the kinase into xenopus oocytes increased the phosphorylation of ribosomal protein S6 (Maller et al., 1986) and a monoclonal antibody which was found to prevent insulin induced oocyte maturation also inhibited the receptor kinase activity (Morgan et al., 1986).

1.3.6.7. The production of an unidentified mediator of insulin action

The existence of an insulin mediator, or group of mediators, was first proposed by Larner et al., (1974, 1976) who provided evidence for the production of such a substance in hind-limb muscle of rats injected intravenously with insulin. The substance(s) which

was trichloroacetic acid extractable, inhibited cAMP-dependent protein kinase activity. Subsequent studies showed that analogous to the action of insulin the mediator substance/s stimulated adipocyte PDH activity (Jarett & Seals, 1979a,b) and inhibited glycogen synthase phosphatase (Larner et al., 1979b,d) which led to the substance/s being proposed as a chemical mediator/s for insulin action (Jarett & Seals, 1979b; Larner et al., 1979b).

The mediator/s is produced in a number of insulin sensitive systems including rat white adipose tissue, skeletal muscle, liver and hepatoma cells (Larner et al., 1974; Jarett & Seals, 1979a,b; Jarett et al., 1980; Parker et al., 1982a,b; Saltiel et al., 1982a,b) and human placenta (Sakamoto et al., 1982). Interestingly in IM-9 lymphocytes, to which insulin binds but appears not to elicit any biological response, the mediator activity is decreased by insulin (Jarett et al., 1980). The activities of a large number of insulin sensitive enzymes are modified by the mediator/s: enzymes stimulated include glycogen synthase phosphatase (Larner et al., 1979b,d) glycogen synthase (Kikuchi et al., 1981), PDH (Jarett & Seals, 1979a,b), PDH-phosphatase (Seals et al., 1979b), high affinity ATPase (McDonald et al., 1981) low K_m cAMP-phosphodiesterase (Kiechle & Jarett, 1981a,b) and ACC (Saltiel et al., 1983) while cAMP-dependent protein kinase (Larner et al., 1974), adenylate cyclase (Larner, 1982) and glucose-6-phosphatase (Suzuki et al., 1984) are inhibited by the mediator substance/s.

The production and action of the insulin mediator/s has been extensively studied using a subcellular system consisting of an adipocyte plasma membrane enriched fraction, which produces the

mediator/s in response to insulin, and isolated mitochondria, which can be used to quantify the amount of mediator activity produced by measuring the stimulation of mitochondrial PDH activity (Jarett, 1974; Seals et al., 1978, 1979a,b; Seals & Jarett, 1980; McDonald et al., 1981; Seals & Czech, 1981a; Begum et al., 1982a). The addition of insulin to isolated mitochondria alone has no effect on PDH activity but the hormone produces an increase in mitochondrial PDH activity when plasma membranes are also present (Seals & Jarett, 1980). Mitochondrial PDH activity is also stimulated by the supernatant collected after centrifugation of plasma membranes which have been exposed to insulin (Seals & Czech, 1980; Kiechle et al., 1981). The mediator is spontaneously produced by plasma membranes in the absence of insulin but the hormone increases its production (Kiechle et al., 1981): the amount of mediator activity produced spontaneously is greater when the plasma membranes are prepared in Tris buffer with EDTA than when phosphate buffer with no EDTA is used (Kiechle et al., 1981). Optimum effects of insulin are observed when the plasma membranes are prepared in phosphate buffer with no EDTA, to minimise the basal production of the mediator, and the proportion of PDH in the active dephosphorylated state in the mitochondria has been reduced by pre-incubation with ATP (Seals & Jarett, 1980; Seals & Czech, 1981a): maximal stimulation of PDH activity is achieved with 50-100μunits/ml of insulin within one minute of treatment (Seals & Czech, 1981a). As described above activation of PDH activity by insulin in vivo and in tissue pieces or cells in vitro occurs via a stimulation of mitochondrial PDH-phosphatase which results in the dephosphorylation and stimulation of PDH (see section 1.2.3.1.1.).

The stimulation of PDH in the subcellular system has been shown to occur via the same mechanism (Popp et al., 1980; Kiechle et al., 1980a,b). The insulin mediator/s stimulates PDH activity in coupled and uncoupled mitochondria (Parker & Jarett, 1985) and activates partially purified PDH and PDH-phosphatase (Newman et al., 1985).

The mediator substance/s has not been fully characterised. Partial purification and fractionation of the substance/s has shown that it has a molecular weight in the range 1000-4000da (Larner et al., 1979b,d; Kiechle et al., 1980b, 1981; Saltiel et al., 1981; Seals & Czech, 1981a; Parker et al., 1982b), has a negative charge at pH 7.4 (Larner et al., 1976; Seals & Czech, 1981a, 1982) and is soluble in aqueous solutions (Larner et al., 1976; Seals & Czech, 1981a; 1982; Parker et al., 1982b) and in acid (Larner et al., 1979a) which suggests that the substance/s is hydrophilic (Larner, 1982; Seals & Czech, 1982). The putative mediator/s is acid stable (Larner et al., 1974, 1976, 1979a,b; Kiechle et al., 1980b; Saltiel et al., 1981; Seals & Czech, 1981a; Parker et al., 1982b) and is stable at 100°C for about 5 min (Larner et al., 1974, 1976, 1979b; Saltiel et al., 1981; Seals & Czech, 1981a; Saltiel et al., 1982a; Parker et al., 1982b) but is stable at 30-37°C and neutral pH for only 30 min (Larner et al., 1978, 1979a,c; Seals & Czech, 1981a) although it can be stored for up to 5 months at -70°C (Kiechle et al., 1981; Larner, 1982; Larner et al., 1982c). Early studies suggested that the mediator/s may be a peptide as it purified with a 230nm ninhydrin-positive peak (Larner et al., 1979b, 1982b; Kiechle et al., 1980a, 1981) and some workers were able to destroy the substance/s with proteases (Sakamoto et al., 1982; Seals & Czech, 1980) although

others have reported little or no effect of these enzymes (Jarett et al., 1983a,b; Larner, 1983). It has been reported that the mediator is generated from plasma membranes by a proteolytic cleavage event (Larner et al., 1982b) and that its production is blocked by various protease inhibitors (Seals & Czech, 1980). However, although amino acid sequences for the mediator/s have been reported (Gainutdinov et al., 1978; Larner et al., 1982b; Seals, 1983) Jarett and co-workers concluded that the mediator contains peptide bonds but these are not essential for its activity (Jarett et al., 1983a,b; Jarett & Kiechle, 1984). The mediator/s is destroyed by neuraminidase and β-D-galactosidase which cleave exposed sialic acid and galactose residues from glycoproteins (Larner, 1982; Begum et al., 1983). It has been suggested that the mediator/s contains phospholipid (Kiechle et al., 1982; Macauley et al., 1982; Parker et al., 1982c; Jarett et al., 1984) yet it is insensitive to phospholipase (Zhang et al., 1983), insoluble in ethanol (Saltiel et al., 1983) and not very soluble in chloroform methanol (Larner, 1982). Clearly then some disagreement has occurred over the nature of the putative insulin mediator/s and this may be due to contaminating substances present in mediator preparations. However very recently it has been claimed that two putative insulin mediator/s which are produced by liver membranes (Saltiel & Cuatrecasas, 1986) and cultured murine myocytes (Saltiel et al., 1986) have been partially purified and characterised. These putative mediators activate adipocyte PDH and high-affinity cAMP-phosphodiesterase and rather than being peptides they are complex carbohydrate-phosphate substances containing inositol and glycosidically linked glucosamine. It was suggested

that insulin activates a selective phospholipase C in plasma membranes which hydrolyses a novel glycolipid present in the membranes resulting in the production of the putative mediators along with DAG (Saltiel & Cuatrecasas, 1986; Saltiel et al., 1986). This is consistent with the observation that insulin increases the amount of inositol phospholipids and phosphatidic acid in various cell types (Farese et al., 1985; Pennington & Martin, 1985).

The activation of PDH, and various other enzymes, by the insulin mediator/s occurs in a biphasic manner hence the activation of the enzymes reaches a peak and then falls off until at high concentrations of mediator/s the enzymes are inhibited (Jarett & Seals, 1979a; Cheng et al., 1980; Seals & Jarett, 1980; Jarett et al., 1981; Kikuchi et al., 1981; Saltiel et al., 1981; Seals & Czech, 1981a; Saltiel et al., 1982b, 1983; Suzuki et al., 1984). This led to the proposal that more than one mediator existed which can regulate PDH activity and indeed it was reported that two such substances had been isolated; one which inhibited PDH and one which stimulated it (Cheng et al., 1980; Saltiel et al., 1982c, 1983). The dose response curves for these separate mediator fractions appeared to be no longer biphasic (Saltiel et al., 1982,b,c) and it was proposed that the inhibitory mediator had a lower affinity for PDH than the stimulatory one (Jarett et al., 1982). However the two putative insulin mediators that have been recently isolated by Saltiel & Cuatrecasas both regulate PDH activity in a biphasic manner and these workers proposed that the two separate stimulatory and inhibitory PDH regulatory fractions which had been previously isolated actually consisted of different concentrations of the same

substances (Saltiel & Cuatrecasas, 1986). Indeed a range of insulin mediators may exist which modulate the activities of different enzymes and indeed this could explain how the diversity of action of insulin is achieved (see Larner et al., 1981, 1982a; Larner, 1982, 1984).

The proposal that insulin binding to target cells results in the production of a novel mediator/s which elicits the biological actions of the hormone is very promising. Mediator production appears to be ubiquitous among insulin sensitive tissues and the substance/s mimics many of the actions of insulin on various enzyme systems. In addition agents which have insulin-like actions such as conconavalin A (Katzen et al., 1981; Begum et al., 1982b) and anti-insulin receptor antibody (Jarett & Seals, 1979a; Popp et al., 1980; Seals & Jarett, 1980; Jarett et al., 1981; Jarett & Kiechle, 1981; Begum et al., 1982b, 1983) also stimulate mediator production. Some physiological conditions which are associated with insulin resistance result in a reduction in the ability of the hormone to stimulate mediator production for example fasting and diabetes mellitus reduce hepatic mediator production (Amatruda & Chang, 1983) and feeding rats a high fat diet reduces mediator production from both white adipocyte and hepatocyte plasma membranes (Begum et al., 1982a, 1983).

The suggestion that the mediator/s is a peptide, perhaps derived by proteolytic cleavage of the insulin receptor, (Seals & Czech, 1980) raised the question as to how the binding of a few molecules of insulin to cell surface receptors could regulate the activity of millions of target enzyme molecules within the cell (see

Houslay, 1985; Houslay et al., 1986). This could be easier explained if the mediator/s is the product of an insulin-stimulated enzymatic reaction that occurs on or near the plasma membrane of target cells, such as the hydrolysis of a novel plasma membrane glycolipid by an insulin-sensitive phospholipase as proposed by Saltiel & Cuatrecasas, (1986). The exact mechanism by which the mediator/s modifies the activity of target enzymes, such as PDH, is not known. The mediator/s activates partially purified PDH-phosphatase (Newman et al., 1985) suggesting that in the intact cell the mediator/s enters mitochondria and interacts with PDH-phosphatase resulting in the dephosphorylation and activation of PDH (see section 1.2.3.1.1.). Full elucidation of the role the putative insulin mediator/s has in insulin action will probably depend on the ability to produce purified mediator preparations and on the identification of its exact chemical nature.

1.3.6.8. Conclusions

Although the mechanism by which the binding of insulin to its plasma membrane receptors is coupled to intracellular metabolic events remains to be elucidated there are a number of promising proposals such as the insulin-receptor tyrosine kinase activity (see section 1.3.6.6.), internalisation of the insulin-receptor complex (see section 1.3.6.1.), the guanine nucleotide binding protein N_{ins} (see section 1.3.6.3.) and a novel, unidentified mediator or group of mediator molecules (see section 1.3.6.7.). It may be that more than one signal is generated after the binding of insulin to its cell surface receptors (see fig. 1.3.2.) and indeed this would explain the diversity of action of the hormone (see Houslay, 1985).

1.4. Catecholamines

The catecholamine adrenaline is a circulating hormone that is released exclusively from the adrenal medulla while noradrenaline is primarily a neurotransmitter released from sympathetic nerve endings (see Barrand & Callingham, 1983; Landsberg & Young, 1985). Noradrenaline that is present in the circulation originates from sympathetic nerve endings but has escaped neuronal reuptake and local metabolism at the adrenergic synapses (see Landsberg & Young, 1985). In the blood the half-life of catecholamines (about 20s) is very short (Vane, 1969) due to uptake by body tissues (Iversen, 1975). In general catecholamines are released during activity or stress (see Barrand & Callingham, 1983) to increase the availability of metabolic fuels to the tissues. Metabolic effects of catecholamines can occur through direct effects on target cells (see Stiles et al., 1984; Exton, 1985) as a consequence of a change in the plasma levels of other hormones, such as insulin (see section 1.3.1.) or via effects on the cardiovascular system which modify blood flow to body tissues (see Nilsson, 1985).

1.4.1. Mechanisms of catecholamine action

The effects of catecholamines on their target tissues depends on the type of receptors present on the tissues. The three main classes of receptors, based on their relative affinities for various agonists and antagonists, are α_1 , α_2 and β (Ahlquist, 1948; Berthelsen & Pettinger, 1977; Langer, 1974; Lafontan et al., 1985). α_1-adrenergic effects are mediated by an increase in cytoplasmic Ca^{++} concentration whereas stimulation of α_2 and β-receptors results in a

decrease or increase respectively in cAMP levels within the target cell (for review see Exton, 1985; Levitzki, 1986).

1.4.1.1. β-Mediated catecholamine action

β-Adrenergic receptors have been subclassed, on the basis of their relative affinities for adrenaline, noradrenaline and other agonists and antagonists, into β_1 and β_2-receptors (Lands et al., 1967; Stiles et al., 1984; Molinoff et al., 1981). β_1-receptors show approximately equal affinity for adrenaline and noradrenaline while β_2-receptors have a greater affinity for adrenaline than noradrenaline. Generally β_1-receptors are found on white adipose tissue and heart and mediate the stimulation of cardiac muscle contraction and of lipolysis in adipose tissue while agonist occupancy of β_2-receptors results in the stimulation of glycogenolysis in skeletal muscle and liver, of gluconeogenesis in liver, of insulin secretion from the pancreas and of amylase secretion from salivary glands and in the relaxation of smooth muscle (see Stiles et al., 1984).

β-adrenergic receptors are coupled to the guanine nucleotide regulatory protein N_s which consists of three subunits α, β and γ (Gilman, 1984). The α-subunit contains a binding site for guanine nucleotides and displays GTPase activity (Northup et al., 1983b). The binding of hormone to β-receptors stabilises the interaction of the receptor with N_s and promotes the exchange of GDP for GTP at the guanine nucleotide binding site (Limbird, 1984) which in turn promotes the dissociation of the α, β and γ subunits (Sternweis et al., 1981). The free α-subunit interacts directly with and stimulates adenylate cyclase resulting in an increase in cAMP

formation (Northup et al., 1983a,b). The deactivation of N_s occurs via the hydrolysis of GTP to GDP by the α-N_s GTPase activity which promotes the re-association of the α, β and γ subunits (Gilman, 1984; Houslay, 1984). The GTPase activity, and hence deactivation of the system, is stimulated by the binding of agonist to the β-receptor (Cerione et al., 1984). Non-hydrolysable analogues of GTP bind to α-N_s and maintain its dissociation resulting in the persistent activation of the system (Northup et al., 1983a) and cholera toxin promotes NAD-dependent ADP-ribosylation of α-N_s, which stimulates the exchange of GDP for GTP and inhibits the GTPase activity, also resulting in the persistent activation of N_s (Northup et al., 1980; Hanski et al., 1981; Sternweis et al., 1981).

1.4.1.2. α_2-Mediated catecholamine action

α_2-Adrenergic agonists generally stimulate aggregation of platelets and contraction of vascular smooth muscle and inhibit lipolysis in white adipose tissue, insulin release from the pancreas, and renin release from the kidney (see Exton, 1985; Lafontan et al., 1985). α_2-adrenergic receptors are coupled to the guanine nucleotide binding protein N_i which consists of a β and a γ subunit, which are identical to the corresponding subunits of N_s, and a distinct α-subunit (Bokoch et al., 1984; Codina et al., 1984). As in N_s the α-subunit of N_i contains a guanine nucleotide binding site and shows GTPase activity (Bokoch et al., 1984). Occupancy of the α_2-receptor with agonist stabilises the interactions of the receptor with N_i and promotes GTP binding which results in the dissociation of the α_i, β and γ subunits (Katada et al., 1984a,b; Kaibuchi et al., 1981). This leads to the inhibition of adenylate cyclase and hence a reduction in

intracellular cAMP production. The precise mechanism by which the dissociation of N_i leads to the inhibition of adenylate cyclase is still debatable (Codina et al., 1983; Northup et al., 1983a,b) but is attributable either to a direct inhibitory effect of α-N_i on adenylate cyclase or to free β-N_i promoting the deactivation of and inhibiting the release of α-N_s (Northup et al., 1983a,b; Katada et al., 1984a,b). As for α-N_s agonist binding to N_i also stimulates the α-subunit GTPase activity which leads to the re-association of the subunits and deactivation of N_i while non-hydrolysable analogues of GTP promote the persistent activation of N_i (Bokoch et al., 1984). Pertussis toxin stimulates the NAD-dependent ADP-ribosylation of α-N_i which inhibits the release of GDP and hence blocks the dissociation of N_i and obliterates the inhibition of adenylate cyclase (Gilman, 1984; Katada et al., 1984a,b).

1.4.1.3. α_1-mediated catecholamine action

α_1-Agonists promote the contraction of various smooth muscle including that in the vascular system, iris, uterus and bladder, and also stimulate the relaxation of gastrointestinal smooth muscle, the contraction of cardiac muscle, glycolysis in the heart, glycogenolysis in the liver and adipose tissue, gluconeogenesis and Na^+ reabsorption in the kidney and secretion from salivary and sweat glands (see Exton, 1985). α_1-adrenergic responses are mediated by a rise in the cytoplasmic free Ca^{++} concentration (Exton, 1980; Charest et al., 1983, 1985) which in most smooth muscle (Deth & Van Breemen, 1974; Bolton, 1979; Bulbring et al., 1981), the liver (Charest et al., 1983; Morgan et al., 1983; Blackmore et al., 1984; Reinhart et al., 1984a,b; Prpic et al., 1984; Charest et al., 1985) and salivary

gland (Putney, 1979) is initially due to the release of Ca^{++} from internal stores, such as the sarcoplasmic reticulum and plasma membrane, and subsequently involves a change in the transport of Ca^{++} across the plasma membrane. In other tissues, such as adipose tissue, the source of Ca^{++} is unknown. Ca^{++} exerts many of its biological effects by binding to the regulatory protein calmodulin which interacts with a variety of cellular proteins and modifies their activity (see Exton, 1985). The rise in the cytoplasmic Ca^{++} concentration can be relayed to the mitochondria where it has various effects such as the stimulation of PDH activity in liver (McCormack, 1985b; McCormack & Denton, 1985).

Calcium mediated hormones affect phosphatidylinositide metabolism (Hokin & Hokin, 1953), diacylglycerol (DAG) and myo-inositol-1,4,5-trisphosphate (IP_3) which are produced through the action of phosphodiesterase on phosphatidylinositol-1,4-bisphosphate (PIP_2) act as second messengers for α_1-adrenergic agonists and other hormones (Berridge, 1983, 1984; Berridge & Irvine, 1984). IP_3 promotes calcium release from intracellular stores in a range of cells including pancreatic acinar cells (Streb et al., 1983) neutrophils (Lapetina, 1983) vascular smooth muscle cells (Surmatou et al., 1984) and hepatocytes (Joseph et al., 1984). DAG stimulates the plasma membrane bound protein kinase C by lowering the concentration of Ca^{++} required to activate it (Takai et al., 1979; Kishimoto et al., 1980; Nishiazuku, 1984). Protein kinase C is also activated by tumor promoting phorbol esters which have a somewhat analogous structure to DAG (Castagna et al., 1982). Phospholipids are also required for protein kinase C activity, with

phosphatidylserine being the most effective (Takai et al., 1979; Kaibuchi et al., 1981) and therefore the enzyme probably only functions when membrane bound. Despite this protein kinase C is capable of phosphorylating myosin light chains located in the cytoplasm (Naka et al., 1983) and some unidentified cytoplasmic proteins in liver (Garrison et al., 1984); other cellular substrates for the enzyme are yet to be defined (see Exton, 1985).

It is not known how the binding of α_1-agonists to receptors in the plasma membrane is coupled to the PIP_2 phosphodiesterase. Agonist binding may simply induce a conformational change in the receptor which leads to perturbation of the membrane and results in PIP_2 becoming more accessible to the phosphodiesterase (Irvine et al., 1984). However evidence is emerging that the α_1-receptor may be coupled to a guanine nucleotide regulatory protein (see Joseph, 1985): guanine nucleotides decrease the affinity of receptors for some α_1-adrenergic agonists (Cantau et al., 1980; Koo et al., 1983) and pertussis toxin inhibits agonist-dependent production of inositol phosphates in mast and HL-60 cells (Molski et al., 1984; Okajima & Ui, 1984; Nakamura & Ui, 1985) suggesting that receptors for α_1-agonists are coupled to a guanine nucleotide regulatory protein which, similarly to N_i, is a substrate for pertussis toxin. It is not clear whether or not this protein is N_i but in chick heart and astrocytoma cells the concentration of pertussis toxin required to inhibit muscarinic-receptor mediated inositol phosphate production is much higher than that required to deactivate N_i suggesting that a separate, as yet unidentified, guanine nucleotide regulatory protein

is involved in the muscarinic response (Joseph, 1985). Further support to the involvement of a guanine nucleotide regulatory protein in the coupling of agonist binding to inositol phosphate metabolism is the observation that non-hydrolysable analogues of GTP stimulate inositol phosphate metabolism in isolated neutrophil plasma membranes (Lockhart & Gomperts, 1985) in a cell-free system from insect salivary glands (Litosch et al., 1985) and in platelets (Haslam & Davidson, 1984).

1.4.2. Variations in catecholamine levels with physiological state

An increase in plasma catecholamine levels in rats is induced by various types of stress including handling and immobilisation (Popper et al., 1977; Kvetnansky et al., 1978; McCarty & Kopin, 1978a; Benedict et al., 1979); the anticipation of stress can result in similar increases (McCarty & Kopin, 1978b). The response to stress differs between strains of rats (McCarty & Kopin, 1978a,b; McCarty et al., 1978) and amongst individual rats (Weick et al., 1980) and can be reduced by repeatedly subjecting rats to the stressful stimulation (Benedict et al., 1979; Kvetnansky et al., 1979).

Exposure of rats to cold results in a rise in circulating catecholamine levels (Benedict et al., 1979; Avakian & Horvath, 1980; Picotti et al., 1981) and an increase in sympathetic activity in various tissues (Landsberg & Young, 1978). During cold exposure catecholamines probably serve to mobilise fuels for increased metabolism (Young & Landsberg, 1977a), adjust blood flow (Gale, 1973) and stimulate shivering (Bulbring, 1976) and non-shivering thermogenesis (Jansky, 1973).

Feeding induces an increase in circulating catecholamine levels in humans (Welle et al., 1980; Young et al., 1980; Shetty et al., 1981), young pigs (Ingram et al., 1980) and, as reported very recently, in rats (Steffens et al., 1986). Rats are nocturnal animals which eat larger and more frequent meals at night (Le Magnen, 1984) when they are normally in positive energy balance; in contrast during the day they may be in negative energy balance (Le Magnen et al., 1973; Le Magnen & Devos, 1982, 1984). The increase in sympathetic activity during feeding in rats will probably serve to dissipate excess energy, at least partly via an increase in thermogenesis (Smith & Horwitz, 1969; Landsberg & Young, 1978; Rothwell & Stock, 1978; Rothwell & Stock, 1980; Himms-Hagen, 1983). In the rat overfeeding results in an increase in basal sympathetic activity (Landsberg & Young, 1984; Young et al., 1982) while fasting results in a decrease in basal sympathetic activity (Young & Landsberg, 1977b); these changes occur within one day of the alteration in dietary intake (Rappaport et al., 1982).

1.4.3. Control of rat adipose tissue metabolism by catecholamines

Catecholamines play a key role in ensuring that the supply of nutrients to the body tissues is adequate to meet the requirements of the physiological state of the animal. These hormones therefore adjust the metabolism of various tissues when the animal finds itself in adverse situations such as starvation, stress and exposure to low temperatures (see Barrand & Callingham, 1983).

Brown and white adipose tissue depots are the major sites of storage of body fat in the rodent and the regulation of fatty acid mobilisation from these tissues and of the dissipation of energy in

the form of heat in brown adipose tissue are essential aspects of the maintenance of energy balance within the body (Le Magnen, 1984). The effects of catecholamines on these tissues in the rat are outlined below.

1.4.3.1. White adipose tissue

Catecholamine effects on rat white adipose tissue metabolism include the stimulation of lipolysis (Turtle & Kipnis, 1967; Himms-Hagen, 1970; Burns & Langley, 1971; Khoo et al., 1973) and long chain fatty acid transport across the plasma membrane (Abumrad et al., 1985), which together increases the net release of fatty acids from the tissue, the inhibition of lipogenesis (Katz et al., 1966; Saggerson & Greenbaum, 1970; Saggerson, 1972a; Smith & Saggerson, 1978) and the stimulation of glucose uptake and glycolytic flux (Katz et al., 1966; Halperin & Denton, 1969; Saggerson & Greenbaum, 1970) the latter being at least partly due to the activation of phosphofructokinase (Sale & Denton, 1985).

The rat white adipocyte possesses a homogenous population of β-receptors which show atypical characteristics and can not be classified as β_1 or β_2 (Harms et al., 1974, 1977; Belfrage & Fredholm, 1978; de Vente et al., 1980).

Some controversy exists as to whether or not rat white adipocytes possess α-receptors (see Lafontan et al., 1985). Direct binding studies have found that the rat adipocyte binds α-agonists very weakly and that this binding does not display the classical characteristics expected of α_1 or α_2-receptors (Dax et al., 1981; Guidicelli et al., 1981; Carpene et al., 1983). In keeping with these results Burns & Langley (1971) could not detect any α_1 or

α_2-mediated effect upon lipolysis in rat white adipocytes under conditions which allowed such effects to be observed in human white adipocytes and this along with the binding studies has lead to the conclusion that rat white adipocytes possess no functional α-receptors. However α_1-mediated effects on phosphatidylinositol turnover (Garcia-Sainz & Fain, 1980) glycogen sythase activity (Lawrence & larner, 1977, 1978) and PDH activity (Cheng & Larner, 1985) in the rat white adipocyte have been reported.

1.4.3.2. Brown adipose tissue

It is now emerging that brown adipose tissue has an essential role in regulating energy balance in the body (James & Trayhurn, 1976; Landsberg & Young, 1978; Rothwell & Stock, 1980; Himms-Hagen, 1983; Bukowiecki, 1985; Trayhurn, 1985). In the rat the tissue possesses β_1 and β_2-adrenergic receptors (Rothwell et al., 1985) and α_1-receptors (Fink & Williams, 1976; Flaim et al., 1977; Girardier & Schneider-Picard, 1983; Foster, 1985). β-receptors are the primary mediators of non-shivering and diet induced thermogenesis (Budowiecki et al., 1980; Nedergaard & Lindberg, 1982; Rothwell et al., 1982; Mohell et al., 1983) while α_1-adrenergic receptors play an auxiliary role and potentiate the β-adrenergic activation of thermogenesis (Foster, 1984; Ma & Foster, 1984; Foster, 1985).

Catecholamines inhibit lipogenesis and ACC activity in brown adipose tissue yet stimulate PDH activity: this suggests that glucose oxidation is important during thermogenesis (Gibbins et al., 1985).

1.5 Aims of the project

As described in section 1.1.2. glucose metabolism is altered during lactation. The aims of this project were to characterise changes in PDH activity and its endocrine control in different tissues which have a major role in glucose metabolism in the lactating rat.

White adipose tissue received the most attention as a clear cut change was found in the insulin responsiveness of this tissue during lactation. During the course of this study it also became apparent that noradrenaline stimulated white adipose tissue PDH activity in rats. This finding is contrary to the results of many published studies *in vitro* and therefore this effect of noradrenaline was further characterised.

CHAPTER 2

2.1. Chemicals

All agents were obtained from Sigma Chemical Co., Poole, Dorset or Boehringer Corp., Ltd., Lewes, East Sussex, unless otherwise stated. Insulin was from Sigma Chemical Co. unless otherwise stated.

2.2. Animals

2.2.1. Rats

Female and male wistar rats from A. Tuck and Son (Rayleigh, Essex) were given Labsure irradiated CRM diet, comprising 570g of carbohydrate, 180g of crude protein, 24g of crude oil and 36g of crude fibre per kg (Labsure, Poole, Dorset, UK) and water ad libitum. Rats were maintained on a 12h light-dark cycle (light phase from 08.00-12.00h) except for one group of female virgin and lactating rats (see chapter 5) which were maintained on a reversed cycle (i.e. darkness between 08.00 and 20.00h) for 4 weeks before use. Female rats were mated from about 10 weeks of age and the day a vaginal plug was found was designated day 0 of pregnancy. The number of pups per mother was adjusted to 8 within 24h after birth. Rats were used at various stages of pregnancy and lactation or at 2 days after litter removal following 14 days lactation. Pregnant rats with less than 8 foetuses were not used. All female rats, including virgin rats, were 13-16 weeks old at time of use. Male rats weighed 200-250g at time of use. Rats were either killed by cervical dislocation or tissue was removed under pentobarbital anaesthesia as stated in the results chapters 3-6. Experiments were carried out between 10.00h and 11.00h

or 12.00h and 13.00h also as specified in the results chapters.

2.2.2. Sheep

All sheep were mature 4 year-old Finn x Dorset Horn cross-bred ewes at about day 18 of lactation. The animals were given hay and water ad libitum plus 1400g per day of a cereal mix. The cereal mix was fed as two meals at approximately 07.00h and 16.30h. Sheep were first anaesthetised by an intrajugular injection of 20ml of Sagatal (given by Dr R.G. Vernon). The animals were bled when unconscious and tissue was removed as quickly as possible.

2.3. Assay of pyruvate dehydrogenase activity

2.3.1. Preparation of tissue extracts

Frozen tissue was powdered in liquid nitrogen using a mortar and pestle. Weighed portions of this powder were homogenised at 4°C in extraction medium containing 100mM KH_2PO_4, 2mM EDTA, 1mM DTT, 1% triton at pH 7.3 using an Ultra-Turrax homogeniser driven at 70% of maximum speed for 30 sec. The homogenate was frozen in liquid nitrogen thawed, then centrifuged in an Eppendorf centrifuge for 30s (0-4°C). The infranatant below the floating fat cake was then removed and assayed. The protein concentration was determined in homogenates by the method of Lowry et al. (1951) as modified by Wang & Smith (1975) using BSA as a standard.

2.3.2. Pyruvate dehydrogenase assay

PDH activity in homogenates prepared from frozen tissue (section 2.3.1.) or isolated adipocytes (section 2.8.1.) was measured as described by Stansbie et al. (1976b) except that the pyruvate and Mg^{++} concentrations in the assay medium were increased to 3mM and 2mM respectively and 6mM pyruvate was added during the activation step prior to measurement of total PDH activity. The acetyl-CoA formed by

the PDH reaction was coupled to the dye p-(p-aminophenylazo) benzene sulphonic acid (AABS) by an arylamine acetyltransferase (AAT) prepared from pigeon livers (see section 2.3.3.) and the reduction in extinction at 460nm was followed. The AABS used was a kind gift from Dr R.M. Denton, Department of Biochemistry, University of Bristol, Bristol. All PDH assays were carried out at 30°C. The reduction in extinction at 460nm is 6.5 for a millimolar solution and 1cm light path.

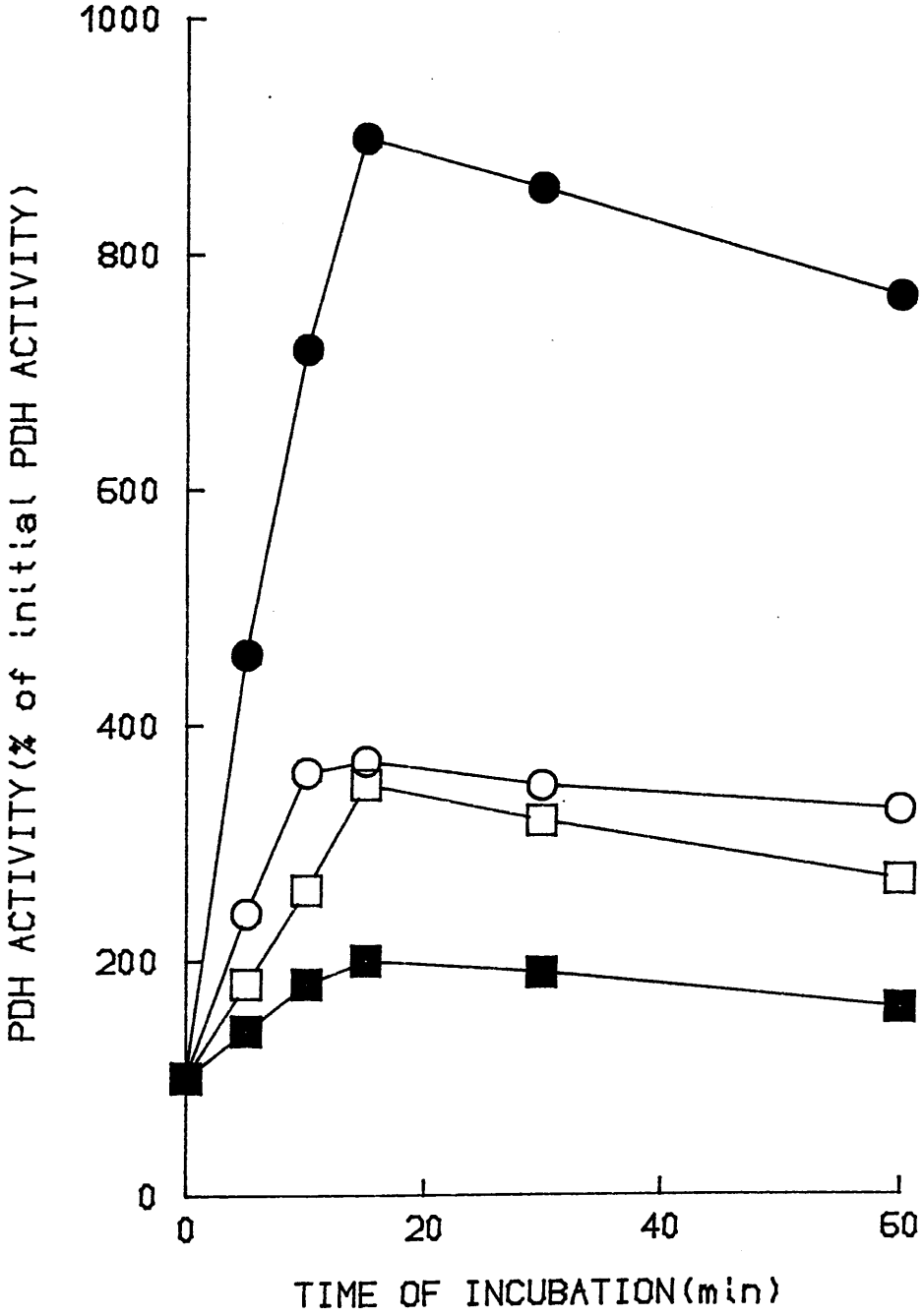
The enzyme activity measured immediately after thawing the homogenates was the initial activity and should reflect the amount of non-phosphorylated form of PDH in the extracts. Samples were incubated at 30°C for 3 min in a total volume of 1.0ml in the presence of 100mM Tris-HCl, pH 7.8, 0.5mM EDTA, 2mM $MgSO_4$, AABS (20μg/ml) and 5mM mercaptoethanol. The reaction was then initiated by addition of 20μl of substrates to give final concentrations in the assay medium of 1.5mM thiamine pyrophosphate, 0.7mM NAD, 3mM pyruvate and 0.2mM CoA.

The total amount of PDH present in the extracts was assayed after converting all of the enzyme to the dephosphorylated (active) form by incubating the homogenate at 30°C for 15 min with 50μl PDH-phosphatase, 20mM $MgCl_2$, 1mM $CaCl_2$ and 6mM pyruvate in a total volume of 150μl. The preparation of PDH-phosphatase is described in section 2.3.4. It was found that 15 min was sufficient to achieve maximal activation of PDH by PDH-phoshatase and ions in all tissues studied (fig. 2.1.). Assay medium (850μl) was then added to give final concentrations of 100mM Tris-HCl, pH 7.8, 0.5mM EDTA, 20μg/ml AABS and 5mM mercaptoethanol. The incubations were continued for 3 min at 30°C then the reaction was initiated by the addition of 20μl

Fig. 2.1. Time course of activation of pyruvate dehydrogenase in rat tissues

Skeletal muscle (●), liver (□), mammary (■) and adipose tissue (○) extracts were incubated with PDH-phosphatase and divalent cations, as described in the text, for the times indicated then assayed for PDH activity.

FIG. 2. 1.


1000
800
600
400
200
0
PDH ACTIVITY(% of initial PDH ACTIVITY)
0
20
40
60
TIME OF INCUBATION(min)

substrates to give final concentrations in the assay of 1.5mM thiamine pyrophosphate, 0.7mM NAD, 3mM pyruvate and 0.2mM CoA.

Background values were obtained by performing the assay exactly as above but in the absence of homogenate. When the assay was performed as described above with homogenate but in the absence of either NAD, thiamine pyrophosphate, pyruvate or CoA no reduction in extinction at 460nm occurred.

PDH activity is expressed in units, one unit being the amount of enzyme that produces 1μmol of acetyl-CoA per min under the assay conditions.

2.3.3. Preparation of pigeon liver arylamine acetyltransferase

Arylamine acetyltransferase (AAT) was prepared from pigeon liver acetone powder (Sigma Chemical Co., Poole, Dorset) by the method of Tabor et al. (1953). The pigeon liver acetone powder was homogenised at 4°C in distilled water (10ml water per 1g powder) containing 0.125mg EDTA/ml using an Ultra-Turrax homogeniser with its thyristor control set at 70% of maximum for 1 min. The homogenate was centrifuged for 15 min at 19,000g at 4°C and the supernatant collected.

Acetone (analytic reagent grade from FSA Laboratory Supplies, Bishop Meadow Road, Loughborough, Leics.) was added dropwise to the supernatant. A new bottle of acetone was used for each preparation of AAT and the acetone was stored at -20°C. Ten ml of acetone was added per 15ml of supernatant which was stirring in an ice bath. When approximately 10% of the acetone had been added solid NH_4Cl was sprinkled over the ice to make a freezing mixture. When all the acetone was added the sample was centrifuged for 15 min at 19,000g at -10°C and the supernatant was collected.

Acetone (7ml acetone per 17ml supernatant) was added dropwise to the supernatant while stirring in an ice bath which had NH_4Cl sprinkled over the ice to make a freezing mixture. The supernatant was centrifuged at 19,000g at -10°C for 15 min. The supernatant was discarded and the pellet suspended in a small volume (approximately 2ml) of 10mM KH_2PO_4, pH 7.5, and dialysed against 1L of the same buffer overnight at 4°C. The dialysate was centrifuged in an Eppendorf centrifuge for 2 min and the supernatant was then assayed for AAT activity. The activity was determined as for PDH activity (see section 2.3.2.) with 2μl of the AAT preparation replacing tissue extract and 10μl of 10mM acetyl-CoA used as the substrate. The AAT preparation was diluted to 4-5 units/ml and stored at -20°C in 250μl aliquots. One unit is the amount of enzyme which catalyses the acetylation of 1μl of AABS per minute.

2.3.4. Preparation of pyruvate dehydrogenase phosphatase from pig heart

Pig heart phosphatase was prepared as described by Siess & Wieland (1972).

Pig hearts were obtained from the abattoir (J. Craig Abattoir, Ayr) as soon as possible after death and transported to the laboratory on ice. The upper chambers and blood vessels were discarded and the ventricles chopped into small pieces. Ventricles from 8 hearts were homogenised in a Waring blender for 30s in 30mM KH_2PO_4, pH 7.6, containing 1mM EDTA and 5mM mercaptoethanol (approximately 100ml buffer/heart). The homogenate was adjusted to approximately pH 6.5 with 10m KOH and centrifuged at 2,000g for 20 min at 4°C. The supernatant was filtered through cheesecloth and kept at 4°C while the pellet was re-extracted as above. The two

supernatants were combined and adjusted to pH 5.4 with 10% acetic acid (v/v).

The mitochondrial fraction was collected by centrifugation at 20,000g for 20 min at 4°C. The mitochondrial pellet was washed with distilled water (approximately 50ml, 4°C) and centrifuged as before. The washed mitochondria were resuspended in a minimum volume (approx. 25ml) of 20mM KH_2PO_4 pH 7.0 containing 5mM mercaptoethanol and adjusted to pH 7.0 with 5M KOH. The mitochondrial suspension was frozen in liquid nitrogen and thawed three times in pyrex round bottomed flasks. The suspension was then centrifuged for 2h at 15,000g at 4°C.

The supernatant was removed, warmed to 30°C, and solid $MgCl_2$ was added (final concentration 10mM $MgCl_2$) and the preparation was incubated for 20 min at 30°C. The preparation was then cooled to 0°C and solid $(NH_4)_2SO_4$ (209g/l) was added over 30 min with constant stirring. The precipitate was collected by centrifugation for 20 min at 12,000g at 4°C, redissolved in a minimum volume (approx. 20ml) Of 20mM KH_2PO_4, pH 7.0 containing 5mM mercaptoethanol and dialysed overnight at 4°C against the same buffer.

The dialysate was adjusted to pH 6.1 using 10% acetic acid and centrifuged for 40 min at 19,000g at 4°C. The supernatant was adjusted to pH 7.0 with 5M KOH and stored in 1ml aliquots at -20°C.

Prior to use the preparation was centrifuged for 90 min at 180,000g at 4°C to remove the PDH complex and leave the PDH-phosphatase in the supernatant. The supernatant was checked for PDH activity by assaying as described in section 2.3.2. and if no PDH activity was found, was then either used immediately or stored frozen at -20°C for up to 1 week.

2.4. Extraction and assay of acetyl-CoA carboxylase activity

ACC was assayed by the incorporation of [^{14}C]bicarbonate into malonyl-CoA as described by Halestrap & Denton (1973) with some modifications. Frozen adipose tissue was powdered in liquid nitrogen using a mortar and pestle. Weighed portions of this powder were homogenised at 4°C in 5 volumes of extraction medium containing 30mM Tris-HCl, pH 7.4, 300mM sucrose, 1mM EDTA and 1mM reduced glutathione, using an Ultra-Turrax homogeniser driven at 70% of maximum speed for 30s. The extracts were centrifuged in an Eppendorf centrifuge for 1 min at 4°C and the infranatant below the floating fat cake removed for assay of ACC activity. The enzyme activity obtained when the extract (150μl) was assayed immediately in the absence of citrate is termed the initial activity. The incubation mix contained (final concentrations): 20mM Tris-HCl, pH 7.4, 20mM $MgCl_2$, 0.2mM EDTA, 2.5mg/ml fatty acid-free BSA, 1mM reduced glutathione, 5mM ATP, 0.2mM acetyl-CoA and 6.25mM (1.25μCi) $NaH^{14}CO_3$ (Amersham International PLC, White Lion Road, Amersham, Bucks. England). The assay was carried out at 37°C in a final volume of 1.0ml and was terminated after 90sec by the addition of 0.1ml of 6M HCl.

Total ACC activity was measured after pre-incubation of a sample of infranatant (150μl) for 30 min at 37°C with 20mM Tris-HCl, pH 7.4, 20mM $MgCl_2$, 0.2mM EDTA, 2.5mg/ml BSA, 1mM reduced glutathione and 20mM citrate in a total volume of 500μl. The assay was then initiated by the addition of 500μl substrates to give final concentrations in the assay of 5mM ATP, 0.2mM acetyl-CoA and 6.25mM (1.25μCi) $NaH^{14}CO_3$ (Amersham International PLC, White Lion Road, Amersham, Bucks., England). The assay was carried out at 37°C and

was terminated after 90s by the addition of 0.1ml of 6M HCl.

After termination of the assay with HCl, samples were heated in scintillation vials at 65°C for 1h 15 min. Ten ml of emulsifier scintillator 299 (Packard Instruments, Caversham, Berks.) was then added to each vial and the acid stable ^{14}C radioactivity was measured using a Packard (Tri-Carb 2405, Packard Instruments, Caversham, Berks.) or LKB (Rackbeta 1215, LKB Instruments, Ltd., South Croydon, Surrey) liquid scintillation counter. Background values were obtained by performing the assays as described for the measurements of initial and total ACC activity but with extraction medium replacing the samples or in the absence of acetyl-CoA.

The enzyme activity is expressed in units, one unit being the amount of enzyme that catalyses the conversion of 1μmol of acetyl-CoA per min under the assay conditions.

2.5. Studies in vivo

To minimise stress effects rats were handled regularly for 5-6 days prior to the experiment. All agents were dissolved in 0.15M NaCl unless otherwise stated and were injected intraperitoneally with fine needles (23 or 26 gauge) and minimum stress. Experiments were carried out between 10.00 and 11.00h or between 12.00 and 13.00h as specified in chapters 4 and 5. Rats rarely showed any obvious sign of stress during the experiments except when injected with prazosin, yohimbine or streptozotocin.

The experimental procedures are shown in fig.s 2.2. and 2.3. Rats were anaesthetised with pentobarbital (60mg/kg body weight) and remained so throughout the experiment. While under anaesthesia rats were kept warm with a lamp. Propranolol was routinely injected along with the anaesthetic, to minimise further any stress effects (fig. 2.2.) except when the rats were subsequently injected

Figure 2.2.

PROCEDURE FOR THE IN VIVO TREATMENT OF RATS WITH INSULIN AND GLUCOSE

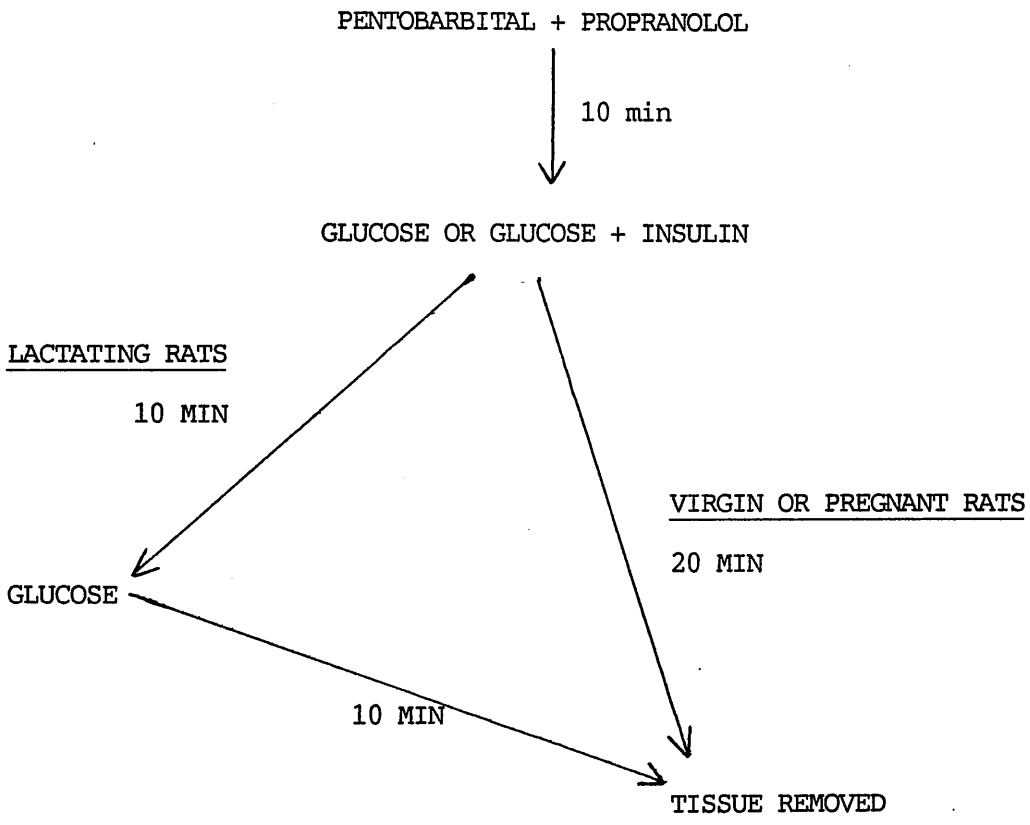
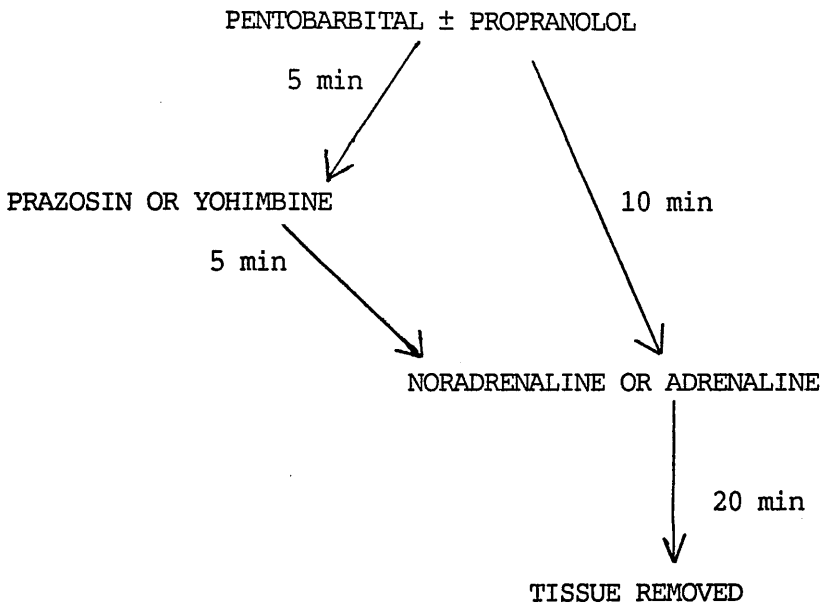


Figure 2.3.

PROCEDURE FOR THE IN VIVO TREATMENT OF RATS WITH ADRENERGIC AGONISTS AND ANTAGONISTS



with noradrenaline or adrenaline. Ten minutes after the anaesthetic rats were injected with either 0.15M NaCl, glucose (1g/kg body wt.) either alone or with insulin (0.4mg in 0.4ml 0.15M NaCl per rat), noradrenaline (1.25mg/kg body wt.) or adrenaline (1.25mg/kg body wt.) and after a further 20 min samples of white adipose tissue (parametrial or epididymal) liver, mammary gland and skeletal muscle (gastrocnemius and biceps femoris from the hind-limb) were removed and immediately frozen in liquid N_2 for subsequent extraction and assay of PDH (see section 2.3.). Samples of blood were also taken from the heart while additional samples of white adipose tissue were taken and stored in isotonic saline at 37°C for determination of cellularity as described in section 2.8.2. Lactating rats were injected with a second, identical, dose of glucose 10 min after the first (fig. 2.2.). When the effects of adrenergic antagonists (propranolol, prazosin and yohimbine) on the actions of adrenaline and noradrenaline were investigated propranolol (1.5mg/kg body wt.) was injected along with the anaesthetic whereas yohimbine (5mg/kg body wt.) or prazosin (5mg/kg body wt.) were injected 5 min after the anaesthetic (fig. 2.3.) as injection of these agents caused obvious stress in conscious rats. Prazosin (a kind gift from Pfizer Central Research, Sandwich, Kent) was dissolved in 40mM lactic acid to give a 10mg/ml solution of the agent which was then diluted with water to give 5mg/ml prazosin.

Rats treated with streptozotocin (Upjohn Comp., Kalamazoo, Michigan, USA) were injected with the agent (100mg/kg body wt.) 19h prior to the start of *in vivo* experiments. The amount of streptozotocin that was to be given to each rat was weighed into a plastic tube then dissolved in 1ml 0.1M sodium citrate, pH 4.5,

immediately prior to the injection.

Serum was prepared from the blood samples as described in section 2.6. and stored frozen at -20°C prior to the assay of insulin (see section 2.7.) and glucose (see section 2.6.) concentrations.

2.6. Preparation of serum

Blood, was collected from the heart and then allowed to clot at room temperature for 1h. Serum was obtained from this by centrifugation at 1000g for 10 min and the glucose concentration was then measured using an Analox GM6 glucose analyser. Serum was stored at -20°C until used for the determination of insulin concentration as described in section 2.7.

2.7. Measurement of serum insulin concentration

Serum insulin concentration was determined by radioimmunoassay. The serum sample to be assayed (25μl or 50μl) was incubated in 50mM Na_2HPO_4 buffer, pH 7.4, containing 0.15M NaCl, 15mM NaN_3, 5mg/ml BSA (RIA grade, fraction V, Sigma Chemical Co., Poole, Dorset) 16mg/ml polyethylene glycol, 12mM EDTA and 100μl of a 1:10 dilution of anti-insulin serum (raised to bovine insulin in guinea pigs by Dr D.J. Flint) at 4°C for 20h in a total volume of 300μl. Five nCi (0.1mg) ^{125}I-insulin was then added in a volume of 100μl and the incubations were continued at 4°C. Porcine insulin (Sigma Chemical Co., Poole, Dorset) was iodinated by Dr D.J. Flint using the lactoperoxidase method (see Morrison & Bayse, 1970). After 20h 300μl of anti-guinea pig precipitating serum (Scottish Antibody Production Unit at Glasgow and West of Scotland Blood Transfusion Service, Law Hospital, Carluke, Lanarkshire, Scotland) was used. The samples were incubated at room temperature for 5h then centrifuged at 3000g for 30 min. The supernatant was removed then the pellet was

counted in a gamma counter (Rackgamma 11, LKB Instruments, Ltd., Selsdon, Surrey). The insulin content of the pellet was determined using a standard curve which was obtained by performing the assay as described above but with a range of concentrations of porcine insulin (0-1000ng/ml) replacing the serum sample. Blank values were obtained by performing the assay with 50mM Na_2HPO_4 buffer replacing the anti-insulin serum.

2.8. Studies in vitro

2.8.1. Studies with isolated adipocytes

Parametrial white adipose tissue was removed from rats under pentobarbital anaesthesia (60mg/kg body wt. injected intraperitoneally as described in section 2.5.) and adipocytes were prepared by the method of Rodbell, (1964) with modifications. The procedure for the preparation and incubation of isolated adipocytes is shown in fig. 2.4. The fat pads from one rat were minced finely then incubated in 10ml Krebs-Ringer bicarbonate buffer pH 7.3, (Krebs & Henseleit, 1932) but containing 1.25mM calcium (i.e. half the original concentration of calcium) and with 5mM glucose, 40mg/ml dialysed fatty acid-free albumin (all albumin used was fraction V, essentially fatty acid-free from Sigma Chemical Co., Poole, Dorset and was dialysed before use as described in section 2.8.4.), 1mg/ml collagenase (Type II, Sigma Chemical Co., Poole, Dorset) and 25mM Hepes and gassed with O_2:CO_2 (95:5). Incubations were carried out for 1h at 37°C in stoppered flasks shaking at 180 oscillations per minute. The resulting cell suspension was filtered through a nylon mesh (about 1mM diameter) to remove undigested material and was then washed three times with 30ml of Krebs-Ringer bicarbonate buffer, pH 7.3 (containing 1.25mM calcium, 5mM glucose, 40mg/ml BSA and 25mM

Figure 2.4.

PROCEDURE FOR IN VITRO STUDIES USING ISOLATED ADIPOCYTES

PARAMETRIAL ADIPOSE TISSUE MINCED

↓

1hr AT 37°C IN BICARBONATE BUFFERED MEDIUM CONTAINING:

5mM	GLUCOSE
40mg/ml	BSA
1mg/ml	COLLAGENASE
25mM	HEPES
O_2:CO_2	95:5

↓

CELL SUSPENSION FILTERED THROUGH NYLON MESH
THEN WASHED THREE TIMES

↓

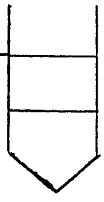
ALIQUOTS OF CELLS INCUBATED AT 37°C WITH AGENTS

↓

ALIQUOTS OF INCUBATION LAYERED ON DINONYL PTHALATE OIL
AND CENTRIFUGED

↓

CUT WITH SCALPEL



TUBES FROZEN IN LIQUID N_2 THEN
CUT WITH SCALPEL

↓

CELL BAND STORED IN LIQUID N_2 PRIOR TO EXTRACTION OF PDH

hepes). Aliquots (300μl) of packed cell suspensions were incubated in Krebs-Ringer bicarbonate buffer, pH 7.3, (containing 1.25mM calcium) in the presence of agents as described in chapters 4-6 in a final volume of 1.3ml. All incubations were carried out in the presence of 40mg/ml dialysed fatty acid-free BSA (see section 2.8.4.), 5mM glucose and 25mM hepes at 37°C in stoppered plastic tubes shaking at 80 oscillations per minute. When adrenergic agonists (adrenaline, methoxamine and isoproterenol) or antagonists (prazosin, yohimbine and propranolol) were used these agents were made up just prior to the start of the incubations and the tubes were wrapped in tin foil throughout the incubations. Prazosin (a kind gift from Pfizer Central Research, Sandwich, Kent) was made up in 40mM lactic acid (to give a solution containing 10mg/ml prazosin) and was then diluted in the Krebs-ringer bicarbonate medium used in the incubations. All other agents were made up in the incubation medium. After incubation cells were separated from the media by layering 250μl aliquots on 100μl of dinonyl pthalate and centrifugation (60s) in an Eppendorf centrifuge. The tubes were frozen in liquid N_2 then the cell band was removed by cutting the tubes with a scalpel (fig. 2.4.). The cell band was frozen in liquid N_2 prior to extraction as described for whole adipose tissue (section 2.3.1.) for subsequent assay of PDH activity as described in section 2.3.2.

2.8.2. Determination of adipocyte volume and number of adipocytes per gram tissue

Cells were prepared and washed as described in section 2.8.1. Samples (5μl) of the cell suspension were transferred to a warm haemocytometer and the diameter of 100 cells was measured using a Projectina microscope at 145 x magnification. The mean cell volume

was then calculated as described by di Girolamo et al. (1971). An estimate of the number of cells per gram tissue was obtained by dividing the total lipid content of the tissue by the average lipid content of the fat cells as described by Di Girolamo et al. (1971) except that the lipid content of adipose tissue was taken to be equal to the dry weight of the tissue. The average lipid content of the fat cells was derived by mean cell volume x density of lipid (density of lipid was taken to be 1.1.).

2.8.3. Studies on adipose tissue pieces

Parametrial fat pads were removed from rats under pentobarbital anaesthesia (60mg/kg body wt., I.P.) and were washed in incubation medium at 37°C. The incubation medium was Krebs-ringer bicarbonate buffer, pH 7.3, (Krebs & Henseleit, 1932) but containing 1.25mM Ca^{++} (half the original calcium concentration), 5mM glucose, 25mM hepes, 40mg/ml BSA (prepared as described in section 2.8.4.) and gassed with O_2:CO_2 (95:5). All subsequent steps were carried out at 37°C. The fat pads from one rat were excised and pooled then cut with scissors into small pieces of tissue (about 5mg). Samples of these adipose tissue pieces (total wt. about 50mg) were pre-incubated in pyrex flasks in 5ml incubation medium at 37°C for 30 min with gentle shaking (80 oscillations/min). Insulin was then added and the incubations were continued at 37°C with gentle shaking (80 oscillations/min). After 20 min the tissue pieces were removed and rapidly blotted on filter paper (Whatman no. 1) then frozen in liquid N_2. Adipose tissue pieces were stored frozen in liquid N_2 for up to 24h prior to the extraction and assay of PDH activity as described in section 2.3.

2.8.4. Preparation of bovine serum albumin

All bovine serum albumin used, except in the measurement of serum insulin concentration (see section 2.7.), was fraction V, essentially fatty acid-free from Sigma Chemical Co., Poole, Dorset and was dialysed prior to use as described by Hanson & Ballard, (1968). A 10% solution of the albumin was dialysed over 3 days against 0.9% NaCl at 4°C. The 0.9% NaCl was changed 5 times over the three days. The albumin was finally dialysed for at least 6h against distilled H_2O to remove NaCl. The dialysed solution was lyophilised and the dry powder stored at 4°C until required.

For the measurement of serum insulin concentration (section 2.7.) RIA grade, fraction V BSA from Sigma Chemical Co., Poole, Dorset, was used.

2.8.5. Studies with subcellular fractions

2.8.5.1. Preparation of adipocyte mitochondria and plasma membrane enriched fractions

Adipocytes were prepared by collagenase digestion of rat parametrial white adipose tissue as described in section 2.8.1. Plasma membrane and mitochondria fractions were prepared as described by Seals & Czech (1981a) with some modifications. The adipocytes obtained from the fat pads of 3-4 rats were washed (see section 2.8.1.) then resuspended in 5-10ml of extraction medium (10mM potassium phosphate, pH 7.4, containing 0.25M sucrose). This suspension was vortexed vigorously for 1 min at room temperature and then centrifuged at 3000g for 15 min at 25°C. The infranatant and pellet were removed onto ice and all subsequent steps were carried out at 4°C. The pellet was resuspended in the infranatant and centrifuged at 20,000g for 30min The pellet from this spin was resuspended in 2ml extraction medium then layered over 12ml of 32% sucrose (made up in

10mM potassium phosphate buffer, pH 7.4) and centrifuged at 100,000g for 1hr. The plasma membranes were collected from the top of the sucrose layer and diluted with about 12ml extraction medium and the mitochondrial pellet was recovered from the bottom of the sucrose layer and resuspended in about 12ml extraction medium. Both fractions were then pelleted by centrifugation at 28,000g for 15 min and the pellets obtained were resuspended in 1ml of 50mM potassium phosphate buffer pH 7.4. The plasma membrane and mitochondria fractions were stored in 100μl aliquots in liquid N_2 for up to 1 week. The protein content of the subcellular fractions was measured by the method of Bradford (1976) using BSA as a standard.

2.8.5.2. Preparation of plasma membrane enriched fraction from mammary tissue

Mammary plasma membranes were prepared essentially as described by Clegg (1981) except that the extraction medium used was 10mM potassium phosphate buffer, pH 7.4; containing 0.25M sucrose. Mammary tissue was removed from 12-14 day lactating rats under pentobarbital anaesthesia (see section 2.5.). The tissue collected from 2 rats was placed in ice-cold extraction medium (approximately 25ml medium per 10g tissue) and all subsequent steps were carried out at 4°C. The tissue was finely chopped with scissors and then strained from the medium which was then replaced. The tissue was agitated in this fresh medium for 2-3 min and then again strained and the operation repeated once more. Fresh extraction medium was added to the tissue (about 25ml medium per 10g tissue) and this suspension was homogenised for 30 sec using an Ultra-Turrax homogeniser with its thyristor control set at 60% of maximum. The homogenate was strained through 6 layers of cheesecloth and then centrifuged at 80g for 10

min. The supernatant was centrifuged at 3,000g for 10 min and the supernatant from this spin was then centrifuged at 105,000g for 45 min and the pellet was resuspended in about 5ml of 40% sucrose and dispensed into 2 tubes. Five ml of each of 32% and 0.9% sucrose were overlayed onto the sample and the tubes were centrifuged at 100,000g for 2h. All sucrose solutions were made up in 10mM potassium phosphate buffer, pH 7.4.

The membrane fractions were removed from between the 0.9% and 32% sucrose layers, pooled and then diluted with about 12ml of 50mM potassium phosphate buffer, pH 7.4. The membranes were then pelleted by centrifugation at 100,000g for 45 min. The pellet was resuspended in 2ml of 50mM potassium phosphate buffer, pH 7.4, and stored in 100μl aliquots in liquid N_2 for up to 1 week. The protein content of the subcellular fractions was measured as described by Bradford (1976) using BSA as a standard.

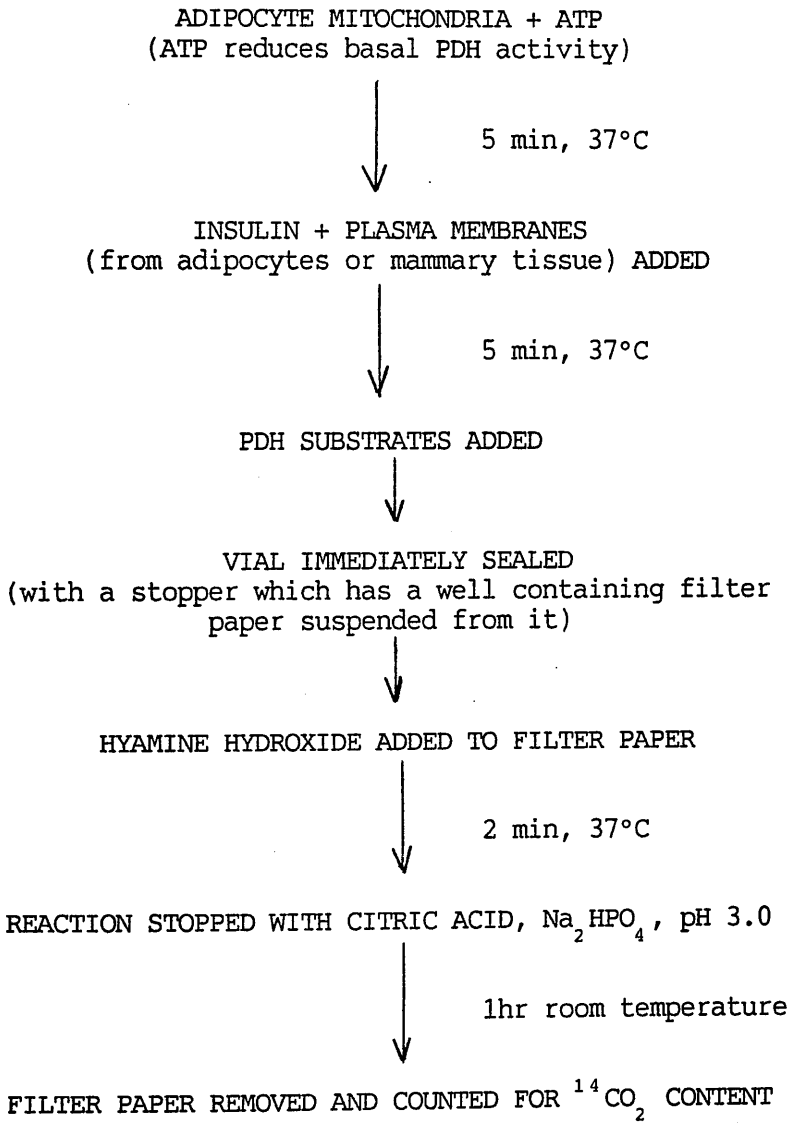
Plasma membrane fractions were prepared from sheep mammary tissue exactly as described above for rat mammary tissue.

2.8.5.3. Generation and assay of unidentified mediator substance/s from plasma membranes

The method of Seals & Jarett (1980) was used with some modifications. The procedure used is shown in fig. 2.5. Rat adipocyte mitochondria (about 75μg protein) were pre-incubated in 50mM potassium phosphate buffer, pH 7.4; with 250μM ATP, 50μM $MgCl_2$ and 50μM $CaCl_2$ for 5 min at 37°C in a total volume of 400μl. Incubation of mitochondria with ATP reduced basal PDH activity. Plasma membranes (about 65μg protein or a range of concentrations) from rat adipocytes, rat mammary tissue or sheep mammary tissue were then added along with insulin (100μunit/ml final concentration or a

Figure 2.5.

ACTIVATION OF PYRUVATE DEHYDROGENASE BY ADDITION OF PLASMA MEMBRANES AND INSULIN TO MITOCHONDRIA



range of concentrations) and the incubation continued for a further 5 min in a total volume of 450μl. The assay of PDH activity was then initiated by the addition of 50μl of an assay mix to give final concentrations of 50mM potassium phosphate, pH 7.4, 1mM dithiothreitol, 0.1mM coenzyme A, 0.1mM cocarboxylase and 0.25mM (1mCi/mmol) [1-^{14}C]-pyruvate (see section 2.8.5.4.) in a final volume of 500μl. The vial was immediately sealed with a rubber stopper that suspended a plastic well (Kontes Scientific Glassware/Instruments, Vineland, new Jersey 08360, USA) containing a 25 x 75mM piece of filter paper (Whatman No. 1) and 250μl of hyamine hydroxide was then added to the well via a syringe inserted through the rubber stopper. Subsequent additions were made by syringe through the rubber cap. The assay was continued for 2 min at 37°C with gentle shaking (80 oscillations/min) then stopped by adding 500μl of 0.08M citric acid, 0.04M Na_2HPO_4 buffer (pH 3.0). The filter paper was allowed to absorb $^{14}CO_2$ at room temperature for 1h. The wells containing the filter paper were then removed into 6ml of a toluene based scintillation fluid (Packard Instrument Co., Caversham, Berks.) and 4ml of methanol and the mixture was shaken vigorously. The [^{14}C] content was measured using a Packard (Tri-Carb 2405, Packard Instruments, Caversham, Berks.) or LKB (Rackbeta 1215, LKB Instruments Ltd., South Croydon, Surrey) liquid scintillation counter. The amount of $^{14}CO_2$ released was linear over at least the first 5 min of the assay (fig. 2.6.) and was proportional to the amount of mitochondria used in the range 0-100μg protein (fig. 2.7.). Background values were determined from samples that were incubated exactly as described above but without the addition of mitochondria or plasma membranes. The PDH activity associated with the plasma

Fig. 2.6. Time course of carbon dioxide release by adipocyte mitochondria during the assay of PDH activity

PDH activity was assayed, as described in the text, in incubations of mitochondria (75μg protein) prepared from adipocytes isolated from parametrial white adipose tissue from virgin rats. Mitochondria were incubated with ATP, as described in the text, prior to the assay of PDH activity.

membrane fractions was also measured in the absence of mitochondria and was consistently less than 10% of the activity in the mitochondria. The specific activity of [1-^{14}C] pyruvate in the assay was determined by measuring the radioactivity of an aliquot of the assay mix.

In a few experiments plasma membranes (40μg protein) were incubated in 50mM potassium phosphate buffer, pH 7.4, along with insulin at 37°C for 5 min in a final volume of 300μl. The samples were then centrifuged in a microfuge at 50,000g for 1 min. The supernatants were removed and tested for their ability to stimulate mitochondrial PDH activity (fig. 2.8.). After the incubation of mitochondria with ATP, as described above, 50μl of supernatant was added and the incubations continued for 5 min at 37°C with gentle shaking (80 oscillations/min). The PDH assay was then initiated, by the addition of assay mix, and performed exactly as described above for the co-incubation of plasma membranes and mitochondria. This procedure is outlined in fig. 2.8.

PDH activity is expressed in units, one unit being the amount of enzyme that produces 1μmol of $^{14}CO_2$ per min under the assay conditions.

2.8.5.4. Storage of [1-^{14}C] pyruvate

[1-^{14}C] pyruvate (Amersham International PLC, White Lion Road, Amersham, Bucks., England) was dissolved in 0.1mM HCl, pH 5.0, to give a solution containing 1μCi/125μl. This solution was snap frozen in liquid N_2 in 125μl aliquots and stored in liquid N_2 until required. To minimise blank values in the PDH assay the [1-^{14}C] pyruvate was not thawed until just prior to use and was then kept on ice until addition to samples.

FIG.2.6.

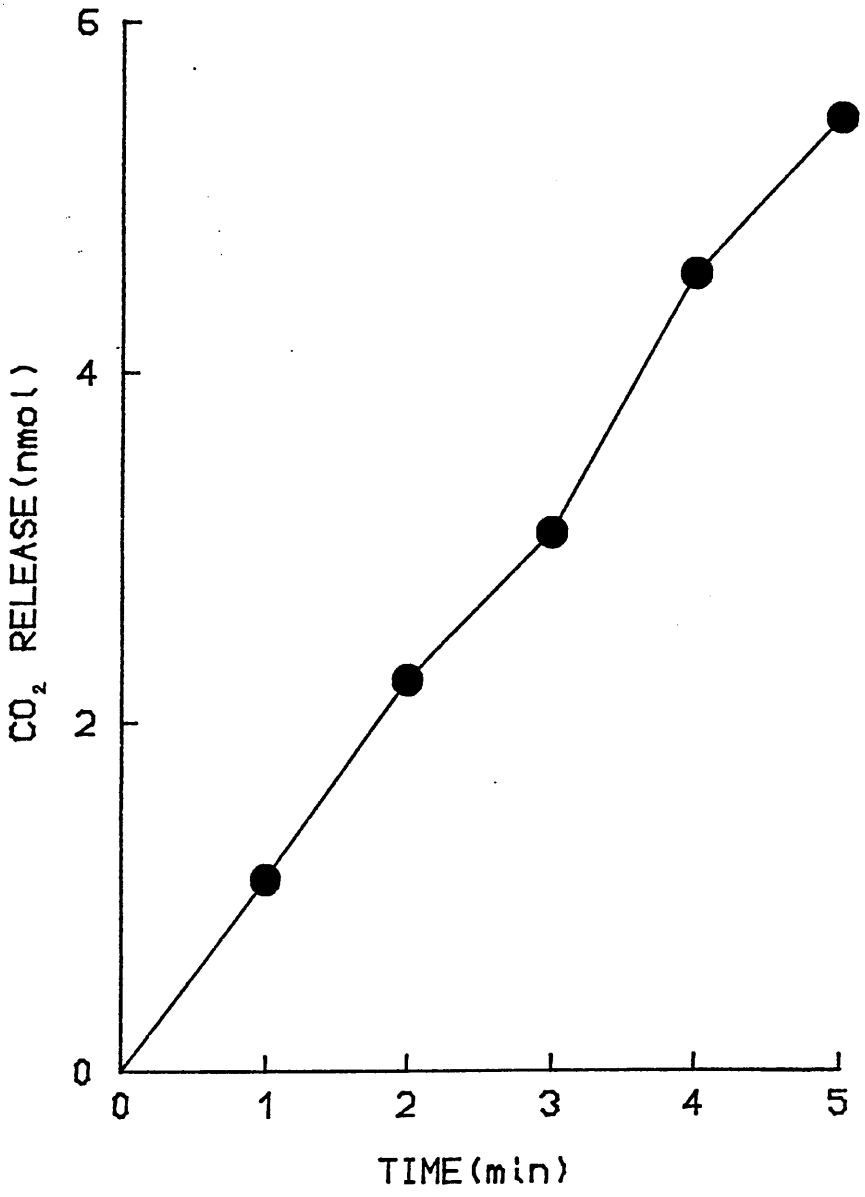


Fig. 2.7. Carbon dioxide release by adipocyte mitochondria during the assay of PDH activity

PDH activity was assayed, as described in the text, in incubations of adipocyte mitochondria which were prepared from parametrial adipose tissue from virgin female rats. The assay was stopped after 2 min. Mitochondria were incubated with ATP, as described in the text, prior to the assay of PDH activity.

FIG.2.7.

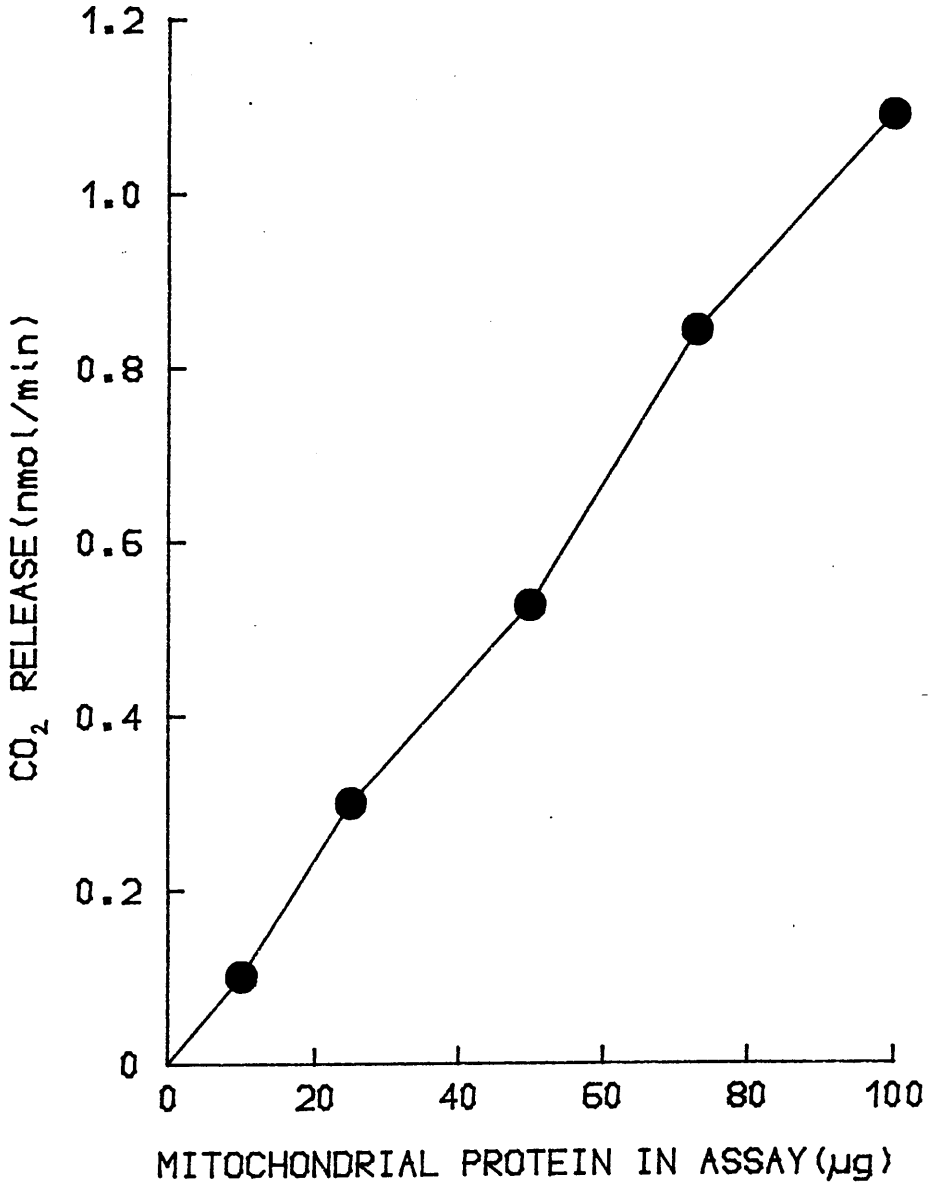
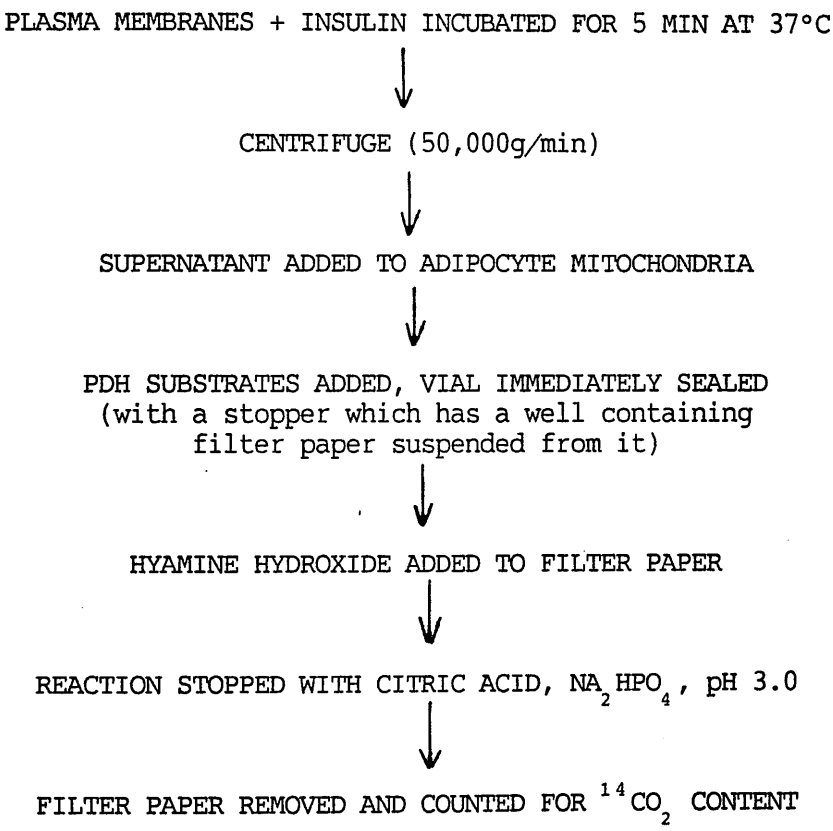

1.2
1.0
0.8
0.6
0.4
0.2
0
CO_2 RELEASE(nmol/min)
0
20
40
60
80
100
MITOCHONDRIAL PROTEIN IN ASSAY(μg)

Figure 2.8.

ACTIVATION OF PYRUVATE DEHYDROGENASE BY ADDITION OF SUPERNATANT FROM INSULIN TREATED PLASMA MEMBRANES TO MITOCHONDRIA



2.8.5.5. Binding of ^{125}I-labelled insulin to plasma membranes

Plasma membranes were prepared from rat white adipocytes and mammary tissue as described in sections 2.8.5.1. and 2.8.5.2. respectively except that the fractions were finally suspended and stored in 10mM Tris-HCl, pH 7.4. Adipocyte (50μg protein/assay) or mammary (25μg protein/assay) plasma membranes were incubated in 10mM Tris-HCl, pH 7.8, containing 1% BSA, 4mg/ml bacitracin (to inhibit insulin degradation), 0.5ng ^{125}I-insulin (0.025μCi) and 0-100ng unlabelled insulin at 4°C in a final volume of 400μl. Porcine insulin was iodinated by Dr D.J. Flint using the lacto-peroxidase method (see Morrison & Bayne, 1970). Incubations were terminated after 16-18h by the addition of 1ml of ice-cold 10mM Tris-HCl, pH 7.8, containing 1% BSA and centrifugation at 2,000g and 4°C for 30 min. The supernatant fractions were removed by decanting and saved for determination of insulin degradation by precipitation in 10% trichloroacetic acid as described below. The radioactivity in the pellets was measured on a gamma counter (Rackgamma 11, LKB Instruments Ltd., Seldon, South Croydon, Surrey). All data were corrected for non-specific binding by subtracting the amount of radioactivity still bound in the presence of 10μg of porcine insulin from all other values.

Results were subjected to Scatchard analysis (Scatchard, 1949). The results were interpreted in terms of a component with a high affinity for insulin and a second component with a lower affinity. The total numbers of both types of insulin receptor were determined from Scatchard plots.

Insulin degradation was determined by precipitation of intact hormone with trichloroacetic acid as described by Freychet et al.

(1972) with some modifications. The sample (75μl) was added to 25μl of 40% TCA and the amount of radioactivity present was measured by gamma counting. Ten per cent TCA (500μl) was then added and the sample was incubated at 4°C. After 10 min the sample was centrifuged in an Eppendorf centrifuge for 5 min then after removing the supernatant the amount of radioactivity in the whole sample minus that in the pellet was taken to represent the amount of insulin that was degraded.

2.8.5.6.1. Assay of 5'nucleotidase activity

5'nucleotidase activity in adipocyte homogenates and subcellular fractions was determined as described by Vernon et al. (1983). Plasma membrane and mitochondria enriched fractions were prepared as described in section 2.8.5.1. except that the extraction medium was 10mM Tris-HCl, pH 7.4, containing 0.25M sucrose and the fractions were finally suspended and stored in 50mM Tris-HCl, pH 7.4. The homogenates and subcellular fractions were stored in liquid N_2 for up to 1 week before 5'nucleotidase was assayed by measuring the conversion of [^{14}C]AMP into [^{14}C]adenosine. The incubation medium consisted of the following (final concentrations in assay): 50mM Tris/maleate, pH 7.4, 10mM $MgCl_2$, 0.4mM EDTA, 1mM DTT, 400μm [8-^{14}C]AMP (0.012μCi; Amersham International PLC, White Lion Road, Amersham, Bucks.) and 0.3mM adenosine. The reactions were initiated by the addition of plasma membrane fraction (5-10μg protein), mitochondria fraction (20μg protein) or adipocyte homogenate (20μg protein) to the incubation medium to give a final volume of 25μl. The assay was performed at 37°C for 10 min after which the reaction was stopped by the addition of 25μl of ethanol followed by 5μl of carrier solution which contained 20mM adenosine, 20mM AMP and

7mM inosine in distilled water. A 25μl sample was taken and 50μl of Dowex-1 resin was added. The procedure for the preparation of Dowex-1 resin is given in section 2.8.5.6.2. The mixture was agitated for 5s and allowed to settle for 10 min; this was repeated three times. Finally the mixture was centrifuged for 5 min in an Eppendorf centrifuge and 25μl of the supernatant was mixed with 10ml of a toluene based scintillation fluid (Packard Instruments, Caversham, Berks.). The [^{14}C] content was measured using a Packard (Tri-Carb 2405, Packard Instruments, Caversham, Berks.) or LKB (Rackbeta, 1215, LKB Instruments Ltd., South Croydon, Surrey) liquid scintillation counter. Background values were obtained by carrying out the assay as described above but with 50mM Tris-HCl, pH 7.4, replacing the plasma membrane and mitochondrial fractions.

5'nucleotidase activity in sheep and rat mammary plasma membranes was determined as described above for adipocytes. Mammary plasma membrane fractions were prepared as described in section 2.8.5.2. except that the extraction medium was 10mM Tris-HCl, pH 7.4, containing 0.25M sucrose and the fractions were finally suspended and stored in 50mM Tris-HCl, pH 7.4. The 5'nucleotidase assay was initiated by the addition of sheep or rat mammary plasma membrane enriched fraction (1-5μg protein) or sheep or rat mammary tissue homogenate (1-5μg protein).

Enzyme activity is expressed in units, one unit being the amount of enzyme that causes the production of 1μmol of adenosine per minute under the assay conditions.

2.8.5.6.2. Preparation of anion-exchange resin

Between 5-10ml of packed Dowex-1 resin (chloride form 8% cross-linkage, 200-400 mesh; Bio-Rad Laboratories Ltd., Caxton Way,

Watford Business Park, Watford, England) was washed with 250ml 0.5N HCl, 500ml 0.5N NaOH, 250ml 0.5N HCl and then repeatedly with distilled H_2O until the eluate reached pH 5.0. The resin was then allowed to settle for at least 1h before 2 volumes of distilled H_2O was added to 1 volume of resin. The resin was stored as an aqueous slurry at room temperture.

2.8.5.7. Assay of succinic dehydrogenase activity

The succinate-dependent reduction of indophenol to leucoindophenol was followed spectrophotometrically at 600nm. Rat adipocyte plasma membrane and mitohondria enriched fractions and rat and sheep mammary plasma membrane enriched fractions were prepared as described for the measurement of 5'nucleotidase activity (see section 2.8.5.6.1.). The assay was performed as described by Bachmann et al., 1966 except that 10μg of 2,6-dichloroindophenol was used instead of 20μg: it was necessary to reduce the amount of 2,6-dichloroindophenol in the assay to obtain a steady baseline. The assay mixture consisted of 10μmol of potassium phosphate, pH 7.4, 0.5mg of dialysed fatty acid-free bovine serum albumin (see section 2.8.4.), 1μmol of KCN, 10μmol of succinate and 10μg of 2,6-dichloroindophenol in a final volume of 0.98ml. The assay was initiated by the addition of 0.02ml of sample (5-10μg protein of adipocyte plasma membrane fraction, 20μg protein of adipocyte or mammary homogenate) and the assay was carried out at 30°C. Background values were obtained by carrying out the assay as described above but with 0.02ml of 50mM Tris-HCl, pH 7.4, replacing the extracts. The molar extinction coefficient was taken to be $16.1 \times 10^3 cm^{-1} M^{-1}$. Enzyme activity is expressed in units, one unit being the amount of enzyme that catalyses the oxidation of 1μmol of succinate per min under the assay conditions.

2.9. Glycerol assay

Glycerol was measured as described by Aitchison et al, (1982). Sample (100μl) was incubated at 37°C for 10 min in a final volume of 1ml of 0.125M triethanolammonium chloride buffer, pH 7.4, containing 0.1mM ATP, 3mM phosphoenolpyruvate, 0.15mM NADH, 10mM $MgCl_2$, 0.5 unit lactate dehydrogenase and 2 unit pyruvate kinase. Glycerokinase (0.1 unit in 5μl) was then added and the reduction in extinction at 340nm was followed. Blank values were obtained by performing the assay with distilled H_2O replacing the sample.

2.10. Expression of results

Results are expressed as means ± SEM and were analysed by Student's t-test for unpaired observations.

CHAPTER 3

Pyruvate dehydrogenase activity in rat white adipose tissue, liver and skeletal muscle during pregnancy and lactation

3.1. Introduction

In the rat mammary gland both the total amount of PDH present and the proportion of the enzyme present in the active form remain low during pregnancy then rise sharply around parturition and are elevated throughout lactation (Coore & Field, 1974; Kankel & Reinauer, 1976). By comparison surprisingly little is known about the activity of PDH in other tissues of the rat during pregnancy and lactation. Sinnett-Smith et al., (1980, 1982) reported that the total amount of PDH present in parametrial white adipose tissue was similar at 10 and 2 days pre-partum and 2 and 7 days post-partum while the proportion of the enzyme in the active state decreased between 2 days pre-partum and 2 days post-partum. However, they did not report the proportion of the enzyme present in the active state in the tissue at any other stage of pregnancy and lactation and the animals used in the study were not anaesthetised and therefore may have been subjected to some stress which, as discussed later (Chapter 5), can result in changes in PDH activity of white adipose tissue: hence the information available on PDH activity of adipose tissue during pregnancy and lactation is limited.

In view of the changes in glucose metabolism which occur in white adipose tissue, skeletal muscle and liver during pregnancy and lactation (see section 1.1.) it is possible that PDH activity in these tissues also changes. The following study was undertaken to ascertain what, if any, changes in PDH activity of rat white adipose tissue, liver and skeletal muscle occur during pregnancy and lactation.

3.2. Experimental Procedure

Age-matched (13-16 week old) virgin, pregnant and lactating rats were injected intraperitoneally (using 23 gauge needles) with pentobarbital (60mg/kg body wt.; dissolved in 0.15M NaCl) and remained under anaesthesia throughout the experiment. Twenty minutes after administration of the pentobarbital samples of liver, parametrial white adipose tissue and skeletal muscle (gastrocnemius and biceps femoris from the hind-limb) were removed and immediately frozen in liquid N_2 for the subsequent extraction and assay of PDH activity as described in section 2.3. Additional samples of white adipose tissue were taken and stored in isotonic saline at 37°C for determination of cellularity as described in section 2.8.2.

3.3. Results

3.3.1. Parametrial white adipose tissue

There was a trend towards an increase in parametrial fat pad weight during pregnancy, although this was not statistically significant (Table 3.1a), then by day 14 of lactation the fat pad weight, relative to the value for virgin rats, was reduced by 36% ($P<0.05$, Table 3.1b). Throughout pregnancy and lactation there was no significant change in the number of adipocytes per fat pad (Table

3.1a,b) but the mean volume of the parametrial adipocytes was significantly reduced at day 14 of lactation ($P<0.01$; Table 3.1b).

In view of changes in the cellularity of parametrial fat pads during lactation it is inappropriate to compare PDH activity in the tissue from virgin, pregnant and lactating animals solely on a per gram of tissue basis. Furthermore unlike the results for liver (section 3.3.3.) and skeletal muscle (section 3.3.2.) PDH activity in the adipose samples is not expressed on a protein basis. Protein concentrations of adipose tissue, being much lower than those of liver and skeletal muscle, can be distorted by contamination of the tissue with blood during the dissection. Preliminary experiments comparing PDH activity in white adipose tissue before and after the preparation of isolated adipocytes from the tissue showed that over 85% of the enzyme activity is associated with the adipocytes. In addition it became apparent that between individual rats, which were in the same physiological state variations in white adipose tissue PDH activity per g tissue were inversely proportional to the mean fat cell volume which is consistent with most of the PDH activity in the tissue being associated with fat cells. The adipose tissue PDH activities in this study are therefore expressed on a per adipocyte basis as well as per fat pad.

There was no change in the total amount of PDH present in white adipocytes during pregnancy (fig. 3.1.) but the proportion of the enzyme present in the active state was greater in adipose tissue from 7 day pregnant rats than from virgin rats ($P<0.01$; fig. 3.2.); this difference was no longer apparent by the fourteenth day of pregnancy (fig. 3.2.). During lactation total PDH activity and the

amount of PDH in the active state in adipocytes declined so that by day 14 of lactation both values were significantly lower than the values for virgin rats ($P<0.05$, <0.01, respectively; fig. 3.1.): a similar decline occurred during lactation in both the total amount of PDH and in the amount of active PDH per fat pad (table 3.1b) which resulted in the active PDH per fat pad being significantly less in 14 day lactating rats than in virgin animals ($P<0.05$, table 3.1b).

3.3.2. Skeletal muscle

No significant change in the protein content of the tissue occurred during pregnancy or lactation (table 3.2a,b). When PDH activity was measured in the muscle samples the assay was found to be non-linear with respect to muscle protein concentration; the activity per mg protein decreased as the amount of muscle protein used in the assay was increased. All the muscle samples displayed this phenomenon when both total and initial PDH activities were measured; a typical result is shown in fig. 3.3. A similar observation was made by Hagg et al., (1976) who suggested that an inhibitor of PDH activity was present in the muscle homogenates, however other workers who have measured PDH activity in skeletal muscle have not commented on the existence of this phenomenon (Hennig et al., 1975; Förster et al., 1984). At low protein concentrations, (less than 100μg per assay), the assay appeared to be linear (fig. 3.3.) possibly due to a diluting out of the inhibitor, and hence aliquots of muscle homogenate containing less than 100μg of protein were used to assay PDH activity.

The total PDH in skeletal muscle was less in 20 day pregnant rats than in virgin rats (fig. 3.4.) and although this difference was

not significant in this study in a subsequent study total PDH of skeletal muscle from 18 day pregnant rats was significantly less than the value for virgin rats ($P<0.001$; table 4.4.). Total PDH activity recovered during lactation (fig. 3.4.). The amount of muscle PDH in the active state (fig. 3.4.) was greater at day 7 ($P<0.01$) and day 14 ($P<0.001$) of pregnancy than in virgin rats and the proportion of the enzyme in the active state was also increased at day 7 of pregnancy ($P<0.02$; fig. 3.5.). By the twentieth day of pregnancy active PDH in skeletal muscle was less than in virgin rats ($P<0.001$; fig. 3.4.) and then both the amount ($P<0.01$, fig. 3.4.) and proportion ($P<0.01$; fig. 3.5.) of the muscle enzyme in the active state were greater at day 1 of lactation than the values for virgin rats. The amount of active PDH in skeletal muscle declined during lactation (fig. 3.4.) and by day 14 of lactation was significantly lower than in virgin rats ($P<0.01$); the proportion of PDH in the active state also declined during lactation (fig. 3.5.).

3.3.3. Liver

There was no significant change in the protein content of the liver during pregnancy or lactation (Table 3.3a,b). Liver weight was significantly increased by day 7 of pregnancy ($P<0.05$) and by the twentieth day of pregnancy was 36% greater than in virgin rats ($P<0.01$) then remained elevated during lactation (table 3.3a,b).

No change occurred in either total PDH, expressed on a protein basis, (fig. 3.6.) or in the proportion of PDH in the active state (fig. 3.7.) during pregnancy or lactation apart from a transient fall in the amount of active enzyme at day 1 of lactation ($P<0.01$; fig. 3.6.) which resulted in a similar fall in the proportion of the

enzyme present in the active state ($P<0.01$, fig. 3.7.). The amount of active PDH per liver, when compared to the value for virgin rats, was reduced on the first day of lactation ($P<0.05$; table 3.3.) and was increased by day 14 of lactation ($P<0.02$; table 3.3.) when the weight of the liver was greatest (table 3.3b).

3.3.4. Discussion

Although lipid accumulation usually occurs in the rat during pregnancy (see section 1.1.2.1.) in the present study there was no significant increase in the mean adipocyte volume in parametrial white adipose tissue from pregnant animals. This was probably due to the large variation between individual rats in mean adipocyte volume which made it difficult to detect overall differences between the relatively small groups of rats used: it would have been necessary to follow the mean adipocyte volume in an individual rat throughout pregnancy and lactation to identify all of the changes which occur. The significant decrease in mean adipocyte volume at day 14 of lactation is in agreement with other studies on parametrial (Flint et al., 1979b) and perirenal (Bershtein & Aleksandrov, 1977) adipocytes. The number of cells per fat pad did not change significantly during pregnancy and lactation which agrees with other studies on parametrial (Flint et al., 1979b) and perirenal (Knopp et al., 1970a; Bershtein & Aleksandrov, 1977) fat pads.

The proportion of PDH in the active state in white adipose tissue of virgin rats in the present study was similar to values reported for epididymal adipose tissue of male rats (Stansbie et al., 1976a,b) but the values for total PDH activity of white adipose tissue in this study were lower than values reported in other studies

(Stansbie et al., 1976a,b). The virgin female rats used in the present study were age-matched with 14 day lactating rats and so were comparatively old (13-16 weeks, 250-300g) while the rats used by Stansbie et al., 1976a,b) were young males (150-220g). As the values for PDH activity in the studies by Stansbie et al., (1976a,b) were quoted on a per gram of tissue basis, and PDH activity of adipose tissue is inversely proportional to adipocyte size (see section 3.3.1.) the reason why the activities per gram of tissue in the present study were lower than those in the other studies could be that the rats in this study were older and so fatter than those used in the other studies: in a subsequent study (chapter 4) the values for total PDH activity in epididymal white adipose tissue from male rats were similar to values reported by Stansbie et al., (1976a,b).

Sinnett-Smith et al. (1980, 1982) found no change in total PDH activity of parametrial white adipose tissue between rats at 10 and 2 days pre-partum and 2 and 7 days post-partum which is confirmed in the present study. However the fall in the proportion of PDH present in the active state between 2 days pre-partum and 2 days post-partum which was reported by Sinnett-Smith et al. (1980, 1982) was not apparent in the present study. The rats used by Sinnett-Smith et al., were killed by cervical dislocation, which would result in a surge of sympathetic nervous activity (see Barrand & Callingham, 1983) and, as PDH in white adipose tissue is less responsive to catecholamines during lactation (see Chapter 5), this may account for the fall in the enzyme activity observed. In the present study the stressful effects of killing conscious animals were avoided as the rats were under anaesthesia throughout the experiment. The decrease

in total PDH activity in parametrial adipose tissue at day 14 of lactation together with the absence of any change in the proportion of the enzyme in the active state during lactation were confirmed in a second study (see chapter 4): as in the rat PDH activity of sheep white adipose tissue is reduced during lactation, due to a fall in total activity while the proportion of the enzyme in the active state remains unchanged (Vernon et al., 1984).

PDH catalyses the irreversible conversion of glucose to acetyl-CoA which can then be oxidised via the citric acid cycle or used as a substrate for fatty acid synthesis (see section 1.2.). Smith (1973b) reported that basal glucose oxidation is increased in rat white adipose tissue in vitro at day 7 of pregnancy: the surge in PDH activity in white adipose tissue at this time could at least partially, account for this increase in glucose oxidation. It has been reported that glucose oxidation in isolated white adipocytes from 19 and 20 day pregnant rats is less than in white adipocytes from virgin rats (Sutter-Dub et al., 1984; Toyoda et al., 1985). In contrast Leturque et al. (1984b) found that the rate of glucose oxidation was similar in isolated white adipocytes from 19 day pregnant rats to that in adipocytes from virgin rats and Smith (1973b) found no difference in the rate of glucose oxidation in pieces of white adipose tissue from virgin and 20 day pregnant rats. Under the experimental conditions used by Sutter-Dub et al. (1984) and Toyoda et al. (1985) - 0.5mM glucose and 2mM glucose respectively - glucose metabolism may have been limited by the rate of glucose transport (Foley et al., 1980) but Leturque et al. (1984b) and Smith (1973b) used higher concentrations of glucose (5mM and 20mM

respectively) which would not result in glucose transport being rate-limiting. Certainly PDH activity in white adipose tissue of 20 day pregnant rats is similar to that in virgin rats and so could not account for a decrease in glucose oxidation. At mid-lactation PDH activity in white adipose tissue is reduced yet the basal rate of glucose oxidation *in vitro* is similar to that in the virgin rat (Smith 1973b; Burnol *et al.*, 1986). This suggests that the flux of acetyl-CoA through the citric acid cycle is unchanged during lactation and hence the proportion of acetyl-CoA directed towards lipogenesis in white adipose tissue may be reduced.

There is a transient increase in the rate of lipogenesis in white adipose tissue around day 9 of pregnancy (see section 1.1.2.1.) which could be at least partly explained by the transient surge in PDH activity at this time. The rate of fatty acid synthesis in white adipose tissue falls around parturition and is low throughout lactation (see section 1.1.2.1.) however the fall in fatty acid synthesis commences 2 days prior to parturition and so precedes the fall in PDH activity. The activities of fatty acid synthetase and ACC in white adipose tissue fall sharply after mid-pregnancy, reach extremely low levels by day 2 of lactation and remain low throughout lactation (Sinnett-Smith *et al.*, 1980, 1982). Changes in the activities of these enzymes thus closely parallel the changes in the rate of fatty acid synthesis in white adipose tissue which occur during the reproductive cycle. In contrast, changes in PDH activity in white adipose tissue are not always paralleled by changes in the rate of fatty acid synthesis, which will be limited during late pregnancy and lactation by the low activities of ACC and fatty acid

synthetase. Therefore it appears that PDH is not as important as ACC and fatty acid synthetase in controlling long term changes in the flux through the fatty acid synthesis pathway in white adipose tissue. The role of PDH in the short term control of this pathway is discussed later (section 4.4.).

In the present study the values for total PDH activity of skeletal muscle from virgin female rats were similar to those reported for male rats by Hennig et al. (1975) but were 5 fold higher than values reported by Hagg et al. (1976). The proportion of PDH in the active state in skeletal muscle from virgin female rats was 32% in the present study which is lower than values reported by Hennig et al. (1975), 40%, and higher than those reported by Hagg et al. (1976), 22%, while in a second study the value (table 4.5.; 10%) was lower than those reported by the above workers. In skeletal muscle from male rats the proportion of PDH in the active state was about 40% (Table 5.5.) which is similar to values reported by Hennig et al. (1975). This suggests that there may be a sex difference in skeletal muscle PDH activity. Both Hennig et al. (1975) and Hagg et al. (1976) studied male rats of a different breed to the animals used in the present studies and these workers used different assays to measure PDH activity which could account for the differences in the values for PDH activity.

The total amount of PDH present in skeletal muscle from 18 day pregnant rats was significantly lower than in muscle from virgin rats ($P<0.001$; table 4.4.). In the present study the proportion of PDH in the active state was lower in lactating than virgin rats (24% compared with 32.5%; fig. 3.5.) but this difference was not

significant while in a subsequent study the proportion of enzyme in the active state was significantly less in skeletal muscle from 14 day lactating rats than in muscle from virgin rats ($P<0.001$; table 4.5.). The total amount of PDH present in the tissue was similar in both studies but the amount and proportion of the enzyme in the active form were lower in the second study for all the groups of rats studied (table 4.3.). The reason for this discrepancy is unknown although it is possible that as the time of day at which the tissue was removed differed between the two studies (10:00h for this study and 12:00h in the second study) variations in circulating hormone and metabolite levels *in vivo* could be involved. In the second study the PDH assay when performed on the muscle homogenates did not display the non-linearity with respect to protein concentration found in the present study and the presence of a PDH inhibitor in the homogenates was not evident hence the results in the second study may be the more reliable. Certainly a decrease in the proportion of muscle PDH present in the active state during lactation, as found in the second study (chapter 4), agrees with findings reported for the sheep (Vernon *et al.*, 1984) and, as discussed later (chapter 4.4.) is consistent with the lower serum insulin concentration during lactation.

Comparatively little is known about skeletal muscle metabolism during pregnancy and lactation (see section 1.1.2.4.). Although PDH activity is suppressed in the rat during late pregnancy basal glucose utilisation by the tissue is unchanged both *in vivo* (Leturque *et al.*, 1986) and *in vitro* (Rushakoff & Kalkhoff, 1981; Leturque *et al.*, 1981b) as is the amount of lactate and pyruvate released *in vitro*

(Rushakoff & Kalkhoff, 1981) and this suggests that the actual flux through PDH is not changed. It is not known if glucose metabolism of skeletal muscle changes during lactation but in the sheep the proportion of glucose carbon released as lactate by the hind-limb *in vivo* increases during lactation (Pethick & Lindsay, 1982) probably largely due to the decrease in the proportion of muscle PDH in the active state (Vernon *et al.*, 1984): the fall in PDH activity of rat skeletal muscle during lactation could have a similar effect. In sheep this lactate is probably used for gluconeogenesis which is increased during lactation (Bergman *et al.*, 1974).

In the present study the values for PDH activity in liver are similar to those reported elsewhere (Wieland *et al.*, 1972; Stansbie *et al.*, 1976a; Oviasu & Whitton, 1984) and the increase in liver weight during pregnancy and lactation agrees with findings in other studies (Herrera *et al.*, 1969; Smith, 1975; Flint, 1980).

Hepatic lipogenesis is elevated during pregnancy and lactation (see section 1.1.2.3.) and this is accompanied by increased activities of ACC and fatty acid synthetase (Smith, 1973a; Zammit & Corstorphine, 1982a), the flux through the lipogenic pathway (Smith, 1973a; Agius & Williamson, 1980b) and PDH activity all undergo a transient fall in the liver around parturition although PDH activity falls from levels found in virgin rats to lower levels while the other activities fall from elevated values to values found in virgin rats. The activities of the glycolytic enzymes, hexokinase and glucokinase, are elevated in the liver during pregnancy and at peak lactation while the activities of key enzymes of gluconeogenesis are either unchanged or reduced and this has led to the suggestion that

glycolysis is elevated in the liver during pregnancy and lactation (see section 1.1.2.3.). It has been suggested that increased hepatic glycolytic activity during lactation serves to increase the supply of carbon for fatty acid synthesis (Williamson, 1980) and this may also be the case during pregnancy. It is therefore somewhat surprising that hepatic PDH activity is unchanged during pregnancy and lactation as the activity of this enzyme determines the proportion of pyruvate that is converted to acetyl-CoA, which can then be used for fatty acid synthesis. However, the flux through hepatic PDH in the virgin rat may not be sufficient to saturate the enzyme and therefore an increase in PDH activity may not be necessary for an increase in the flux of pyruvate to acetyl-CoA to occur in the liver during pregnancy and lactation.

It is clear from the present study that tissue specific changes occur in PDH activity during pregnancy and lactation in the rat. However the mechanisms underlying these changes are not certain. Insulin has been implicated in the control of PDH activity in a number of tissues (see section 1.2.3.1.). Short-term exposure to insulin both *in vitro* and *in vivo* increases the proportion of PDH in the active state in white adipose tissue but has no effect on total PDH in the tissue (see section 1.2.3.1.1.). PDH activity in 48h-starved or diabetic rats is reduced in liver (Wieland *et al.*, 1972; Patzelt *et al.*, 1973), skeletal muscle (Hennig *et al.*, 1975; Hagg *et al.*, 1976) and white adipose tissue (Wieland *et al.*, 1973; Stansbie *et al.*, 1976b) due to a decrease in the proportion of the enzyme in the active state while little change occurs in total PDH in these tissues. In addition changes in the serum insulin

concentration have been connected with some of the metabolic adaptations that occur during pregnancy and lactation (see section 1.1.). During pregnancy the serum insulin concentration increases (see section 1.1.1.) and this is probably responsible for the increase in the proportion of PDH in the active state in skeletal muscle and white adipose tissue in early pregnancy. The insulin binding capacity of white adipocytes is also increased during pregnancy (see section 1.1.2.1.). Although the serum insulin concentration and the insulin binding capacity of white adipocytes both remain elevated throughout pregnancy (see section 1.1.) PDH activity of white adipose tissue returns to values similar to those found in virgin rats by day 14 of gestation which suggests that during the latter half of pregnancy the tissue becomes less responsive to insulin; the ability of insulin *in vivo* to stimulate glucose utilisation by white adipose tissue is reduced during late pregnancy (Leturque *et al.*, 1986). Despite the serum insulin concentration being greater in pregnant than in virgin rats the amount of active PDH in skeletal muscle is reduced at day 20 of pregnancy and the ability of insulin to stimulate glucose uptake by skeletal muscle is also reduced in late pregnancy (Rushakoff & Kalkhoff, 1981; Leturque *et al.*, 1986) hence at this time the tissue is resistant to insulin. The reason for the fall in total PDH in skeletal muscle at day 20 of pregnancy is not certain. It seems improbable that changes in the serum insulin concentration or resistance of the tissue to insulin are responsible as total PDH of skeletal muscle is not changed in 48h starved or in diabetic rats (Hennig *et al.*, 1975; Hagg *et al.*, 1976).

Curiously the fall in PDH activity in both rats and sheep (Vernon et al., 1984) during lactation is due to a fall in the proportion of the enzyme in the active state in skeletal muscle, which is probably due to the fall in serum insulin concentration, and to a fall in the total PDH in white adipose tissue; the reason why the proportion of PDH in the active state in white adipose tissue does not fall in parallel to the serum insulin concentration during lactation will be discussed later (chapter 4.4.). The fall in total PDH of white adipose tissue during lactation parallels the fall in the serum insulin concentration (see section 1.1.1.). However low serum insulin concentrations are unlikely to be responsible for this decrease in total PDH as starvation for 48h and alloxan-diabetes have little effect on total PDH of adipose tissue (Stansbie et al., 1976b). Feeding rats a high-fat diet for more than 6 days results in a decrease in total PDH of white adipose tissue (Stansbie et al., 1976b; Begum et al., 1982a) but this may be attributable to an increase in fat cell size as the enzyme activities in these studies were not expressed on a cellular basis (see section 3.3.1.). Despite the variations in the serum insulin concentration during pregnancy and lactation hepatic PDH activity showed little change suggesting that insulin is less important in controlling PDH activity in liver than it is in white adipose tissue or skeletal muscle. The transient fall in hepatic PDH activity at day 1 of lactation may be due to the reduction in food intake around parturition (see section 1.1.) or to some of the many endocrine changes which occur at this time (see section 1.1.1.).

3.5. Conclusions

The diminished PDH activity in white adipose tissue and skeletal muscle of the lactating rat will limit the use of glucose and lactate carbon by these tissues and hence facilitate the preferential use of these substrates by the mammary gland. Also the proportion of glucose utilised by these tissues that is released as lactate should increase during lactation. This lactate may be used for gluconeogenesis, particularly during re-feeding after a period of food deprivation (Williamson et al., 1985). Nothing is known about lactate output by skeletal muscle during lactation and Burnol et al. (1986) found no evidence of increased lactate output by isolated white adipocytes from lactating rats but in their study the percentage of glucose uptake that was released as lactate by adipocytes from virgin rats was relatively high. In another study the proportion of glucose utilised by white adipose tissue in vitro that was released as lactate was higher when the tissue was from lactating rather than virgin rats (R. G. Vernon et al., unpublished observation) which is in accordance with the lower PDH activity in white adipose tissue during lactation.

Changes in the serum insulin concentration (see section 1.1.1) could explain the corresponding changes in the proportion of PDH in the active state in both skeletal muscle and white adipose tissue during early pregnancy and in skeletal muscle during lactation. However although the serum insulin concentration is greater in 20 day pregnant rats than in virgin rats (see section 1.1.1.) there is no such increase in PDH activity in white adipose tissue or skeletal muscle suggesting that the response of these tissues to insulin is

suppressed. Also there is no change in the proportion of PDH in the active state in white adipose tissue during lactation despite the falling serum insulin concentration (see section 1.1.1.) suggesting that the tissue is unresponsive to insulin. The reason for the changes in total PDH activity in white adipose tissue and skeletal muscle during the reproductive cycle are not known.

Fig. 3.1. Pyruvate dehydrogenase activity of parametrial white adipose tissue from virgin, pregnant and lactating rats

Parametrial white adipose tissue was removed from age-matched rats under pentobarbital anaesthesia and immediately frozen in liquid N_2. PDH activity was extracted from the tissue and assayed as described in chapter 2. Initial PDH activity (O) is the active PDH in the tissue homogenate immediately after thawing and the total PDH activity (●) was obtained after incubation of the homogenate with $MgCl_2$, $CaCl_2$ and PDH-phosphatase as described in Chapter 2. Results are means ± SEM for 3 to 7 observations; *, *** indicate the value is significantly different from the value for virgin rats: $P<0.05$, <0.01 respectively.

fig.3.1.

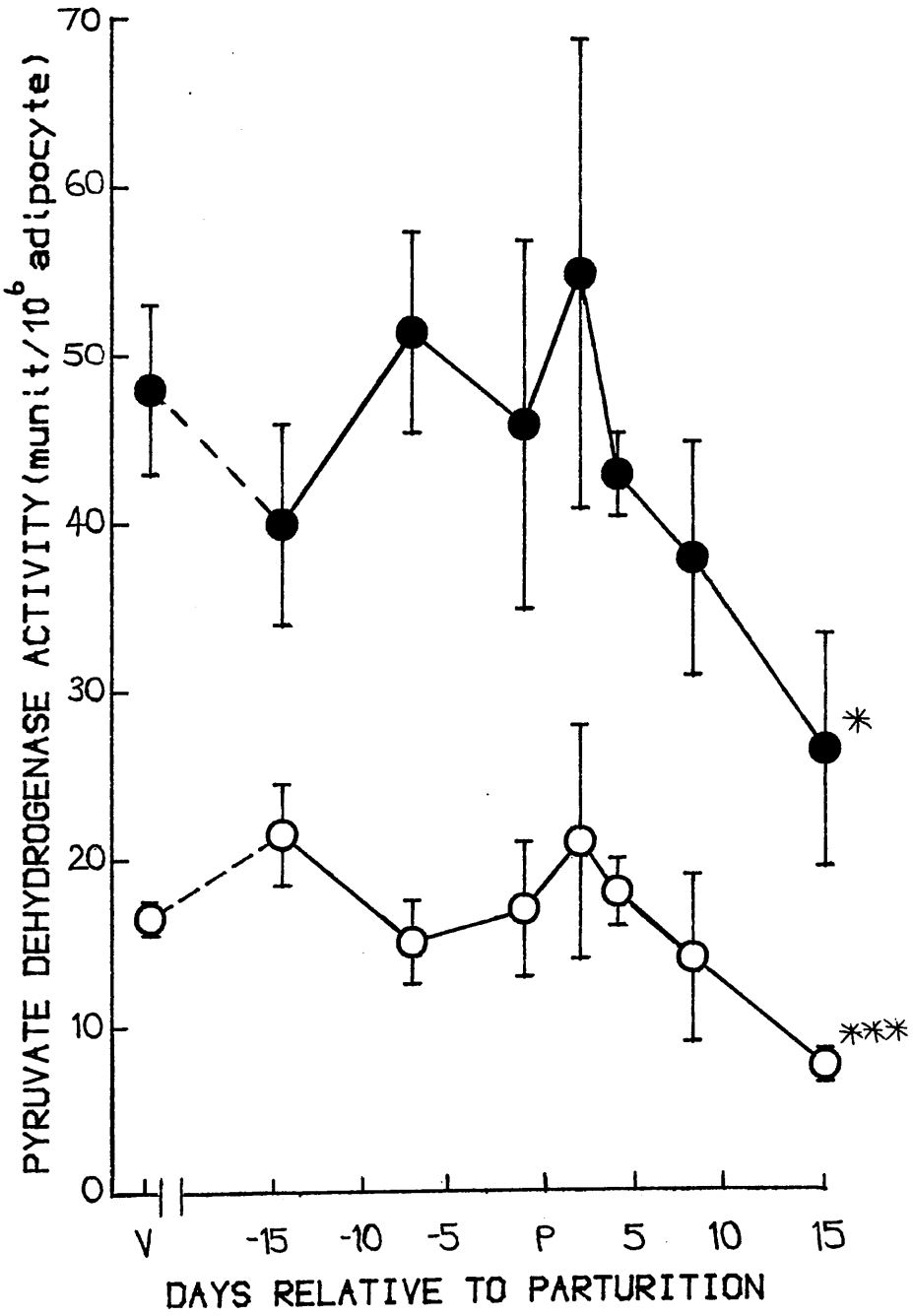

PYRUVATE DEHYDROGENASE ACTIVITY (munit/10^6 adipocyte)
70
60
50
40
30
20
10
0
*
***
V
-15
-10
-5
P
5
10
15
DAYS RELATIVE TO PARTURITION

Fig. 3.2. The proportion of pyruvate dehydrogenase present in the active state in parametrial white adipose tissue from virgin, pregnant and lactating rats

For experimental protocol see legend to fig. 3.1. PDH activity is expressed as:

$$\frac{\text{initial activity}}{\text{total activity}} \times 100$$

Results are means ± SEM for 3 to 7 observations; *** indicates the value is significantly different from the value for virgin rats: $P<0.01$.

FIG.3.2.

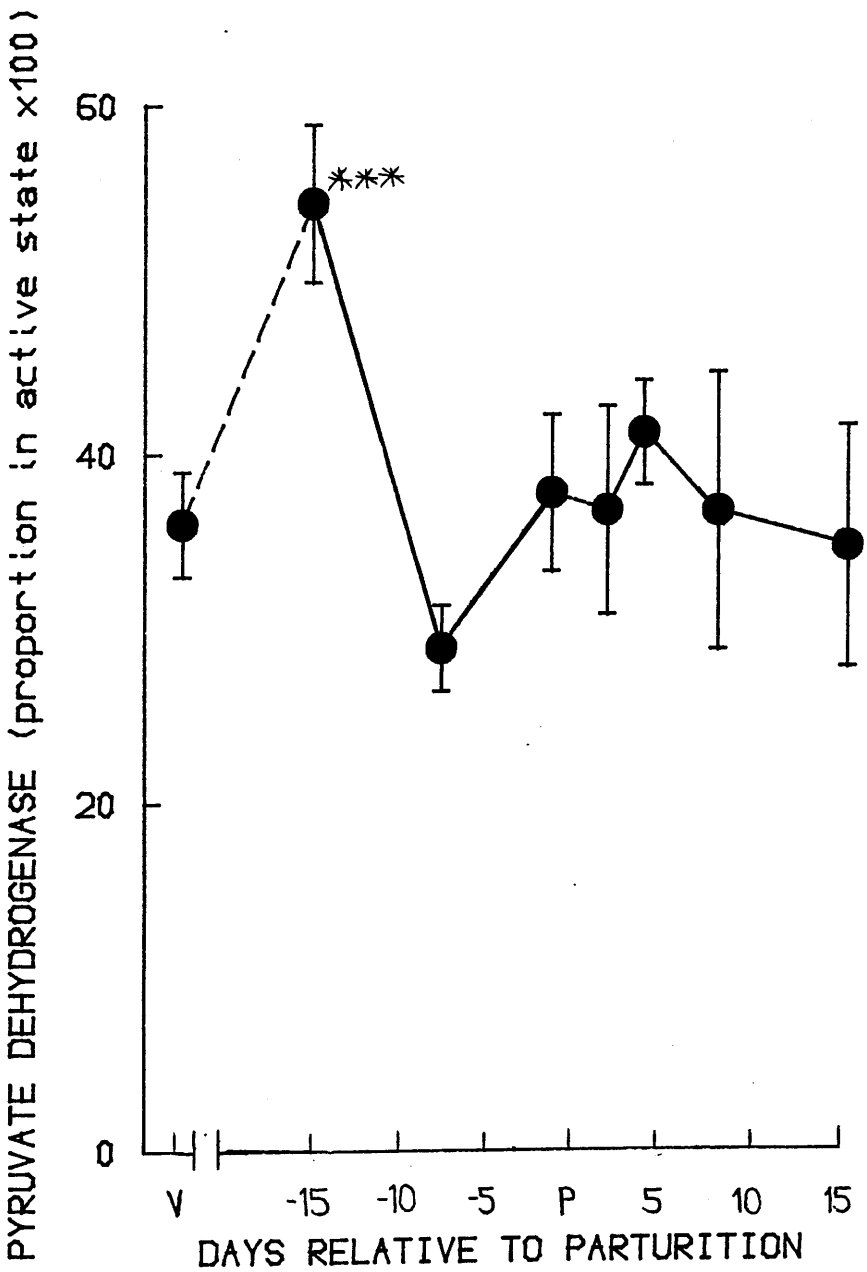

PYRUVATE DEHYDROGENASE (proportion in active state x100)
60
40
20
0
***
V
-15
-10
-5
P
5
10
15
DAYS RELATIVE TO PARTURITION

Fig. 3.3. Pyruvate dehydrogenase activity in rat skeletal muscle homogenates measured at various protein concentrations

Initial PDH activity in homogenates of skeletal muscle from a virgin (○), 20 day pregnant (□) and 1 day lactating (●) rat are shown. Skeletal muscle was removed from the hind-limb of rats under pentobarbital anaesthesia and immediately frozen in liquid N_2. Homogenates were prepared from the frozen tissue and PDH activity was assayed as described in chapter 2. Initial PDH activity is the amount of active PDH in the homogenate immediately after thawing.

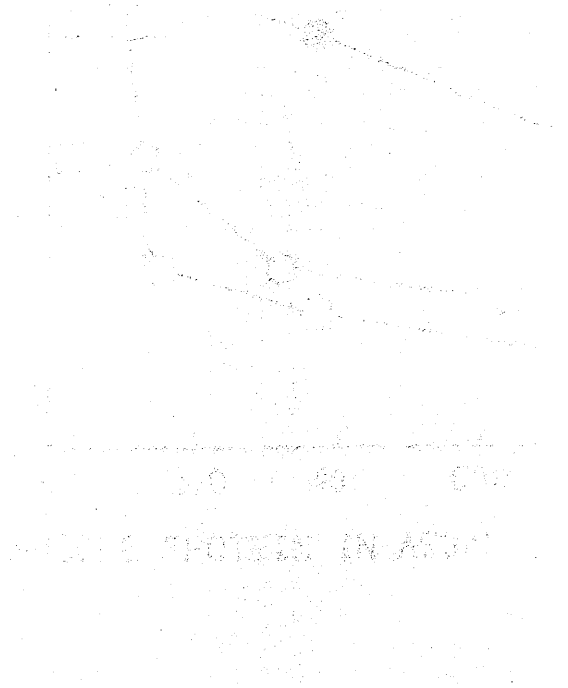


FIG.3.3.

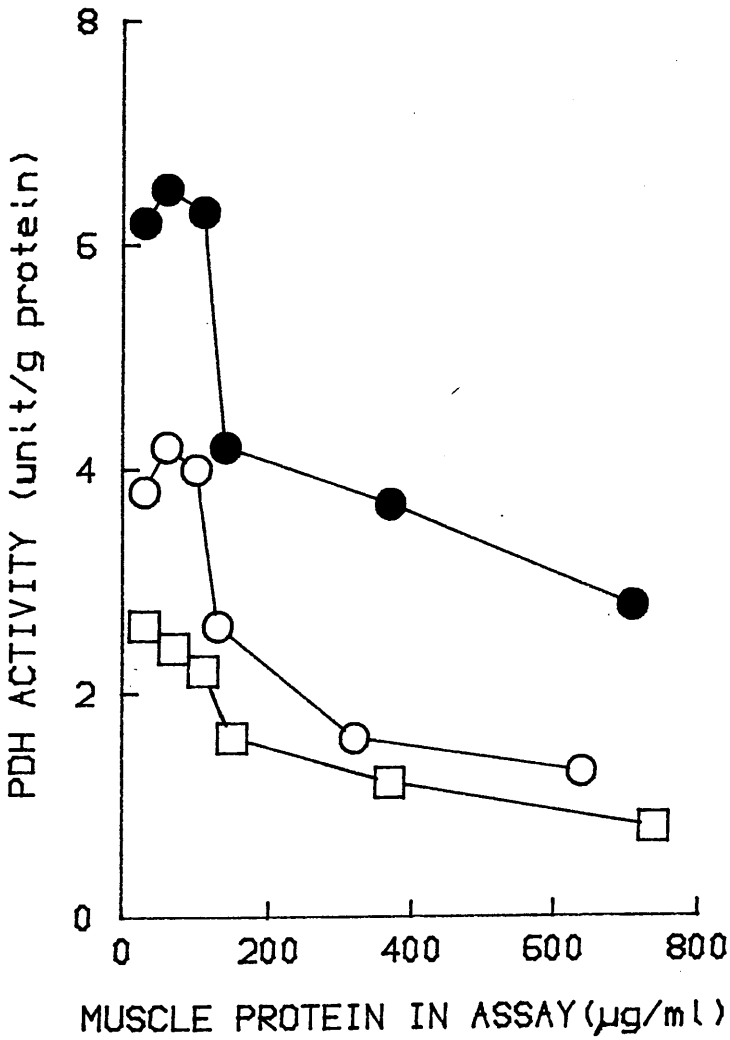

8
6
4
2
0
PDH ACTIVITY (unit/g protein)
0
200
400
600
800
MUSCLE PROTEIN IN ASSAY(μg/ml)

Fig. 3.4. Pyruvate dehydrogenase activity of skeletal muscle from virgin, pregnant and lactating rats

Skeletal muscle was removed from the hind-limb of age-matched rats under pentobarbital anaesthesia and immediately frozen in liquid N_2 for determination of PDH activity as described in chapter 2. Initial PDH activity (●) is the activity in the tissue homogenate immediately after thawing and the total PDH activity (○) was obtained as described in the legend to Fig. 3.1. Results are mean ± SEM for 3 to 7 observations; ***, **** indicate the value is significantly different from the value for virgin rats: $P<0.01$, <0.001 respectively.

FIG.3.4.

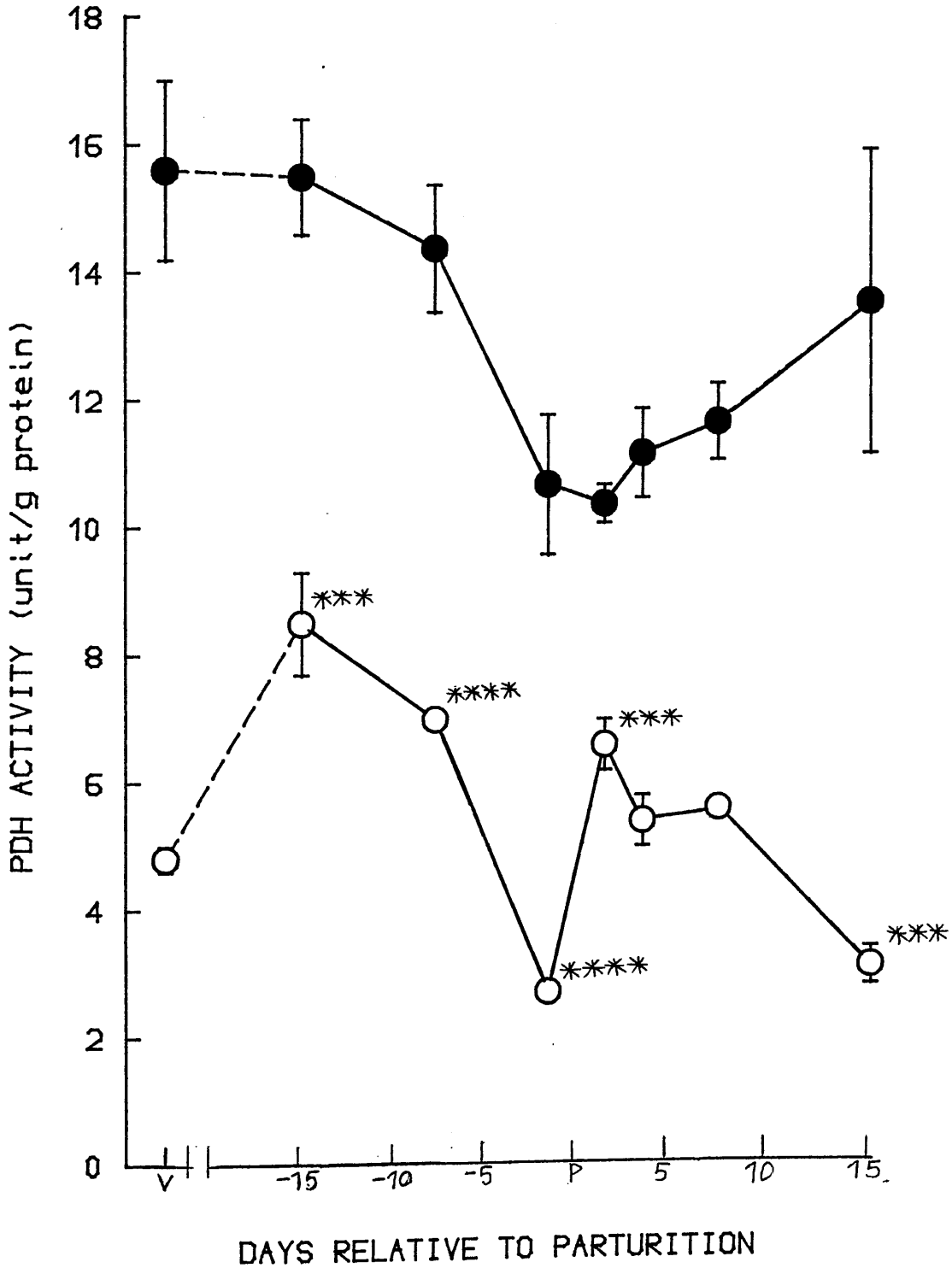

PDH ACTIVITY (unit/g protein)
18
16
14
12
10
8
6
4
2
0
***
****
***
****
***
V
-15
-10
-5
P
5
10
15
DAYS RELATIVE TO PARTURITION

Fig. 3.5. The proportion of pyruvate dehydrogenase present in the active state in skeletal muscle from virgin, pregnant and lactating rats

For experimental protocol see the legend to Fig. 3.4.

PDH activity is expressed as: $\frac{\text{initial activity}}{\text{total activity}} \times 100$

Results are means ± SEM for 3 to 7 observations; **, *** indicate the value is significantly different from the value for virgin rats: $P<0.02$, <0.01 respectively.

FIG.3.5.

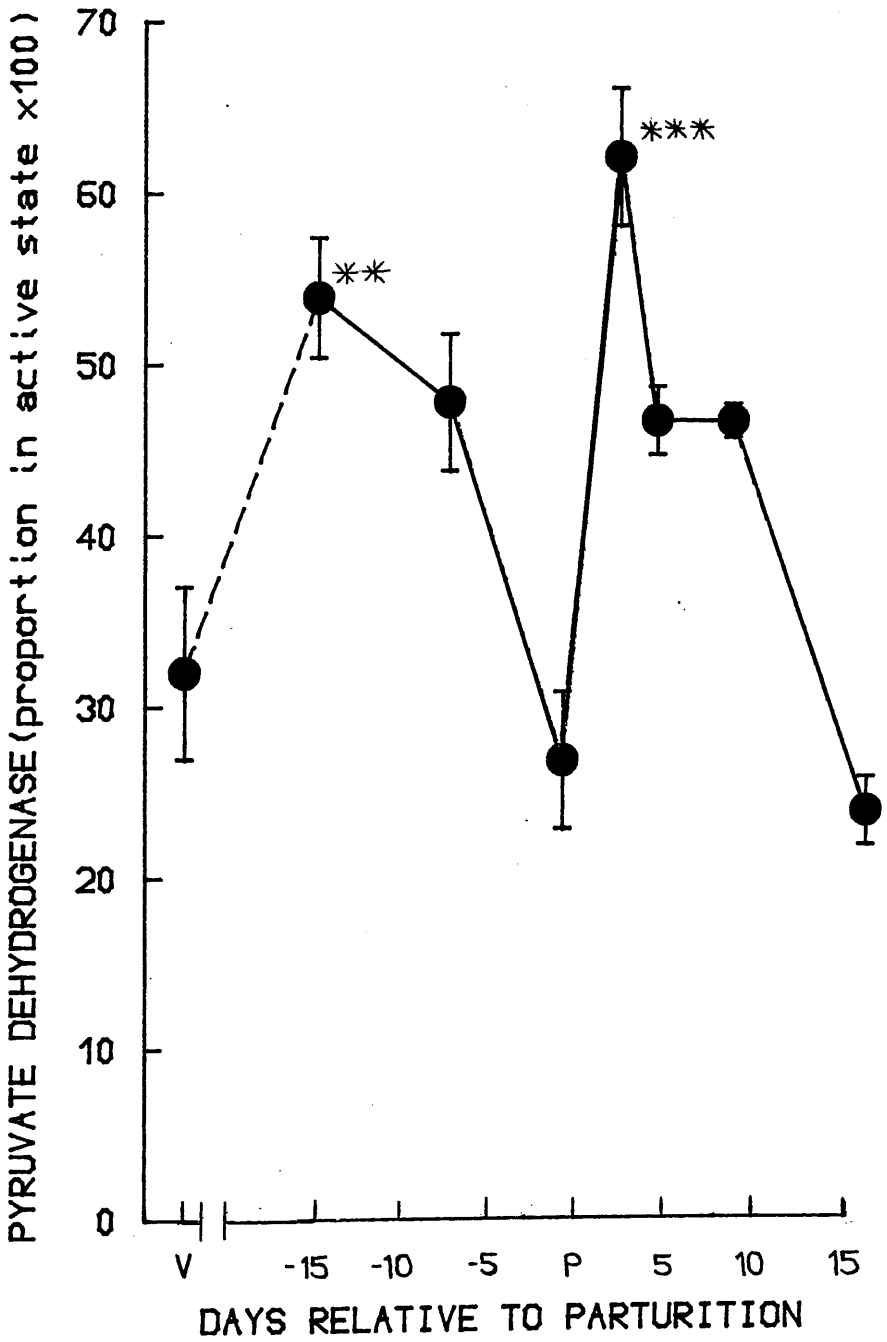

PYRUVATE DEHYDROGENASE (proportion in active state x100)
70
60
50
40
30
20
10
0
**
***
V
-15
-10
-5
P
5
10
15
DAYS RELATIVE TO PARTURITION

Fig. 3.6. Pyruvate dehydrogenase activity of liver from virgin, pregnant and lactating rats

Liver was removed from age-matched rats under pentobarbital anaesthesia and immediately frozen in liquid N_2 for determination of PDH activity as described in chapter 2. Initial PDH activity (●) is the active PDH in the tissue homogenate immediately after thawing and the total PDH activity (○) was obtained as described in the legend to fig. 3.1. Results are means ± SEM for 3 to 7 observations; *** indicates the value is significantly different from the value for virgin rats: $P<0.01$.

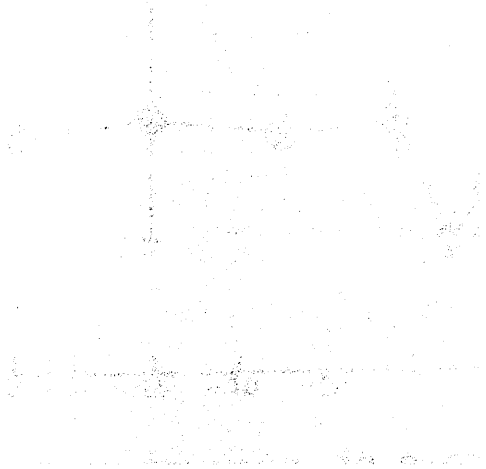
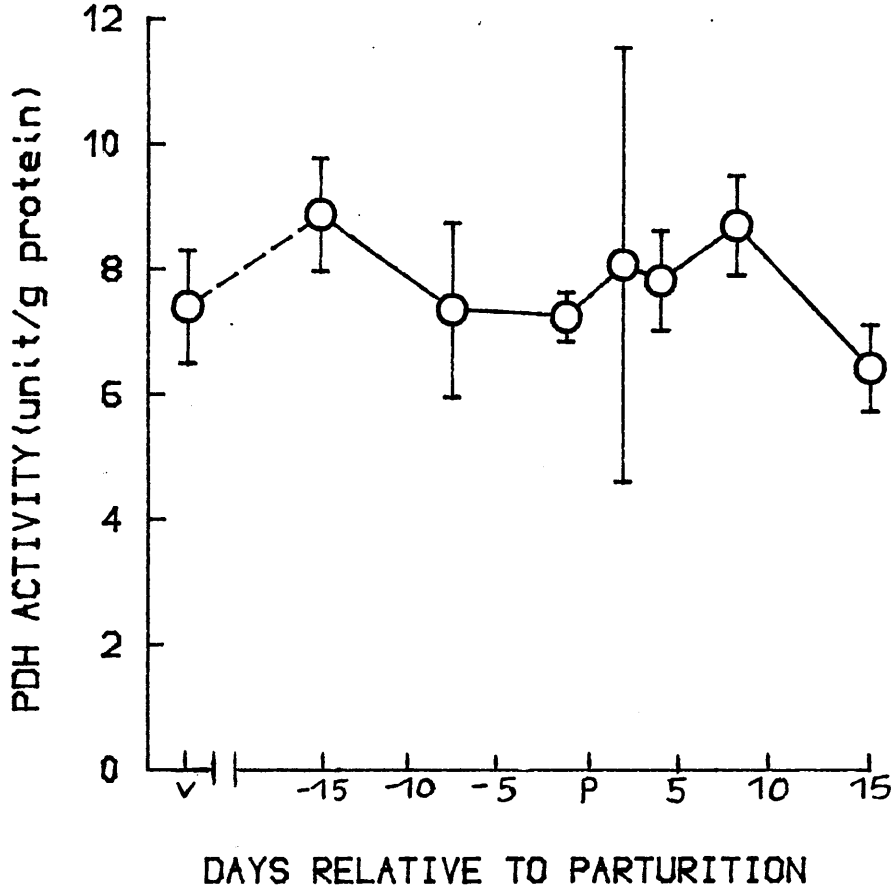


FIG.3.6.


PDH ACTIVITY(unit/g protein)
12
10
8
6
4
2
0
V
-15
-10
-5
P
5
10
15
DAYS RELATIVE TO PARTURITION

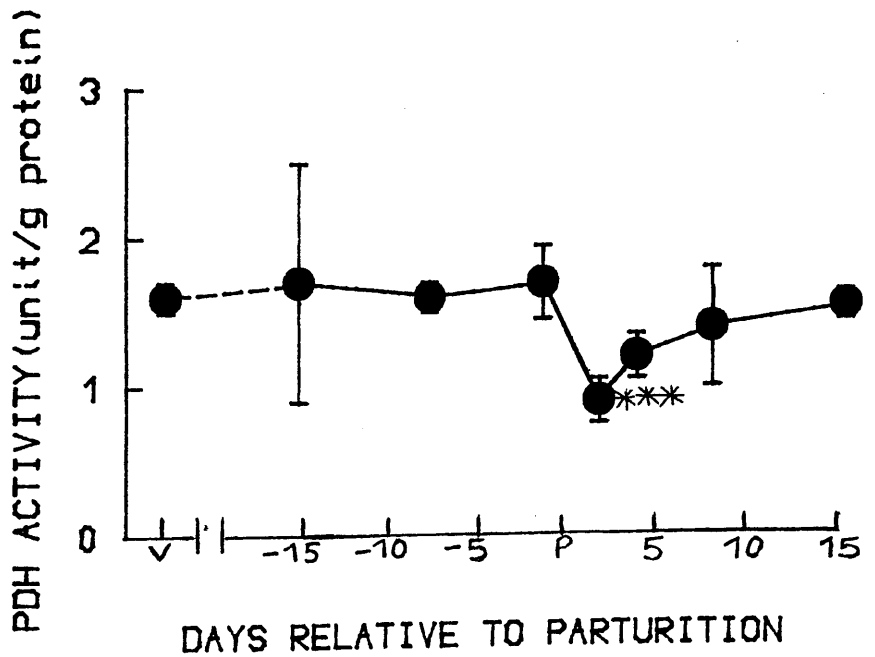

PDH ACTIVITY(unit/g protein)
3
2
1
0
V
-15
-10
-5
P
5
10
15
***
DAYS RELATIVE TO PARTURITION

Fig. 3.7. The proportion of pyruvate dehydrogenase present in the active state in liver from virgin, pregnant and lactating rats

For experimental protocol see the legend to Fig. 3.6.

PDH activity is expressed as: $\frac{\text{initial activity}}{\text{total activity}} \times 100$

Results are means ± SEM for 3 to 7 observations *** indicates the value is significantly different from the value for virgin rats: $P<0.01$.

FIG.3.7.

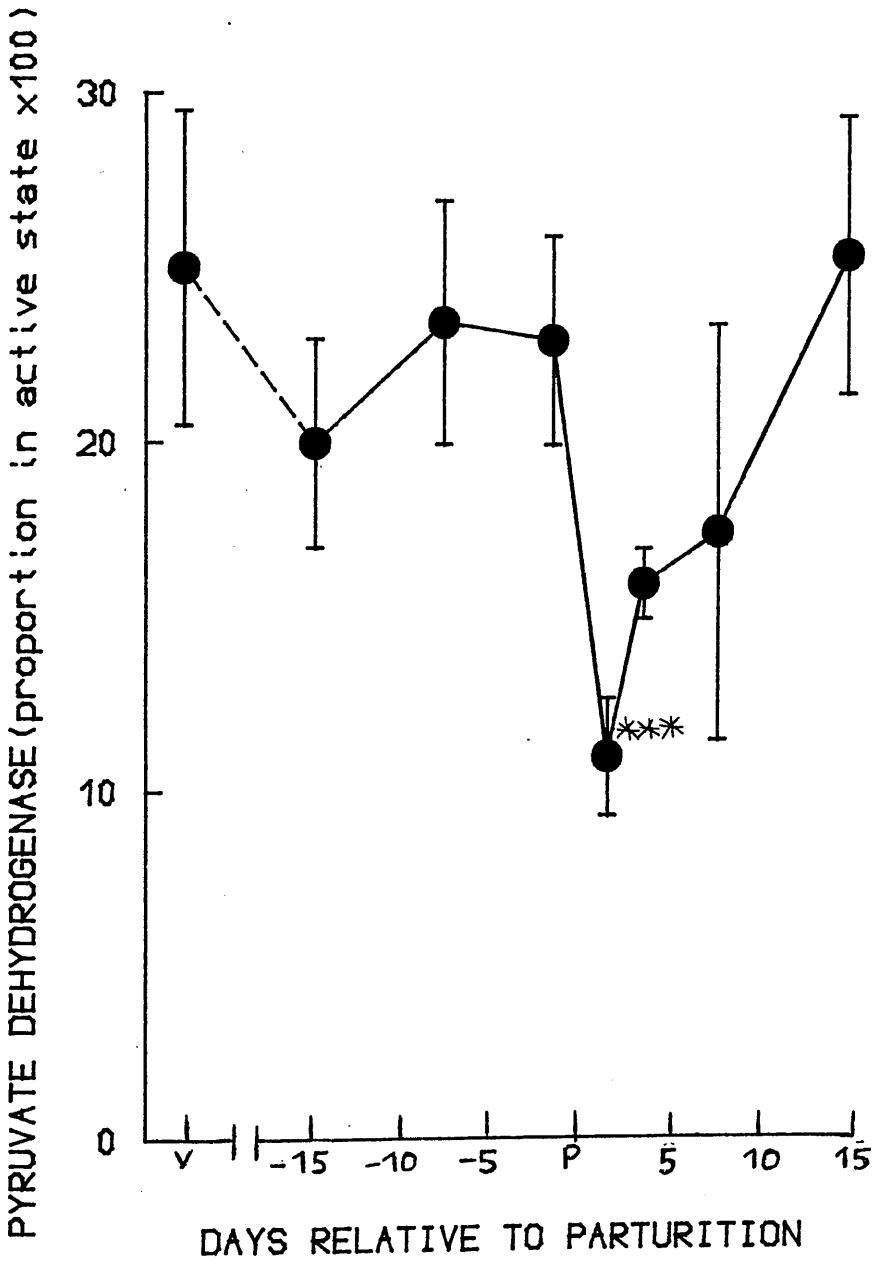

PYRUVATE DEHYDROGENASE(proportion in active state x100)
30
20
10
0
V
-15
-10
-5
P
5
10
15
***
DAYS RELATIVE TO PARTURITION

Table 3.1a. Pyruvate dehydrogenase activity, adipocyte mean volume, fat pad weight and adipocytes per fat pad in parametrial white adipose tissue from virgin and pregnant rats

Parametrial white adipose tissue was removed from age-matched rats under pentobarbital anaesthesia and samples of the tissue were immediately frozen for the subsequent extraction of PDH activity as described in chapter 2. Initial PDH activity is the active PDH in the homogenates immediately after thawing and total PDH activity was measured after pre-incubation with $MgCl_2$, $CaCl_2$ and PDH-phosphatase as described in chapter 2. The number of adipocytes per fat pad was calculated as described in chapter 2. Results are means ± SEM. There were no significant differences between any of the values for pregnant rats and the values for virgin rats.

State..	VIRGIN	PREGNANT		
Day....	-	7	14	20
Mean adipocyte volume (pl)	444± 62	474± 134	455 ± 69	421 ± 60
Fat pad weight (g)	2.2± 0.2	2.4± 0.5	2.8 ± 0.3	2.7 ± 0.2
Adipocytes per fat pad (x 10^{-6})	4.5± 0.6	5.6± 0.6	5.5 ± 0.6	6.3 ± 0.9
PYRUVATE DEHYDROGENASE ACTIVITY				
Initial (munit/g tissue)	38.8± 6.3	55.9±12.7	33.5 ± 9.4	38.9± 9.6
Total (munit/g tissue)	108.9±16.3	97.6±17.3	110.9±20.1	99.7±14.8
Initial (munit/fat pad)	82.8±12.3	120.1±22.0	94.2±28.0	104.7±27.5
Total (munit/fat pad)	236.3±36.0	224.2±39.6	313.2 ± 63.5	267.1± 40.8
Number of observations	7	5	4	5

Table 3.1b. Pyruvate dehydrogenase activity, adipocyte mean volume, fat pad weight and adipocytes per fat pad in parametrial white adipose tissue from virgin and lactating rats

For experimental procedure see the legend to table 3.1a. Results are means ± SEM; *, *** indicate the value is significantly different from the value for virgin rats; $P<0.05$, <0.01 respectively. Values for age-matched pregnant rats are given in table 3.1a.

State..	VIRGIN	LACTATING			
Day...	-	1	3	7	14
Mean adipocyte volume (pl)	444 ± 62	385±30	618 ± 71	467 ± 48	208 ± 25***
Fat pad weight (g)	2.2 ± 0.2	2.1 ± 0.1	2.6 ± 0.2	2.0 ± 0.4	1.4 ± 0.2*
Adipocytes per fat pad ($\times 10^{-6}$)	4.5 ± 0.6	5.2 ± 0.3	4.0 ± 0.2	4.2 ± 0.9	5.8 ± 0.6
PYRUVATE DEHYDROGENASE ACTIVITY					
Initial (munit/g tissue)	38.8 ± 6.3	48.4±19.1	26.4 ± 1.9	24.5 ± 7.4	36.3 ± 9.1
Total (munit/g tissue)	108.9±16.3	121.3±31.5	64.5 ± 5.4	67.4±11.0	125.0±31.4
Initial (munit/fat pad)	82.8±12.3	112.0±44.3	69.4±9.6	43.3± 6.6	43.9± 6.6*
Total (munit/fat pad)	236.3±36.0	281.0±74.9	164.5±13.3	136.2±45.1	150.4±38.8
Number of observations	7	3	4	3	5

Table 3.2a. Pyruvate dehydrogenase activity and protein concentration in skeletal muscle of virgin and pregnant rats

Skeletal muscle was removed from the hind-limb of age-matched rats under pentobarbital anaesthesia and was immediately frozen in liquid N_2 for the determination of PDH activity as described in the legend to table 3.1a. Results are means ± SEM, ** indicates the value is significantly different from the value for virgin rats: $P<0.02$.

State..	VIRGIN	PREGNANT		
Day...	-	7	14	20
Protein concentration (mg/g tissue)	118 ± 8	124±13	127± 7	137± 7
PYRUVATE DEHYDROGENASE ACTIVITY				
Initial (munit/g tissue)	555±117	993±46**	963±46**	377±22
Total (munit/g tissue)	1765±170	1820±65	1798±140	1555±178
Number of observations	7	5	5	4

Table 3.2b. Pyruvate dehydrogenase activity and protein concentration in skeletal muscle of virgin and lactating rats

For the experimental procedure see the legend to table 3.2a. Results are means ± SEM. There were no significant differences between the values for lactating rats and those for virgin rats. Values for age-matched pregnant rats are given in table 3.2.a.

State...	VIRGIN	LACTATING			
Day..	-	1	3	7	14
Protein concentration (mg/g tissue)	118 ± 8	141 ± 5	125 ± 6	139 ± 6	97 ± 1
PYRUVATE DEHYDROGENASE ACTIVITY					
Initial (munit/g tissue)	555±117	909±155	660 ± 43	737 ± 25	304 ± 34
Total (munit/g tissue)	1765±170	1537± 89	1385 ± 53	1568 ± 17	1298±240
Number of observations	7	3	4	3	5

Table 3.3a. Hepatic pyruvate dehydrogenase activity and protein concentration and liver weight of virgin and pregnant rats

The liver was removed from age-matched rats under pentobarbital anaesthesia and a sample was immediately frozen in liquid N_2 for determination of PDH activity as described in the legend to table 3.1a. Results are means ± SEM; *, ***, ****, indicate the value is significantly different from the value for virgin rats: $P<0.05$, <0.01, <0.001 respectively.

State...	VIRGIN	PREGNANT		
Day...	-	7	14	20
Liver weight (g)	10.6± 0.4	11.9± 0.3*	14.8± 0.7****	14.4± 0.9***
Protein concentration (mg/g tissue)	183 ± 14	131 ± 22	167 ± 15	162 ± 16
PYRUVATE DEHYDROGENASE ACTIVITY				
Initial (munit/g tissue)	310.0± 22.8	226.8± 29.4*	256.7±36.6	268.7± 46.1
Total (munit/g tissue)	1353±157	1154± 140	1084± 27	1175± 122
Initial (unit/liver)	3.3± 0.2	2.7± 0.3	3.7± 0.6	4.0± 0.9
Total (unit/liver)	14.3± 1.8	13.7± 1.5	15.5± 0.9	17.2± 2.5
Number of observations	7	5	5	5

Table 3.3b. Hepatic pyruvate dehydrogenase activity and protein concentration and liver weight of virgin and lactating rats

For the experimental procedure see the legend to table 3.3a. Results are means ± SEM; **, ***, **** indicate the value is significantly different from the value for virgin rats, P<0.02, <0.01, <0.001 respectively. The results for age-matched pregnant rats are given in table 3.3a.

State..	VIRGIN	LACTATING			
Day	–	1	3	7	14
Liver weight (g)	10.6 ± 0.4	12.8 ± 0.6**	14.1 ± 0.5***	14.9 ± 0.6****	15.6 ± 0.4****
Protein concentration (mg/g tissue)	183 ± 14	184 ± 3	192 ± 17	183 ± 13	204 ± 5
PYRUVATE DEHYDROGENASE ACTIVITY					
Initial (munit/g tissue)	310.0±22.8	166.5±26.7 ***	226.2±27.7	255.4 ± 40.6	319.3 ± 33.9
Total (munit/g tissue)	1353 ± 157	1497 ± 72	1418 ± 201	1658 ± 268	1346 ± 182
Initial (unit/liver)	3.3 ± 0.2	2.2 ± 0.4*	3.1 ± 0.4	3.6 ± 0.5	4.9 ± 0.6**
Total (unit/liver)	14.3 ± 1.8	19.4 ± 1.9	19.4 ± 3.0	22.5 ± 5.2	20.9 ± 3.2
Number of observations	7	3	4	3	5

CHAPTER 4

The effect of insulin *in vivo* on pyruvate dehydrogenase activity of white adipose tissue, liver, skeletal muscle and mammary gland during the reproductive cycle in the rat.

4.1. Introduction

By no means all of the tissue specific changes in PDH activity during the reproductive cycle in the rat can be explained by changes in the serum insulin concentration or insulin binding capacity of the tissues (see chapter 3): some of the changes in PDH activity suggest that the response of the tissues involved to insulin is modified during pregnancy and lactation (see section 3.4.).

It is already known that tissue specific adaptations occur in the endocrine control of glucose metabolism in the rat during pregnancy and lactation (see section 1.1.) and in view of this the effects of insulin on PDH activity of rat white adipose tissue, skeletal muscle, liver and mammary gland during the reproductive cycle were investigated.

4.2. Experimental Procedure

Age-matched (13-16 week old) virgin, 18 day pregnant, 14 day lactating and 2 day weaned (pups removed at day 14 of lactation) rats were under anaesthesia throughout the experiment (see section 2.5.). All rats received propranolol (1.5mg/kg body wt.), to block the effects of endogenous catecholamines on white adipose tissue PDH activity (see chapter 5), and care was taken to ensure the rats were not stressed: all animals were handled regularly prior to the

experiment and were injected with fine needles (26 gauge). Experiments were carried out between 12.00h and 13.00h. Propranolol (1.5mg/kg body wt.) was injected intraperitoneally along with the pentobarbital anaesthetic (60mg/kg body wt.). Ten minutes later rats were injected intraperitoneally with either glucose (1g/kg body wt.), either alone or along with insulin (0.4mg in 0.4ml 0.15M NaCl per rat) or 0.15M NaCl and after a further 20 min samples of parametrial white adipose tissue, skeletal muscle from the hind-limb, liver and mammary gland were removed and immediately frozen in liquid N_2 for subsequent extraction and assay of PDH activity as described in section 2.3. When lactating rats were given glucose alone or along with insulin they received a second identical glucose load (1g/kg body wt.) 10 min after the first and after a further 10 min the tissues were removed.

Samples of blood were also taken from the heart and serum was prepared as described in section 2.6. Additional samples of parametrial white adipose tissue were also taken and stored at 37°C in isotonic saline for determination of cellularity as described in section 2.8.2. Further details of the experimental procedure are given in section 2.5.

4.3. Results

4.3.1. Serum insulin and glucose concentration

There was no significant difference in the basal serum glucose concentration (measured 20 min after injection of saline) between any of the groups of rats studied (table 4.1a,b). The basal serum insulin concentration was lower in 14 day lactating rats than in virgin rats ($P<0.01$, table 4.1a,b); weaning increased the basal serum insulin concentration ($P<0.001$ compared with lactating rats). The

insulin:glucose ratio in saline treated rats (table 4.1a,b) was greater in 18 day pregnant animals ($P<0.05$, table 4.1a) and lower in lactating rats ($P<0.01$, table 4.1b) than in virgin rats (table 4.1a); weaning increased this ratio ($P<0.001$ compared with lactating rats; table 4.1b).

Twenty minutes after injection of glucose the serum glucose concentration was elevated in all the groups of rats studied (table 4.1a,b) but this increase was significant in the virgin ($P<0.01$) and pregnant ($P<0.01$) rats only. At the same time the serum insulin concentration, was increased in all the groups of rats studied (table 4.1a,b) and this increase was significant in the pregnant ($P<0.01$) and lactating ($P<0.01$) rats. Twenty minutes after receiving a glucose load the serum insulin concentration was greater in the pregnant rats ($P<0.02$) than in virgin rats and was lower in lactating rats than in either the virgin ($P<0.01$) or weaned rats ($P<0.01$) even though the lactating animals had received two glucose loads while the others were given only one. The serum insulin concentration was increased to values greater than 0.5munit/ml in both virgin ($P<0.001$, table 4.1a) and lactating ($P<0.001$, table 4.1b) rats twenty minutes after injection of insulin plus glucose.

4.3.2. Parametrial white adipose tissue

Neither injection of glucose or glucose plus insulin had any effect on total PDH in white adipose tissue in any of the groups of rats studied and so the pooled values for total PDH activity in each group of rats are given in table 4.2. In agreement with the results in chapter 3 total PDH activity in parametrial white adipose tissue from 14 day lactating rats was lower than in virgin rats ($P<0.001$; table 4.2.). Total PDH had recovered by 2 days after litter removal ($P<0.001$; compared to lactating rats; table 4.2.) and weaning also induced an increase in the proportion of PDH in the active state when

compared to values for either virgin ($P<0.001$) or lactating ($P<0.01$) rats (table 4.3.).

Injection of glucose increased the proportion of PDH in the active state in white adipose tissue in virgin ($P<0.001$; table 4.3.) and 18 day pregnant ($P<0.02$; table 4.3.) rats by 2-3 fold: injection of glucose plus insulin had a similar effect in virgin rats ($P<0.001$; table 4.3.). Glucose injections had no effect on PDH activity of white adipose tissue in 14 day lactating or 2 day weaned rats (table 4.3.) and the injection of insulin along with glucose had no effect on the enzyme activity in white adipose tissue of lactating rats (table 4.3.).

4.3.3. Skeletal muscle

Total PDH in skeletal muscle was unaffected by the injection of glucose or glucose plus insulin and so the pooled values for each group of rats are given in table 4.4. Total PDH was lower in 18 day pregnant rats than in virgin rats ($P<0.001$; table 4.4.) and the proportion of PDH in the active state was reduced during lactation ($P<0.001$ compared to virgin rats; table 4.5.): these results are compared to those in chapter 3 and discussed in section 3.4. Weaning resulted in an increase in the proportion of PDH in the active state when compared to the values for both virgin and lactating rats ($P<0.01$; table 4.5.) and in addition there was a small but significant decrease in total PDH in skeletal muscle of weaned rats when compared to virgin ($P<0.05$) and lactating ($P<0.01$) rats (table 4.4.).

Injection of glucose increased the proportion of PDH in the active state by 2-3 fold in virgin ($P<0.001$), 18 day pregnant

($P<0.001$) and 14 day lactating ($P<0.001$) rats but had no effect on PDH activity in skeletal muscle in weaned rats (table 4.5.). Injection of insulin plus glucose increased the proportion of PDH in the active state, to a similar value, in virgin ($P<0.001$) and 14 day lactating ($P<0.001$) rats (table 4.5.).

4.3.4. Liver

Total PDH in liver was not affected by the injection of glucose or glucose plus insulin in any of the groups of rats studied and so the pooled values for each group of rats are given in table 4.4. As found in chapter 3 both total PDH (table 4.4.) and the proportion of the enzyme in the active state (table 4.5.) were the same in liver from virgin and lactating rats.

Injection of glucose had no effect on hepatic PDH activity in virgin, lactating or weaned rats (table 4.5.) while the injection of glucose plus insulin increased the proportion of hepatic PDH in the active state in virgin ($P<0.05$) and 14 day lactating ($P<0.05$) rats (table 4.5.) to a similar extent.

4.3.5. Mammary gland

The protein concentration in the mammary gland increased significantly between day 18 of pregnancy and day 14 of lactation ($P<0.001$; table 4.4.) then fell on weaning ($P<0.001$; table 4.4.). Total PDH activity ($P<0.001$; table 4.4.) and the proportion of the enzyme in the active state ($P<0.001$; table 4.5.) increased substantially between day 18 of pregnancy and the fourteenth day of lactation; both then fell on weaning ($P<0.001$).

Neither injection of glucose or glucose plus insulin had any effect on total PDH activity in the mammary gland and the pooled

values for each group of rats are given in table 4.4. Injection of glucose resulted in a significant increase in the proportion of PDH in the active state in mammary gland of 14 day lactating ($P<0.02$) and 18 day pregnant ($P<0.02$) rats (table 4.5.): injection of insulin along with the glucose had no greater effect on mammary PDH activity in 14 day lactating rats ($P<0.01$; table 4.5.) than injecting glucose alone.

4.4. Discussion

The response of PDH to insulin was assessed by injecting a large dose of insulin plus glucose to avoid concomitant hypoglycaemia and secondly by injecting glucose alone to induce the release of endogenous insulin and so expose the tissues to a more physiological surge of the hormone. The amount of glucose used was the same as that used by Stansbie et al. (1976a) and by Burnol et al. (1983). Intraperitoneal injection of glucose was used as this leads to a rise in serum insulin which appears to remain constant between 10 and 30 min after injection (Bonner-Weir et al., 1983). Whereas intravenous injection leads to a much more rapid (and larger) rise and then fall in serum insulin (Burnol et al., 1983; Bonner-Weir et al., 1983). The concentrations of insulin and glucose were measured in serum prepared from blood samples, collected at the time of tissue removal, to ascertain that a rise in the circulating insulin concentration had actually been achieved and that hypoglycaemia had not occurred.

The relatively high basal serum insulin:glucose ratio and serum insulin concentration after the glucose load in the pregnant rats as compared to virgin rats (table 4.1a) is in agreement with the findings of Leturque et al. (1980) and is probably due to the greater

responsiveness of the pancreas to insulinotropic agents during pregnancy (Malaisse et al., 1969; Kalkhoff & Kim, 1978). The low basal serum insulin concentration in the lactating rats (table 4.1b) is in agreement with other studies (Robinson et al., 1978; Agius et al., 1979; Flint et al., 1979b; Burnol et al., 1983). Jones et al. (1984) suggested that the high rate of glucose utilisation by the mammary gland of lactating rats leads to a fall in the plasma glucose concentration and thereby to a secondary fall in plasma insulin. It was necessary to give lactating rats a second dose of glucose 10 min after the first to ensure that the serum insulin concentration was elevated at the time of tissue removal: Burnol et al. (1983) found that serum insulin concentration declined much more rapidly after a glucose load in lactating than non-lactating rats. This was probably due to the greater rate of glucose clearance and hence more rapid fall in serum glucose levels that occurs during lactation (Burnol et al., 1983). In addition there is a 12-fold increase in the uptake of insulin by the mammary gland during lactation (Jones et al., 1984). The increase in the serum glucose and insulin concentrations upon weaning is consistent with the results of others (Agius et al., 1979; Flint et al., 1981; Burnol et al., 1983) and is probably primarily due to the decrease in glucose utilisation by the mammary gland (Burnol et al., 1983).

The time interval between injection and sampling (20 min) was based on a study by Stansbie et al. (1976a) which showed that the increase in PDH activity of white adipose tissue induced by a glucose load did not vary between 10 and 30 min after sampling. In addition a preliminary experiment showed that the serum glucose concentration

was greatest between 10 and 15 min after intraperitoneal injection of glucose (fig. 4.1.). The 2-3 fold increase in the proportion of PDH in the active state in white adipose tissue of virgin and pregnant rats following injection of glucose is similar to the effect reported in male rats (Stansbie et al., 1976a). In contrast to the effect in virgin and pregnant rats injection of glucose failed to stimulate PDH in white adipose tissue of lactating rats. The lack of effect in lactating rats was not due to the lower serum insulin concentration as injection of a high dose of insulin plus glucose, which increased serum insulin concentration to over 0.5munit/ml, also had no effect on PDH activity of white adipose tissue showing that the tissue had become completely refractory to insulin. As the number and affinity of insulin receptors of white adipocytes is similar in virgin and lactating rats (Flint et al., 1979b) this loss of response to insulin must involve either a systemic factor or a post-receptor defect in the insulin transducing system of the tissue. This loss of ability of insulin to stimulate PDH of white adipose tissue could account for the failure of the hormone to stimulate fatty acid synthesis in the tissue in vivo during lactation (Burnol et al., 1983; Jones et al., 1984) although ACC and fatty acid synthetase activities in the tissue are extremely low during lactation (Sinnett-Smith et al., 1980) which will limit the rate of fatty acid synthesis and may account for the inability to stimulate the pathway. The inability of insulin to stimulate PDH during lactation would account for the findings of Burnol et al. (1986) that insulin stimulation of glucose conversion to CO_2 and fatty acids, but not to lactate and acylglycerol glyceride is impaired in isolated adipocytes from lactating rats.

Injection of glucose also failed to stimulate PDH of white adipose tissue in weaned rats: the proportion of PDH in the active state was similar in white adipose tissue of weaned rats given saline to that of virgin rats given glucose suggesting that the enzyme was already activated to the maximum extent possible in response to insulin. Glucose has a greater insulinotropic effect in weaned rats than in virgin rats (Burnol et al., 1983; table 4.1.) so the lack of stimulation of PDH activity was not due to a lower serum insulin concentration. Burnol et al. (1983) showed that the rate of lipogenesis in white adipose tissue from weaned rats did not respond to insulin in vivo and that the rate of white adipose tissue lipogenesis in saline treated weaned animals was similar to that of non-lactating rats exposed to insulin. The increase in white adipose tissue PDH activity which occurs after the removal of pups probably contributes to the increase in the rates of glucose oxidation (Smith, 1973b) and fatty acid synthesis (Smith, 1973b; Scow et al., 1977; Agius et al., 1979; Flint et al., 1981) which also occur in the tissue.

Injection of glucose resulted in a 2-3 fold increase in the proportion of PDH in the active state in skeletal muscle of virgin, pregnant and lactating rats. During pregnancy the proportion of PDH in the active state in skeletal muscle of both saline and glucose injected animals was lower than in virgin rats given the same treatment even though the serum insulin concentration in the pregnant rats was similar to or greater than in virgin animals and this suggests that the effects of insulin on skeletal muscle are muted during pregnancy. The ability of insulin to stimulate glucose

utilisation by the perfused rat hind-limb (Rushakoff & Kalkhoff, 1981) and by rat skeletal muscle in vivo (Leturque et al., 1986) is muted during late pregnancy and a diminished effect of insulin on muscle PDH activity could account for this. The proportion of muscle PDH in the active state was lower in lactating rats than in virgin rats after the injection of either saline or glucose. After the injection of glucose plus insulin, which raised the serum insulin concentration to over 0.5munit/ml, the proportion of PDH in the active state was similar in skeletal muscle from virgin and lactating rats. Thus the response of the tissue to insulin is not lost during lactation but it is not clear from this study whether the sensitivity of muscle PDH to insulin changes during lactation. Injection of glucose had no effect on skeletal muscle PDH activity in weaned rats and, as in white adipose tissue, the proportion of PDH in the active state in skeletal muscle of weaned rats given saline was similar to the value in virgin rats given glucose. However the proportion of muscle PDH in the active state in virgin rats can be further increased by the administration of glucose plus insulin and therefore either the tissue becomes completely resistant to insulin during weaning or the serum insulin concentration achieved after injection of weaned rats with the glucose load was insufficient to increase further muscle PDH activity.

The increase in the proportion of PDH in the active state in skeletal muscle and also white adipose tissue after the removal of pups for 48h may be due to the increase in the serum insulin concentration and insulin binding capacity of adipocytes (Flint et al., 1981); this implies that the resistance of white adipose tissue

PDH activity to insulin which exists during lactation is lost within 48h after removal of pups.

Glucose or glucose plus insulin both increased the proportion of mammary PDH in the active state to a similar extent in lactating rats. A similar effect of insulin has been found in 24h-fasted and diabetic lactating rats in which the proportion of PDH present in the active state was markedly reduced compared to that of fed lactating rats (Field & Coore, 1976; Baxter & Coore, 1978) but studies to date on the effect of insulin *in vitro* on mammary PDH have been hindered by the lack of a suitable preparation of mammary tissue (see Goheer & Coore, 1977). Further studies *in vitro* are necessary to establish if the stimulation of mammary PDH by insulin *in vivo* is a direct effect of the hormone upon the mammary gland or is secondary to some other effect of insulin such as the lowering of the circulating free fatty acid concentration. Even if the effect of insulin is a direct effect upon the gland, the stimulation of PDH may be secondary to the stimulation of glucose uptake by the gland (see Field & Coore, 1976).

Injection of glucose had no effect on hepatic PDH activity in virgin, lactating or weaned rats which confirms the results of Stansbie *et al.* (1976a) who made a similar observation for male rats. The administration of insulin plus glucose was required to stimulate hepatic PDH activity in virgin and lactating rats: the increase in hepatic PDH activity in virgin rats after this treatment was much less than the increase in skeletal muscle and white adipose tissue PDH activity. Activation of PDH by insulin appears to be less important in the liver than in white adipose tissue or skeletal muscle (see section 1.2.3.1.2.) which may explain the lack of change

in hepatic PDH activity during pregnancy, lactation and weaning (see chapter 3) when variations occur in the serum insulin concentration and insulin binding capacity of hepatocytes (Flint, 1980). Although weaning had no effect on hepatic PDH activity the rates of glucose oxidation (Smith, 1973a) and of fatty acid synthesis (Smith, 1973a; Agius et al., 1979) in liver are increased and this is accompanied by increases in the activities of ACC (Smith, 1973a; Zammit & Corstorphine, 1982a) and the glycolytic enzyme pyruvate kinase (Smith, 1975). This suggests that the flux through hepatic PDH during lactation is insufficient to saturate the enzyme and so it is not necessary for PDH activity in the liver to increase to accommodate increases in the rate of conversion of glucose to CO_2 and fatty acids during weaning.

4.5. Conclusions

The results presented in this chapter along with those in chapter 3 show that not only do tissue specific changes occur in PDH activity during the reproductive cycle in the rat but also tissue specific adaptations occur in the reponse of PDH to insulin. The complete loss of response of white adipose tissue PDH activity to insulin during lactation will augment the effects of the decrease in total PDH in this tissue (see chapter 3). Together these adaptations will help to prevent the increase in the flux of glucose to acetyl-CoA which would normally occur in white adipose tissue when the circulating glucose concentration is increased on feeding. Also an increase should occur in the proportion of glucose utilised by white adipose tissue that is released as lactate: Burnol et al. (1986) found no change in the ability of insulin to stimulate the

conversion of glucose to lactate in isolated adipocytes from lactating rats but another study *in vitro* has shown that this effect of insulin on white adipose tissue is muted during lactation (R. G. Vernon, unpublished observation). The lactate released by white adipose tissue *in vivo* during lactation is probably used for gluconeogenesis in the liver and will ultimately be directed to the mammary gland for milk production (Williamson *et al.*, 1985). The resistance of white adipose tissue PDH to activation by insulin during lactation and the decrease in PDH activity in both white adipose tissue and skeletal muscle along with the reciprocal rise in PDH activity in the mammary gland will thus facilitate the preferential utilisation of glucose carbon by the mammary gland for milk production.

Fig. 4.1. The serum glucose concentration after intraperitoneal injection of rats with saline or glucose

Virgin female rats were under pentobarbital anaesthesia throughout the experiment. Pentobarbital (60mg/kg body wt.) was injected intraperitoneally and 10 min later glucose (1g/kg body wt.) or 0.15M saline were injected intraperitoneally then during the next 20 min blood samples from the tail vein were collected over each 5 min period. Shaded areas indicate the serum glucose concentration after treatment with glucose. The experiments were performed between 10.00h and 11.00h. Procedures for the preparation of serum and assay of glucose are given in chapter 2. Only one rat received each treatment.

FIG.4.1.

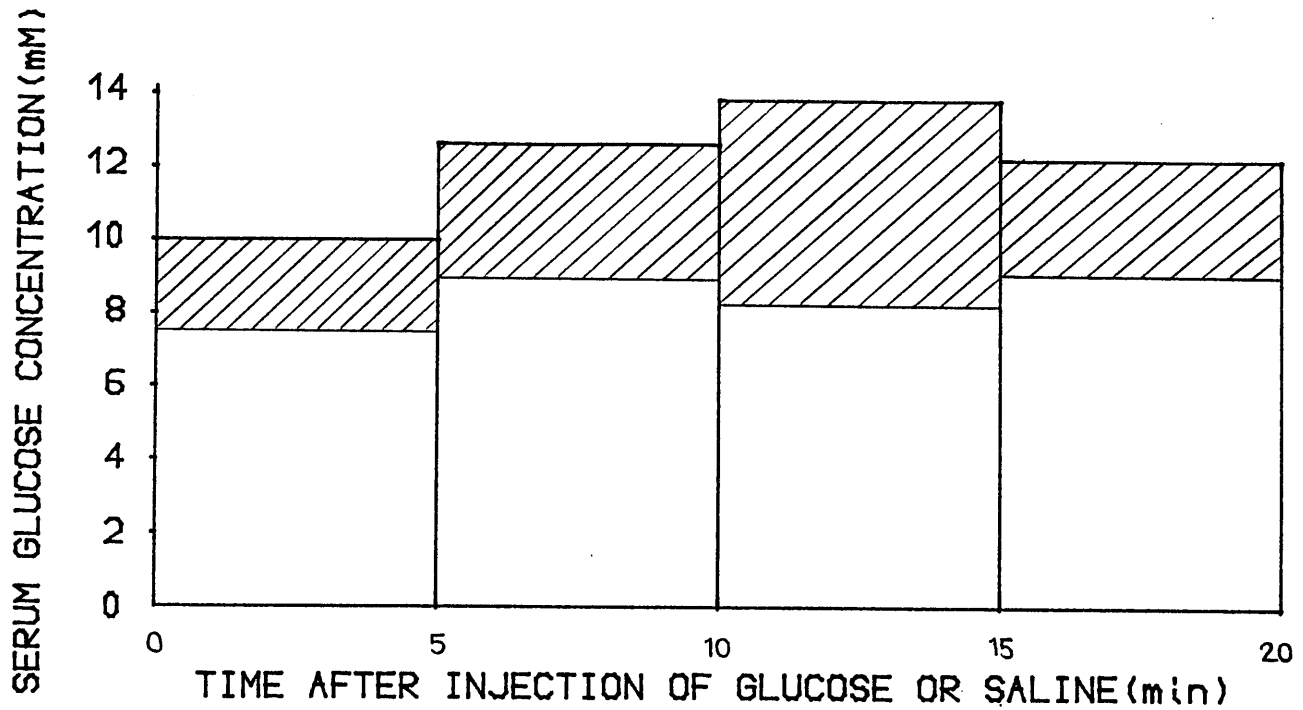

SERUM GLUCOSE CONCENTRATION(mM)
14
12
10
8
6
4
2
0
0
5
10
15
20
TIME AFTER INJECTION OF GLUCOSE OR SALINE(min)

Table 4.1a. The serum insulin and glucose concentrations of virgin and pregnant rats

Rats were under pentobarbital anaesthesia throughout the experiment. All rats received propranolol (1.5mg/kg body wt) which was injected along with the pentobarbital (60mg/kg body wt) 10 min prior to glucose (1g/kg body wt.), 0.15M saline or insulin (6units per rat) plus glucose (1g/kg body wt.) which were injected intraperitoneally 20 min prior to a blood sample being collected from the heart. Procedures for the preparation of serum and the assay of insulin and glucose are given in chapter 2. Results are mean ± SEM; ***, **** indicate the value is significantly different from that of the appropriate rats given saline: P<0.01, <0.001 respectively.

State.....	Virgin			18 day pregnant	
Treatment...	Saline	Glucose	Insulin + glucose	Saline	Glucose
Glucose concentration (mM)	8.7±0.8	16.8±2.2***	10.5±3.6	6.6±0.7	16.4±2.4***
Insulin concentration (μunit/ml)	72.5±15.0	115.0±22.5	665.0±27.5****	82.5±12.5	257.5±40.0***
Ratio of insulin:glucose (μunit/nmol)	8.2±1.5			13.2±1.0	
Number of observations	5	5	3	4	4

Table 4.1b. The serum insulin and glucose concentrations of lactating and weaned rats

The experimental procedure is described in the legend to table 4.1a. When lactating rats were given glucose alone or along with insulin they received a second glucose injection (1g/kg body wt.) 10 min later and the blood sample was collected after a further 10 min. Results are means ± SEM; ***, **** indicate the value is significantly different from that of the appropriate rats given saline: P<0.01, <0.001 respectively.

State....	14 day lactating			2 day weaned	
Treatment..	Saline	Glucose	Insulin + glucose	Saline	Glucose
Glucose concentration (mM)	7.0±0.6	13.9±3.1	8.5±1.6	7.9±0.4	13.3±2.9
Insulin concentration (μunit/ml)	15±2.5	40±7.5 ***	617.5±60.0 ****	90.0±15.0	147.5±25.0
Ratio of insulin:glucose (μunit/nmol)	2.5±0.5			10.2±1.0	
Number of observations	6	6	4	4	5

Table 4.2. Total pyruvate dehydrogenase activity, mean adipocyte volume and adipocyte content in parametrial white adipose tissue of rats at different stages of the reproductive cycle

Parametrial white adipose tissue was obtained from rats given the various treatments in table 4.1.; as these treatments had no effect on total PDH activity, mean adipocyte volume or the adipocyte content of the tissue the values were pooled. Total PDH activity was measured after incubating the tissue with $MgCl_2$, $CaCl_2$ and PDH-phosphatase; further details of the extraction and assay of PDH activity are in chapter 2. Results are means ± SEM; *, **** indicate the value is significantly different from the value for virgin rats, $P<0.05$, <0.001 respectively.

State...	Virgin	Pregnant (18 day)	Lactating (14 day)	Weaned (2 day)
Mean adipocyte volume (pl)	646±94	750±99	410±36*	426±46
Adipocytes per gram tissue ($x10^{-6}$)	1.7±0.2	1.5±0.3	2.0±0.2	1.9±0.2
Total PDH activity (munit/10^6 adipocytes)	50.5±4.5	49.3±10.5	25.7±2.7****	61.4±7.6
Number of observations	14	8	16	10

Table 4.3. The effect of injection of glucose or glucose plus insulin on pyruvate dehydrogenase activity in parametrial white adipose tissue from rats at different stages of the reproductive cycle

Rats were under pentobarbital anaesthesia throughout the experiment. All rats received propranolol (1.5mg/kg body wt.) which was injected intraperitoneally along with the pentobarbital (60mg/kg body wt.) ten minutes prior to the intraperitoneal injection of glucose (1g/kg body wt.), 0.15M saline or glucose (1g/kg body wt.) plus insulin (6 unit per rat) and after a further 20 min samples of tissue were removed and immediately frozen in liquid N_2. When lactating rats were injected with glucose or glucose plus insulin they received a second glucose injection (1g/kg body wt.) 10 min after the first and the tissue was removed after a further 10 min. Further details of the experimental procedure and the methods of extraction and assay of PDH activity are given in the methods section and chapter 2. Results are means ± SEM for the number of observations in parenthesis; **, **** indicate the value is significantly different from the value for rats given saline, $P<0.02$, <0.001 respectively.

		pyruvate dehydrogenase (proportion in active state x 100)			
Treatment	State.....	Virgin	Pregnant (18 day)	Lactating (14 day)	Weaned (2 day)
Saline		21.0±3.1(5)	29.9±5.8(4)	27.2±3.7(6)	58.7±5.7(4)
Glucose		61.7±8.0(5)****	57.9±5.9(4)**	26.6±3.4(6)	53.8±4.8(5)
Glucose + insulin		71.2±1.4(4)****	-	28.5±2.4(4)	-

Table 4.4. Total pyruvate dehydrogenase activity of liver, skeletal muscle and mammary gland and the protein concentration in mammary gland in rats at different stages of the reproductive cycle

Tissues were obtained from rats given the treatments in table 4.1.; as these treatments had no effect on total PDH activity or the protein concentration in the tissue the values were pooled. No significant change in protein concentration in liver or skeletal muscle occurred during the reproductive cycle: the pooled values were 204 ± 3(31) mg protein/g tissue for liver and 109 ± 1(38) mg protein/g tissue for skeletal muscle. Total PDH was measured after incubation of the tissue homogenates with $MgCl_2$, $CaCl_2$ and PDH-phosphatase as described in chapter 2. Results are means ± SEM for the number of observations in parenthesis; *, **** indicate the value is significantly different from the value for virgin rats: $P<0.05$, <0.001 respectively.

Tissue	State....	Virgin	Pregnant (18 days)	Lactating (14 days)	Weaned (2 days)
Liver	Total PDH activity (unit/g protein)	8.9±0.4(13)	-	9.0±0.3(12)	9.0±0.1(6)
Skeletal muscle	Total PDH activity (unit/g protein)	21.2±0.4(11)	5.2±0.4(7)****	22.7±0.8(10)	18.2±1.2(10)*
Mammary gland	Total PDH activity (unit/g protein)	-	4.9±0.5(8)	8.1±0.3(16)	4.0±0.5(9)
	Protein concentration (mg/g tissue)	-	99±2(8)	130±1(16)	87±4(9)

Table 4.5. The effect of injection of glucose or glucose plus insulin on pyruvate dehydrogenase activity in liver, skeletal muscle and mammary gland of rats at various stages of the reproductive cycle

For the experimental procedure see the legend to table 4.3. Results are means ± SEM for the number of observations in parenthesis; *, **, ***, **** indicate the value is significantly different from the value for rats given saline: P<0.05, <0.02, <0.01, <0.001 respectively.

			Pyruvate dehydrogenase (proportion in active state x 100)			
Tissue	Treatment	State....	Virgin	Pregnant (18 day)	Lactating (14 day)	Weaned (2 day)
Liver	Saline		27.4±1.6(5)	-	32.8±1.4(4)	32.2±1.8(3)
	Glucose		30.9±2.3(5)	-	35.0±1.0(4)	30.1±1.2(3)
	Glucose + insulin		36.6±3.3(3)*	-	40.5±1.5(6)*	-
Skeletal muscle	Saline		10.0±0.1 (4)	7.1± 0.5(4)	3.4±0.5(3)	24.8±3.6(5)
	Glucose		27.0±0.9(4)****	17. 1±0.3(3)****	8.4±0.4(3)****	22.8±3.2(5)
	Glucose + insulin		70.1±3.3(3)****	-	60.9±6.4(3)****	-

Table 4.5. continued

Mammary gland	Saline	–	8.6±0.3(4)	55.4±2.1(7)	13.4±3.0(4)
	Glucose	–	21.4±3.9(4)**	66.7±3.7(5)**	30.6±9.9(5)
	Glucose + insulin	–	–	71.6±4.0(4)***	–

CHAPTER 5

Tissue specific control of pyruvate dehydrogenase activity by catecholamines in the rat

5.1. Introduction

While studying the effects of insulin on PDH activity in rat white adipose tissue both *in vitro* (chapter 6) and *in vivo* (chapter 4) it became apparent that the proportion of PDH in the active state in the tissue was increased by the effects of stress on the animals: this interfered with the characterisation of the effects of insulin on the enzyme. Severe stress results in a massive surge in sympathetic nervous activity (see Callingham & Barrand, 1979) suggesting that catecholamines may be responsible for the stress induced stimulation of PDH activity in white adipose tissue. Previous studies on the effects of catecholamines on PDH of white adipose tissue have produced conflicting results as the hormones have been reported to inhibit (Coore *et al.*, 1971); activate (Taylor *et al.*, 1973; Weiss *et al.*, 1974) or have biphasic effects on (Sica & Cuatrecasas, 1973; Smith & Saggerson, 1978; Cheng & Larner, 1985) the enzyme. However all of these experiments were performed *in vitro*. Therefore I carried out studies to establish the effects of catecholamines on white adipose tissue PDH activity *in vivo* and then further characterised these effects using isolated adipocytes *in vitro*. The results show that catecholamines activate PDH of white adipose tissue both *in vivo* and *in vitro*.

In chapter 4 it was found that tissue specific changes occur in the control of PDH activity by insulin during lactation in the rat. In the present chapter evidence is presented to show that tissue specific adaptations occur also in the control of PDH activity by catecholamines in the lactating rat.

5.2. Experimental procedure

Rats were maintained on a 12h light-dark cycle (light phase from 08.00-20.00h) except for one group which was maintained on a reversed cycle (darkness between 08.00 and 20.00h) for 4 weeks prior to use. All rats were handled regularly prior to the experiments and experiments were performed between 12.00h and 13.00h unless otherwise stated in the table legends.

Studies in vivo

All agents were dissolved in 0.15M NaCl unless otherwise stated and were injected intraperitoneally with fine needles (26 gauge) and minimum stress. Rats were anaesthetised with pentobarbital (60mg/kg body wt.) and remained anaesthetised throughout the experiment. Propranolol (1.5mg/kg body wt.) was administered along with the pentobarbital whereas yohimbine (5mg/kg body wt.) or prazosin (5mg/kg body wt.) were injected 5 min after the pentobarbital as injection of these agents into conscious rats caused obvious distress. Prazosin was dissolved in 40mM lactic acid and then diluted with water. Glucose (1g/kg body wt.), noradrenaline (1.25mg/kg body wt.), adrenaline (1.25mg/kg body wt.) or 0.15M NaCl were injected 10 min after the pentobarbital and 20 min later samples of parametrial or epididymal white adipose tissue, liver, skeletal muscle from the hind-limb and mammary gland were removed and

immediately frozen in liquid N_2 for the subsequent extraction and assay of PDH and ACC activities as described in chapter 2. A blood sample was taken from the heart and serum was prepared as described in section 2.6. Additional samples of adipose tissue were also taken and stored at 37°C in isotonic saline for determination of cellularity as described in section 2.8.2. Further details of the experimental procedure are in section 2.5.

Studies in vitro

Parametrial white adipose tissue was removed from virgin or 14 day lactating rats under pentobarbital anaesthesia (achieved as described in the preceding section) and adipocytes were prepared as described in section 2.8.1. Adipocytes were incubated in Krebs-Ringer bicarbonate buffer containing 5mM glucose and 40mg/ml BSA (see section 2.8.1.) in the presence of agents as described in the legends to tables and figures. Incubations were carried out at 37°C in stoppered tubes wrapped in aluminium foil. When propranolol, yohimbine or prazosin were used they were added 2 min prior to adrenaline. After incubation cells were separated from the media, as described in section 2.8.1., and immediately frozen in liquid N_2 for the subsequent extraction and assay of PDH activity as described in section 2.3. Further details of the expeimental procedure are given in section 2.8.1.

5.3. Results

5.3.1. Studies in vivo - white adipose tissue

No significant changes in total PDH were observed in response to any of the treatments described below; the mean total activity for the virgin female rats was 121.1 ± 7.9munit per g wet wt of tissue

(mean ± SEM of 68 observations), for the male rats it was 584.0 ± 35.0munit per g wet wt of tissue (mean ± SEM for 8 observations) and for 14 day lactating rats it was 82.0 ± 5.4munit per g wet wt of tissue (mean ± SEM of 27 observations).

Intraperitoneal injection of noradrenaline increased the proportion of PDH in the active state in parametrial white adipose tissue of virgin female rats ($P<0.001$, table 5.1a). It took 15 min for the increase in PDH activity to reach a maximum after which it remained so elevated for at least a further 10 min (fig. 5.1.). This effect was prevented by the prior administration of the β-antagonist propranolol (table 5.1a): propranolol by itself had no effect on white adipose tissue PDH activity (table 5.1a). Injection of adrenaline into female rats raised PDH activity of parametrial white adipose tissue to the same extent as noradrenaline; similar to noradrenaline, this effect of adrenaline was blocked by the β-antagonist propranolol and the α_1-antagonist prazosin (table 5.2.). Noradrenaline also activated PDH in epididymal white adipose tissue in male rats ($P<0.001$; table 5.1a) to the same extent as in female rats. Although noradrenaline increased PDH activity of white adipose tissue in lactating rats ($P<0.05$) the increase was less than in virgin rats ($P<0.05$; table 5.1a).

Noradrenaline also increased the serum glucose concentration ($P<0.02$; table 5.1b) and the serum insulin concentration (table 5.1b); these increases were not prevented by prior administration of the β-antagonist propranolol (table 5.1b). The initial activity of ACC, measured in the absence of citrate, was significantly decreased in white adipose tissue of virgin female rats after injection of

noradrenaline ($P<0.05$; table 5.1a); there was no significant change in total ACC activity, measured after pre-incubation of the tissue extract with citrate, following the injection of rats with noradrenaline (table 5.1a).

The serum glucose concentration in streptozotocin treated rats was consistently greater than 25mM indicating that the rats were diabetic. Noradrenaline increased the proportion of PDH in the active state in parametrial white adipose tissue of the diabetic rats ($P<0.02$; table 5.2.); this effect of noradrenaline was completely prevented by the prior administration of the β-antagonist propranolol ($P<0.01$) or the α_1-antagonist prazosin ($P<0.02$) and was partially blocked by the α_2-antagonist yohimbine (table 5.2.).

Cervical dislocation of conscious rats increased the proportion of PDH in the active state in white adipose tissue of virgin rats ($P<0.001$) and this increase was prevented by the prior administration of the β-antagonist propranolol ($P<0.01$; table 5.3.). In lactating rats cervical dislocation increased PDH activity of white adipose tissue ($P<0.01$; table 5.3.) but the increase was less than in virgin rats ($P<0.01$; table 5.3.).

White adipose tissue PDH activity was greater in virgin rats during the dark-phase than the light-phase ($P<0.05$; table 5.4.). PDH activity was still elevated 2h into the light-phase (10.00h) but by 12.00h it had decreased significantly ($P<0.05$; experiment 2, table 5.4.): administration of the β-antagonist propranolol to rats prior to sampling at 10.00h significantly reduced PDH activity ($P<0.05$) to values similar to those found at 12.00h (experiment 2; table 5.4.). In contrast to virgin rats no significant difference was found in PDH

activity of white adipose tissue in lactating rats between the light-phase and the dark-phase (table 5.4.). At 4h into the dark phase the proportion of PDH in the active state in lactating rats was lower than in virgin rats ($P<0.01$).

5.3.2. Studies in vivo - Liver, skeletal muscle and mammary gland

No significant changes in total PDH were observed in response to any of the treatments described below, the mean total activities, expressed as mean ± SEM for the number of observations in parenthesis, were 2.5 ± 0.04 (13) and 1.8 ± 0.14 (8) units per g wet wt of tissue for skeletal muscle from female and male rats respectively, 1.7 ± 0.06 (13), 1.8 ± 0.08 (10) and 2.1 ± 0.12 (8) units per g wet wt of tissue for liver from virgin and lactating female rats and male rats respectively and 1.1. ± 0.06 (14) units per g wet wt of tissue for mammary gland from lactating rats.

Intraperitoneal injection of noradrenaline increased the proportion of PDH in the active state in liver of virgin and lactating female rats ($P<0.001$; table 5.5a) and male rats ($P<0.001$; table 5.5b): the increase in hepatic PDH activity induced by noradrenaline was greater in lactating than in virgin rats ($P<0.05$; table 5.5a). This effect of noradrenaline on hepatic PDH activity was not blocked by the β-antagonist propranolol (table 5.5a). The proportion of PDH in the active state in mammary gland of lactating rats was increased after injection of noradrenaline ($P<0.001$; table 5.5a). In contrast noradrenaline had no effect on PDH activity of skeletal muscle in either female or male rats (table 5.5a,b).

5.3.3. Studies in vitro - white adipose tissue

Incubation with insulin increased the proportion of PDH in the active state in isolated adipocytes from parametrial white adipose tissue of virgin rats (fig. 5.2.): maximal stimulation of PDH activity occurred at 200μunit/ml insulin. In the presence of sub-maximal concentrations of insulin adrenaline increased the proportion of PDH in the active state (fig. 5.3.). Maximal stimulation of PDH activity, in the presence of 50μunit/ml insulin, occurred with 30nM noradrenaline which increased the proportion of PDH in the active state to between 70 and 80% (fig. 5.4.). The maximal effect of adrenaline on adipocyte PDH was achieved within 15 min of incubation and persisted for at least a further 15 min (fig. 5.5.). Under the incubation conditions concentrations of adrenaline greater than 30nM increased the amount of glycerol released by the adipocytes (fig. 5.4.).

Adrenaline also stimulated the proportion of PDH in the active state in adipocytes isolated from parametrial white adipose tissue of 14 day lactating rats (fig. 5.6.): maximal stimulation occurred at 30-100nM adrenaline. The maximal increase in PDH activity in response to adrenaline in adipocytes from lactating rats was less than in adipocytes from virgin rats ($P<0.01$).

The β-antagonist propranolol (fig. 5.7.) and the α_1-antagonist prazosin (fig. 5.8.) inhibited the activation of adipocyte PDH by adrenaline after 5 and 30 min incubations with maximal effects of both agents occurring at 10μM. In contrast the α_2-antagonist yohimbine at concentrations less than 10μM had no effect on the activation of adipocyte PDH by adrenaline although at high

concentrations of yohimbine (1mM) some inhibition of the adrenaline effect was apparent (fig. 5.9.). The combination of both prazosin and propranolol was required to completely block the activation of adipocyte PDH by adrenaline after 5 or 30 min (table 5.6.): inhibition of the adrenaline effect by the α_1-antagonist (prazosin) predominated at 5 min and the by β-antagonist (propranolol) predominated at 30 min (table 5.6.).

Adipocyte PDH activity was activated by the specific agonists methoxamine (α_1) and isoproterenol (β); a combination of both of these agonists was required to achieve maximal stimulation of PDH activity (table 5.7.). Again α_1-activation predominated after 5 min and β-activation after 30min (table 5.7.).

5.4. Discussion

5.4.1. Studies in vivo

Intraperitoneal injection of noradrenaline increased the proportion of PDH in the active state in white adipose tissue and liver of female rats but had no effect on PDH in skeletal muscle. The stimulation of hepatic PDH activity by catecholamines is well established *in vitro* and occurs via the activation of α_1-receptors (Assimacopoulos-Jeannet *et al.*, 1983; Oviasu & Whitton, 1984): in agreement with this prior administration of the β-antagonist propranolol did not prevent the activation of liver PDH by noradrenaline *in vivo*. Noradrenaline also appeared to increase the serum insulin concentration, probably via the increase in the serum glucose concentration but although glucose-induced insulin release is known to stimulate PDH activity of white adipose tissue (see chapter 4) this did not seem to be the reason for the effects of

noradrenaline observed in the present study for, in contrast to white adipose tissue, injection of the catecholamine had no effect on skeletal muscle PDH activity whereas injection of glucose, to increase serum insulin, did increase the activity of PDH in skeletal muscle (see chpater 4). To confirm this point, studies were also carried out with streptozotocin diabetic rats. Noradrenaline was as effective in increasing the proportion of PDH in the active state in white adipose in diabetic rats as in normal rats clearly demonstrating that the effect was not mediated by an increase in the serum insulin concentration. In both diabetic and normal rats this effect of noradrenaline was completely prevented by prior administration of the β-antagonist propranolol. In addition the α_1-antagonist prazosin also prevented the activation of PDH by noradrenaline in diabetic rats whereas the α_2-antagonist yohimbine only partly prevented this effect of noradrenaline. However in vitro studies are necessary to establish if the specific antagonists prazosin, yohimbine and propranolol are acting directly upon white adipose tissue or if the effects observed in vivo are secondary to some other action of the antagonists. The effects of the α-antagonists yohimbine and prazosin on the stimulation of hepatic PDH activity by noradrenaline were not established as streptozotocin treatment of rats reduced the proportion of liver PDH in the active state to only 4.3 ± 0.4% (7) and rendered the enzyme resistant to activation by noradrenaline. Oviasu & Whitton, (1984) found similar effects of starvation on liver PDH and Wieland et al. (1972) reported that streptozotocin diabetes reduced PDH activity in liver.

Injection of noradrenaline significantly reduced initial ACC activity (measured in the absence of citrate) in white adipose tissue of virgin female rats and although some doubt now exists as to the validity of the assay used in the present study to measure this enzyme activity (Haystead & Hardie, 1986) the inhibition of ACC activity by catecholamines is consistent with the findings of other studies performed *in vitro* (Halestrap & Denton, 1974; Brownsey *et al.*, 1979; Zammit & Corstorphine, 1982b) and *in vivo* (Lee & Kim, 1978): this inhibition of activity would be expected to be paralleled by a decrease in the rate of fatty acid synthesis. Adrenaline *in vitro* generally (Katz *et al.*, 1966; Saggerson & Greenbaum, 1970; Smith & Saggerson, 1978) but not invariably (Saggerson, 1972a) inhibits the conversion of glucose to fatty acids in white adipose tissue. In contrast studies *in vivo* have found little evidence for an inhibitory effect of catecholamines on the rate of fatty acid synthesis in white adipose tissue. Thus injection of noradrenaline into rats had no effect on lipogenesis in white fat although it did inhibit the activation of lipogenesis by insulin (Agius & Williamson, 1980b). In addition Takahashi & Shimazu (1982) found that increasing sympathetic activity, which will increase noradrenaline in white adipose tissue, has no effect on lipogenesis in the tissue. More surprisingly, James *et al.* (1986) found that infusion of adrenaline into rats actually increased the conversion of glucose to unesterified fatty acids and glyceride glycerol but this may reflect the activation of hydrolysis of newly esterified fatty acids by lipase. The inhibition of fatty acid synthesis by adrenaline *in vitro* can be mimicked by the addition of free fatty acids to isolated

adipocytes (Saggerson & Tomassi, 1971; Saggerson, 1972a,b; Sooranna & Saggerson, 1975) suggesting that the effect of adrenaline could be secondary to the accumulation of non-esterified fatty acids in incubations (see Saggerson, 1985): free fatty acids would be less likely to accumulate *in vivo* and this may account for the lack of inhibition of fatty acid synthesis by catecholamines in studies *in vivo* (Agius & Williamson, 1980b; Takahashi & Shimazu, 1982; James *et al.*, 1986). It is still surprising that while catecholamines appear to inhibit ACC activity in white adipose tissue *in vivo* (the present study; Lee & Kim, 1978) they do not appear to inhibit fatty acid synthesis in the tissue *in vivo*. It appears that changes in ACC activity in white adipose tissue *in vivo* are not necessarily paralleled by changes in the rate of fatty acid synthesis in the tissue. However the rate of fatty acid synthesis in the studies *in vivo* was measured as the total incorporation of $^{3}H_{2}O$ (Agius & Williamson, 1980b; Takahashi & Shimazu, 1982) or ^{14}C-glucose (James *et al.*, 1986) into fatty acids during the whole experimental period (30-60 min) while the activity of ACC was only measured at one (the present study) or two (Lee & Kim, 1978) time points after the injection of hormone and so the results for the rate of fatty acid synthesis and for ACC activity are not directly comparable.

Perirenal injection of noradrenaline leads to its transport to adipose tissue via the blood whereas *in vivo* it is released within the tissue itself from sympathetic nerve endings. Severe stress, such as cervical dislocation, causes a massive surge of sympathetic activity and provides a means of stimulating the release of catecholamines *in vivo* within adipose tissue itself (Callingham &

Barrand, 1979). Cervical dislocation of conscious rats resulted in a similar increase in PDH activity of white adipose tissue to that found following injection of noradrenaline: as before injection of propranolol prior to cervical dislocation prevented the rise in PDH activity.

Further circumstantial evidence for a role of the sympathetic nervous system in modulating PDH activity of white adipose tissue was provided from diurnal changes in the enzyme activity. The proportion of PDH in the active state in white adipose tissue was higher during the dark-phase than the light-phase. PDH activity was still elevated 2h into the light-phase (10.00h) but by 12.00h had decreased significantly. Plasma insulin concentrations in rats exhibit diurnal variation with the concentration of insulin falling during the early daylight hours and reaching peak levels during the dark phase (Kaul & Berdanier, 1972) which could account for the diurnal changes in PDH activity of white adipose tissue. However the administration of the β-antagonist propranolol to rats prior to sampling at 10:00h decreased the proportion of PDH in the active state to values found at 12.00h suggesting that increased sympathetic activity was responsible for the higher PDH activity at 10.00h. Sympathetic nervous activity is stimulated by feeding (see Landsberg & Young, 1985); rats being nocturnal creatures normally feed and are more active at night, then become quiescent during the day (see Le Magnen, 1984). This pattern of behaviour is reflected in the whole body oxygen consumption which tends to increase during the dark-phase and then decreases during the first few hours of the light-phase (Rothwell & Stock, 1982; Maxwell et al., 1985), at the same time as PDH activity in white adipose tissue decreases.

In analogy with the control of PDH activity by insulin (Chapter 4) tissue specific changes occur in the ability of catecholamines to stimulate PDH activity during lactation. Although injection of both lactating and virgin rats with noradrenaline increased the proportion of PDH in the active state in white adipose tissue and liver and had no effect on skeletal muscle PDH the increase in white adipose tissue PDH activity was less in lactating than in virgin rats ($P<0.05$) whereas in liver the increase in PDH activity was greater in lactating than in virgin rats ($P<0.05$). Cervical dislocation of lactating rats increased white adipose PDH activity but, in parallel to the response to noradrenaline in vivo, the increase in the proportion of PDH in the active state following cervical dislocation was less in lactating than in virgin rats ($P<0.01$). The response of white adipose tissue PDH to catecholamines was therefore muted during lactation. Further evidence in support of this is that in lactating rats, in contrast to virgin rats, white adipose tissue PDH activity was not significantly different between the dark and light phases. The proportion of PDH in the active state at 4h into the dark phase was also lower in lactating rats than in virgin rats ($P<0.01$).

Injection of noradrenaline also increased the proportion of PDH in the active state in the mammary gland of lactating rats. Rat mammary epithelial cells possess β-adrenergic receptors but do not increase their cAMP content significantly in response to β-adrenergic stimulation except when simultaneously exposed to phosphodiesterase inhibitors (Clegg & Mullaney, 1985) and the effect of noradrenaline on mammary PDH activity may be indirect as there was an accompanying

increase in serum insulin. However, Williamson et al. (1983) reported that adrenergic agents in vitro caused a small but significant increase in glucose utilisation by mammary acini which was accompanied by a similar increase in lactate ouptut and glucose conversion to lipid and other workers have found small but consistent increases in the rate of fatty acid synthesis and the activity of ACC in mammary acini exposed to β-adrenergic agents in vitro (Clegg et al., 1986). It is possible that the activation of PDH is at least partially responsible for the stimulation of glucose metabolism in the mammary gland by adrenergic agents.

To conclude it is apparent from the above studies that catecholamines in vivo stimulate PDH activity in white adipose tissue and liver of male and female rats but have no effect on skeletal muscle PDH activity. Noradrenaline in vivo also increases the proportion of PDH in the active state in the mammary gland of lactating rats although this may be secondary to a rise in the serum insulin concentration. During lactation tissue specific adaptations occur in the control of PDH activity by catecholamines as well as by insulin in vivo.

5.4.2. Studies in vitro

The studies in vivo showed that activation of both α_1 and β-receptors are involved in the stimulation of white adipose tissue PDH activity by noradrenaline. White adipocytes of the rat possess α_1 and β-receptors but not α_2-receptors (see section 1.4.) hence the modest inhibitory effects of yohimbine in vivo must be indirect. Cheng & Larner (1985) using isolated adipocytes concluded that

catecholamines activate PDH via an α_1-linked mechanism but inhibit via a β-linked mechanism. As the in vivo studies clearly showed that β-blockade with propranolol prevented rather than enhanced the effect of noradrenaline and adrenaline on PDH activity the effects of catecholamines in vitro were investigated; adrenaline rather than noradrenaline was used in these studies to facilitate direct comparison with the findings of Cheng & Larner (1985) and also Smith & Saggerson (1978).

The stimulation by insulin in vitro of PDH activity in isolated white adipocytes, with the maximal effect on PDH activty occurring at 200μunit/ml insulin is consistent with the findings of other studies (see chapter 6). Smith and Saggerson (1978) found that in the presence of insulin, adrenaline at concentrations below about 100nM enhanced PDH activity of white adipocytes; at higher concentrations of adrenaline there was a fall in PDH activity with a concomitant rise in the rate of lipolysis. In their experiments, Smith & Saggerson (1978) used a high concentration of insulin (20munit/ml). In the present study the effects of adrenaline on adipocyte PDH were studied in the presence of a sub-maximal concentration of insulin (50μunit/ml); this concentration was sufficient to increase the proportion of PDH in the active state from 20 to 30%. In the presence of 50μunit/ml insulin adrenaline increased the proportion of adipocyte PDH in the active state; maximum effect (80% of PDH in the active state) was achieved with 30nM adrenaline after 30 min of incubation. At higher concentrations of adrenaline the proportion of PDH in the active state decreased along with a rise in glycerol release. These findings, with a

physiological concentration of insulin, are thus in good agreement with those of Smith & Saggerson (1978) and show that insulin and adrenaline have a synergistic effect on PDH activity. Various studies have shown that concentrations of adrenaline above about 0.1μM, which also stimulate lipolysis, decrease the proportion of PDH in the active state in isolated adipocytes (Coore et al., 1971; Sooranna & Saggerson, 1976b; Smith & Saggerson, 1978). It is likely that this decrease in PDH activty is secondary to the accumulation of free fatty acids in the incubations which is known to inhibit PDH activity (see Saggerson, 1985).

The effects of specific antagonists on the activation of PDH were investigated by incubating adipocytes with 30nM adrenaline, 50μunit/ml insulin and the appropriate antagonist. Cheng & Larner (1985) found that the α_1-mediated effects were transient and were lost by 30 min so in the present study the effects were investigated over 5 min and 30 min incubations; a 5 min incubation increased the proportion of PDH in the active state to about 60% of that found after 30 min (Fig. 5.5.). preliminary experiments showed that maximum effects of prazosin and propranolol were achieved at 10μM for both, in agreement with Cheng & Larner (1985). Yohimbine had no effect on the stimulation of PDH by adrenaline at either 5 or 30 min apart from when very high concentrations (1mM) of the α_2-antagonist were used. Both prazosin and propranolol inhibited the activation of PDH by adrenaline and the combination of the two was required to completely inhibit the activation. Curiously, the contributions of the α_1 and β-antagonists to the inhibition reversed over the period of incubation, with α_1-inhibition predominating at 5 min (in

agreement with the findings of Cheng & Larner, 1985) whereas β-inhibition was the major cause at 30 min.

The above findings were confirmed in further experiments using more specific agonists (methoxamine as an α_1-agonist and isoproterenol as a β-agonist). The concentrations of these agonists used were the same as those used by Cheng & Larner, (1985). Maximum activation was again achieved with the combination of both agonists. Again α_1-activation predominated after 5 min and β-activation after 30 min.

The findings in the present study that activation of PDH is mediated by α_1-receptors agrees with the conclusions of Cheng & Larner (1985). In contrast the results both *in vitro* and *in vivo* differ from those of Cheng & Larner (1985) as they found that propranolol enhanced the activation caused by α-agonists: the reason for this difference is not certain. However Cheng & Larner (1985) did not normally include insulin in their incubations and used a lower (2mM) glucose concentration; also only 5-10% of adipocyte PDH was in the active state prior to stimulation in their study compared to 20-30% in both the *in vitro* and *in vivo* experiments in the present study and in other studies *in vitro* (Jungas, 1970; Coore *et al.*, 1971; Weiss *et al.*, 1974; Sooranna & Saggerson, 1976a,b); Cheng & Larner (1985) attribute the low proportion of PDH in the active state to the use of 2mM glucose in their studies. A lower glucose concentration and the absence of insulin could have led to increased fatty acid accumulation in their adipocytes. Fatty acids are potent inhibitors of PDH (Smith & Saggerson, 1978) hence the increase in the proportion of PDH in the active state in the presence of the

β-antagonist propranolol found by Cheng & Larner (1985) could have arisen from the agent inhibiting lipolysis thereby lowering the intracellular fatty acid concentration with concomitant relief of PDH from fatty acid-induced inhibition.

In agreement with the in vivo studies adrenaline in vitro increased the proportion of PDH in the active state in parametrial white adipocytes from lactating rats as well as from virgin animals. The increase in PDH activity induced by adrenaline in vitro was significantly less ($P<0.01$) in the adipocytes from lactating rats than from virgin rats which also confirms the results of the in vivo studies.

The present studies in vitro are therefore in good agreement with the findings in vivo in that catecholamines activated adipocyte PDH activity and this involves both α_1 and β-receptors. The findings differ however in that either α_1 or β-blockade is sufficient to prevent completely the effect of catecholamines on PDH in vivo but both are required in vitro.

5.4.3. General discussion

The results of the present study show that catecholamines activate PDH in white adipose tissue and liver but have no effect on PDH of skeletal muscle. Skeletal muscle contains substantial energy reserves which are chiefly used to provide fuel for the tissue during exercise. Catecholamines play a role in increasing the availability of local and distal fuel stores for muscle function and can influence the recycling of substrates released from muscle for conversion into glucose by the liver (see Young & Landsberg, 1977b). Skeletal muscle

glycogenolysis and glycolysis are stimulated by catecholamines (Soderling & Park, 1974; Cohen, 1976; Dietz et al., 1980; Nescher et al., 1980; Young et al., 1985) and this is accompanied by an increase in lactate output (Saitoh et al., 1974; Nescher et al., 1980; Richter et al., 1982). This lactate can be used as a precursor for glucose synthesis in the liver and hence an effect of the stimulation of skeletal muscle glycogen breakdown by catecholamines is an increase in glucose output by the liver (see Young & Landsberg, 1977b): the failure of catecholamines to stimulate PDH activity in skeletal muscle will favour the release of lactate from the tissue.

The stimulation of PDH activity in white adipose tissue by catecholamines will increase the conversion of pyruvate to acetyl-CoA which can be used for oxidation or as a substrate for fatty acid synthesis. In accordance with the stimulation of PDH activity catecholamines also stimulate glycolysis in white adipose tissue (Katz et al., 1966; Halperin & Denton, 1969; Saggerson & Greenbaum, 1970) at least in part via the stimulation of phosphofructokinase activity (Sale & Denton, 1985). However as discussed in section 5.4.2. most studies in vitro and in vivo have found that catecholamines do not stimulate fatty acid synthesis in white adipose tissue. It therefore appears that, in contrast to insulin, catecholamines may have a selective effect on glucose metabolism in white adipose tissue promoting glucose oxidation but not fatty acid synthesis. Recently it was reported that noradrenaline in vivo activates PDH but inhibits ACC activity in brown adipose tissue of the rat implying that the hormone also has a selective effect on glucose metabolism in brown adipose tissue (Gibbins et al., 1985).

Activation of white adipose tissue PDH by catecholamines probably relates to the sympathetic nervous system having a more subtle role in the control of body function in addition to its well defined role in stress. Feeding, for example, results in increased sympathetic nervous activity which is even modulated by the nature of the diet (see Landsberg & Young, 1985). Eating promotes triacylglycerol turnover in white adipose tissue *in vivo* (Brooks *et al.*, 1983), almost certainly due to increased sympathetic nervous activity (the increase was blocked by propranolol). This creates a demand for glycerol-3-phosphate and the stimulation by catecholamines of glucose metabolism through the initial stages of glycolysis is in accordance with this (see Saggerson, 1985). Increased triacylglycerol turnover also requires ATP; this could be derived from fatty acid oxidation but the present study suggests that the increased sympathetic nervous activity (probably in concert with serum insulin which is also increased on eating) will activate PDH and so increase glucose oxidation. Although increased triacylglycerol turnover might be expected to raise the fatty acid concentration in the cell *in vivo* this is unlikely to be sufficient to inhibit PDH for the concentration of catecholamines injected in the present study would markedly raise the plasma free fatty acid concentration (Brodie *et al.*, 1965); dissipation of fatty acid may be more difficult for adipocytes *in vitro* than *in vivo*.

Increased triacylglycerol turnover coupled with increased PDH activity and glucose oxidation constitutes an 'ATPase' with the concomitant production of heat. A similar mechanism appears to exist in brown adipose tissue in which catecholamines also activate PDH *in*

vivo (Gibbins et al., 1985). Brown adipose tissue has additional mechanisms for producing heat and is thought to be primarily responsible for the increased heat production on eating in rats (see Stock & Rothwell, 1986); the present study suggests that white adipose tissue, as a result of increased sympathetic activity, will make some contribution to increased heat production on eating. In support of this microcalorimetry studies have recently shown that heat production of adipocytes isolated from rat epididymal white adipose tissue is stimulated by both noradrenaline and insulin and analogous to the activation of adipocyte PDH activity in vitro (cf Fig. 5.3.) maximal stimulation occurs in the presence of a combination of insulin and noradrenaline (Nilsson-Ehle & Nordin, 1985). In addition to its possible contribution to heat production on eating white adipose tissue, through increased glucose oxidation, will help to diminish the hyperglycaemia which normally accompanies a meal.

Tissue specific changes occur during lactation in the ability of catecholamines to stimulate PDH activity: this effect of catecholamines on white adipose tissue is muted while in the liver the effect is potentiated. The reduced ability of catecholamines to stimulate white adipose tissue PDH activity along with the refractoriness of the tissue to insulin (chapter 4) will mean that the increased availability of glucose on feeding, which also increases serum insulin concentration and sympathetic nervous activity (see Le Magnen, 1984; Landsberg & Young, 1985) will not result in an increased flux of glucose to acetyl-CoA in white adipose tissue. Rather an increased lactate output by the tissue is to be

expected which may be used for gluconeogenesis in the liver ultimately resulting in an increase in the availability of glucose in the circulation for use by the mammary gland. Hepatic gluconeogenesis is particularly important in the immediate period after re-feeding a starved lactating rat when the rapid stimulation of mammary gland lipogenesis that occurs appears to be dependent on continued gluconeogenesis in the liver (Williamson et al., 1985). Catecholamines stimulate hepatic gluconeogenesis (Kneer et al., 1974; Veneziale et al., 1976) and ketogenesis (Himms-Hagen, 1972) although they appear to have little effect on hepatic fatty acid synthesis in normal fed rats (Raskin et al., 1974; Agius & Williamson, 1980b). The production of glucose and ketone bodies in the liver from precursors orginating elsewhere (such as white adipose tissue, skeletal muscle and mammary gland) is therefore increased by catecholamines: this will ultimately increase the availability of glucose and ketone bodies in the circulation to meet the requirements of the whole animal.

Fig. 5.1. Time course for the activation of pyruvate dehydrogenase in white adipose tissue by noradrenaline in virgin female rats

Rats were anaesthetised with pentobarbital throughout the experiment. Pentobarbital (60mg/kg body wt.) was injected intraperitoneally 10 min prior to the intraperitoneal injection of noradrenaline (1·25mg / kg body wt.) and samples of parametrial white adipose tissue were removed between 10 min and 25 min later as indicated in the figure. The tissue samples were immediately frozen in liquid N_2 for the subsequent measurement of PDH activity. Further details of the experimental procedure are given in chapter 2. Results are from one experiment only.

FIG.5.1.

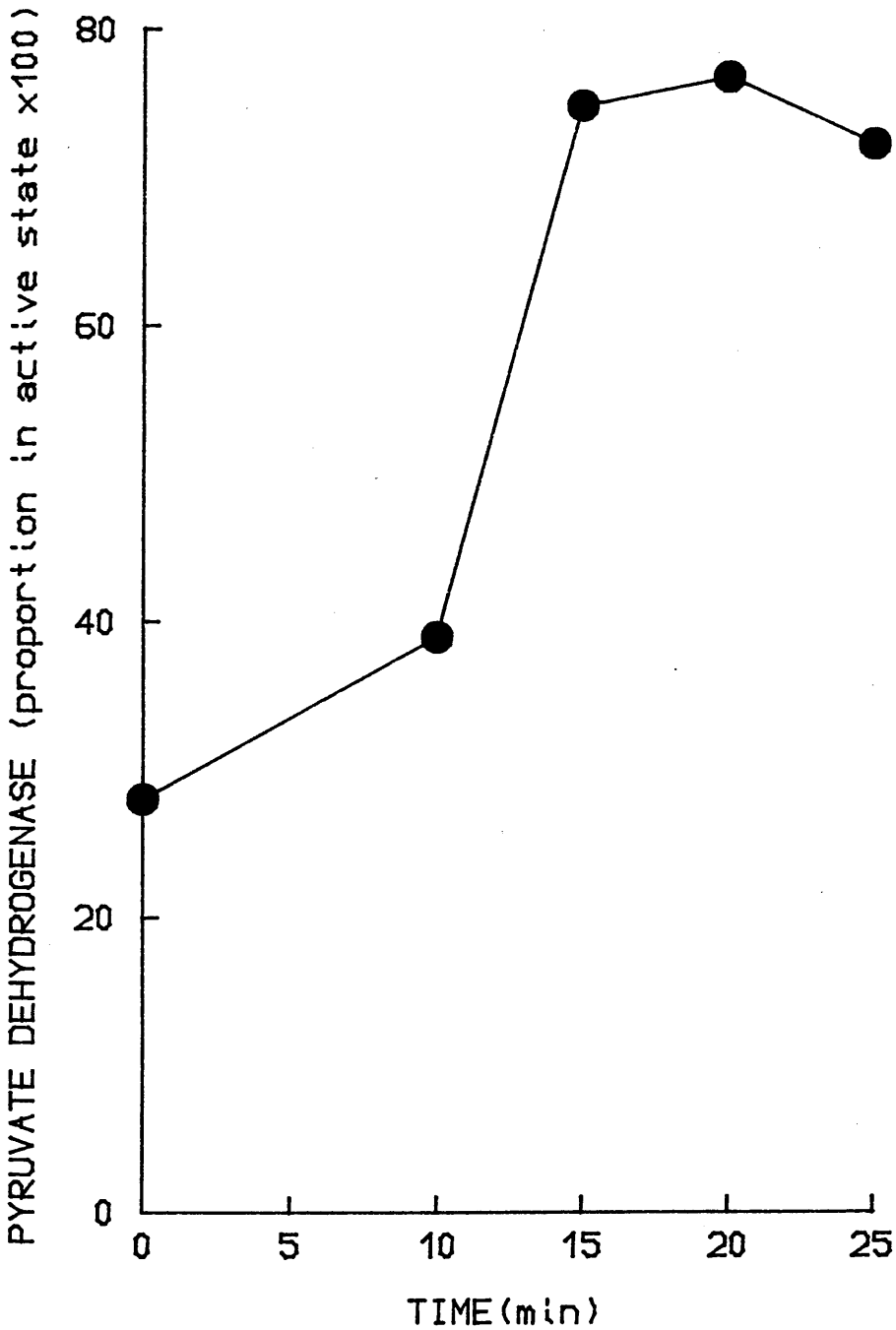

PYRUVATE DEHYDROGENASE (proportion in active state x100)
80
60
40
20
0
0
5
10
15
20
25
TIME(min)

Fig. 5.2. The effect of insulin In Vitro on pyruvate dehydrogenase activity of white adipocytes from virgin female rats

A dose response curve is shown which was obtained when adipocytes isolated from parametrial white adipose tissue were incubated with the concentrations of insulin indicated in the presence of 5mM glucose at 37°C for 30 min after which pyruvate dehydrogenase activity was measured. The experiment was carried out three times with similiar results. Further details of the experimental procedure are in the methods section and chapter 2.

FIG.5.2.

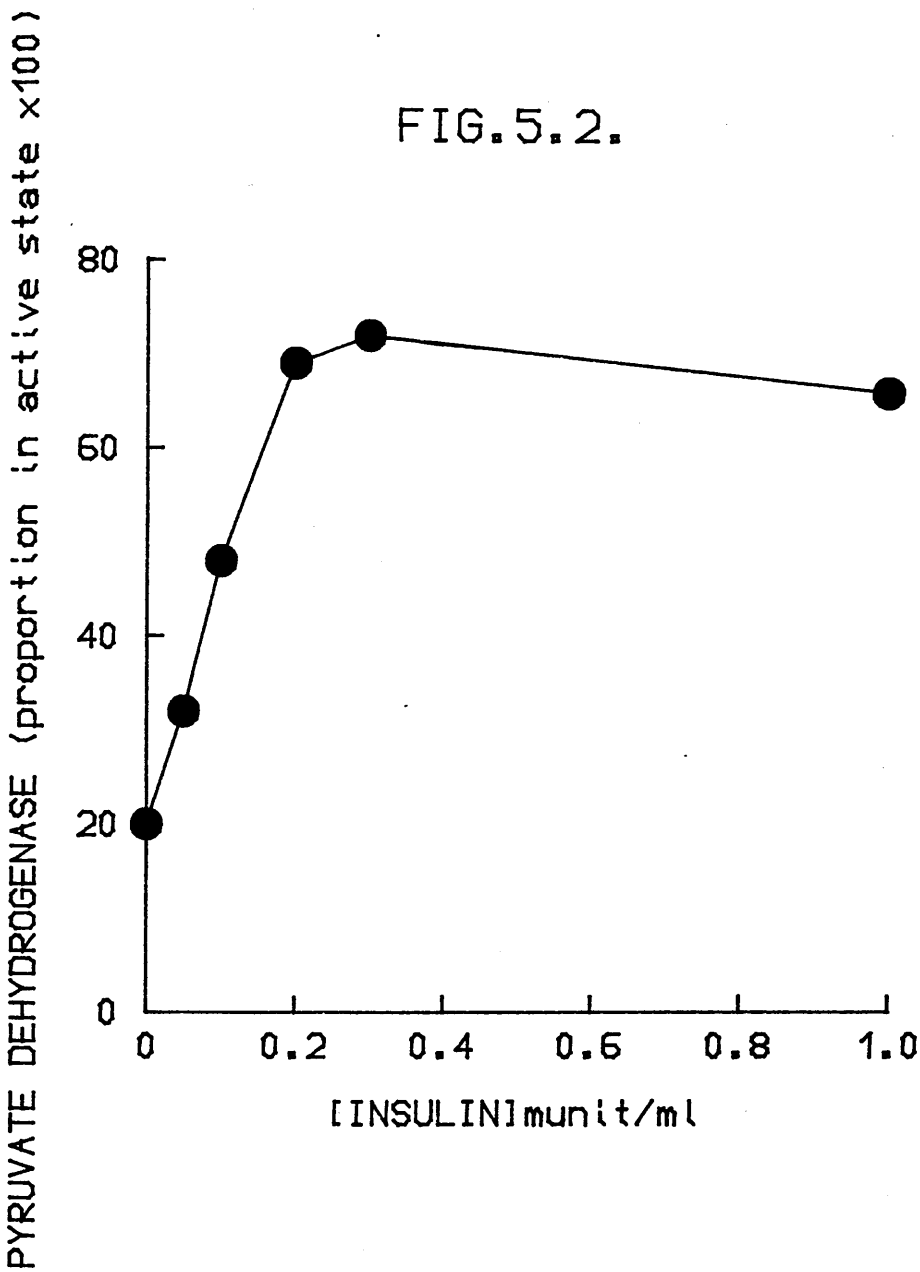

PYRUVATE DEHYDROGENASE (proportion in active state x100)
80
60
40
20
0
0
0.2
0.4
0.6
0.8
1.0
[INSULIN]munit/ml

Fig. 5.3. The effect of insulin on the stimulation of pyruvate dehydrogenase activity in white adipocytes from virgin female rats by adrenaline In Vitro

A dose response relationship is shown which was obtained when adipocytes from parametrial white adipose tissue were incubated with the concentrations of insulin indicated in the presence of 5mM glucose plus no further additions (○) or 30nM adrenaline (●) for 30 min at 30°C after which pyruvate dehydrogenase activity was measured. The experiment was carried out three times with similar results. Further details of the experimental procedure are in the methods section and chapter 2.

FIG.5.3.

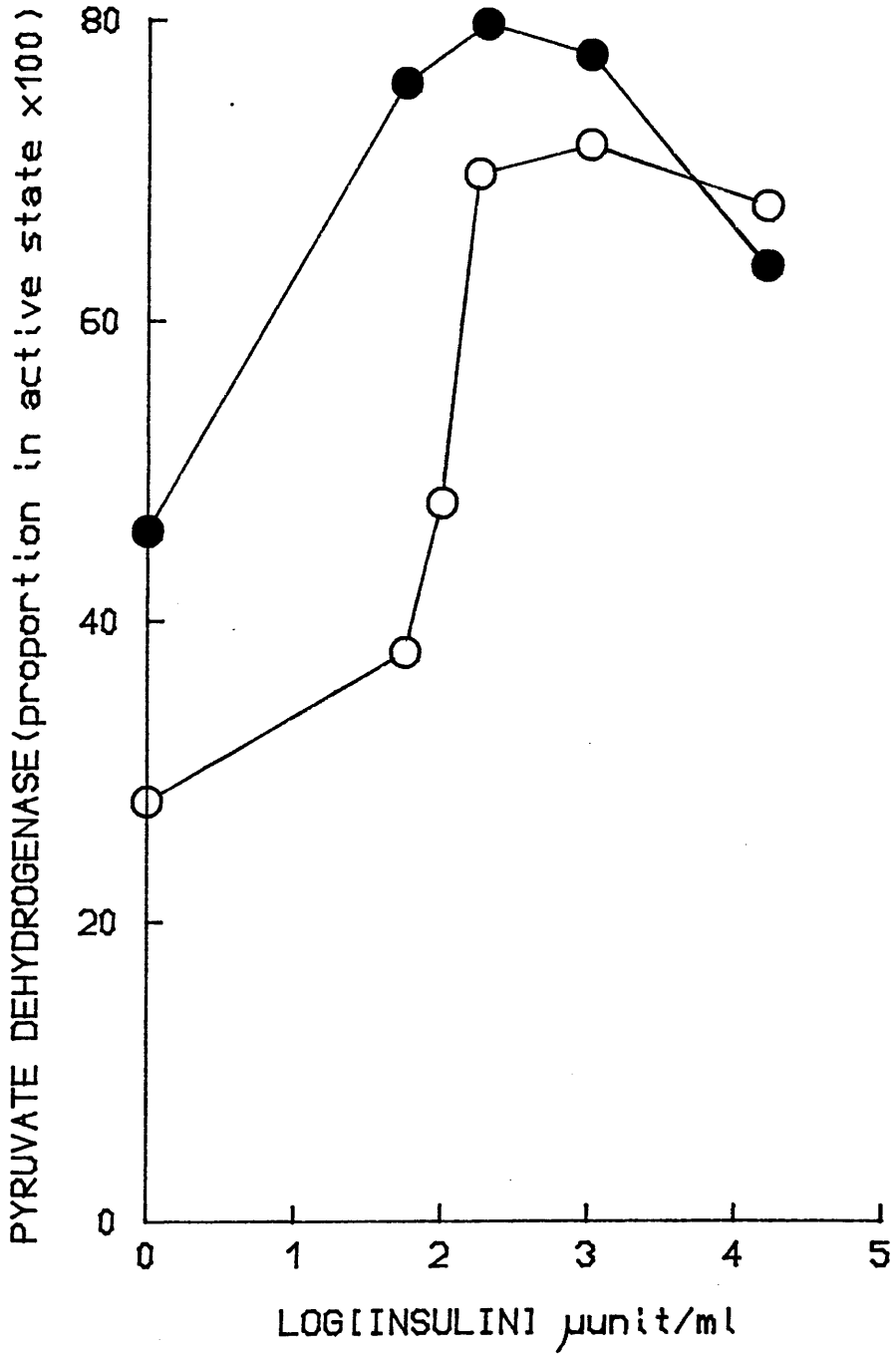

PYRUVATE DEHYDROGENASE(proportion in active state x100)
80
60
40
20
0
0
1
2
3
4
5
LOG[INSULIN] μunit/ml

Fig. 5.4. The effect of adrenaline on pyruvate dehydrogenase activity and glycerol release In Vitro in white adipocytes from virgin female rats

Adipocytes, isolated from parametrial white adipose tissue, were incubated with the concentrations of adrenaline indicated in the presence of 50μunit/ml insulin and 5mM glucose for 30 min at 37°C after which PDH activity in the adipocytes (●) and the amount of glycerol in the incubation medium (○) were measured. Further details of the experimental procedure are in the methods section and chapter 2. Results are means ± SEM for values from three separate experiments. After incubation of adipocytes at 37°C for 30 min in the absence of insulin and adrenaline 52± 10nmol/10^6 cells of glycerol had been released into the medium and 28 ± 2% of total PDH in the adipocytes was in the active state.

FIG.5.4.

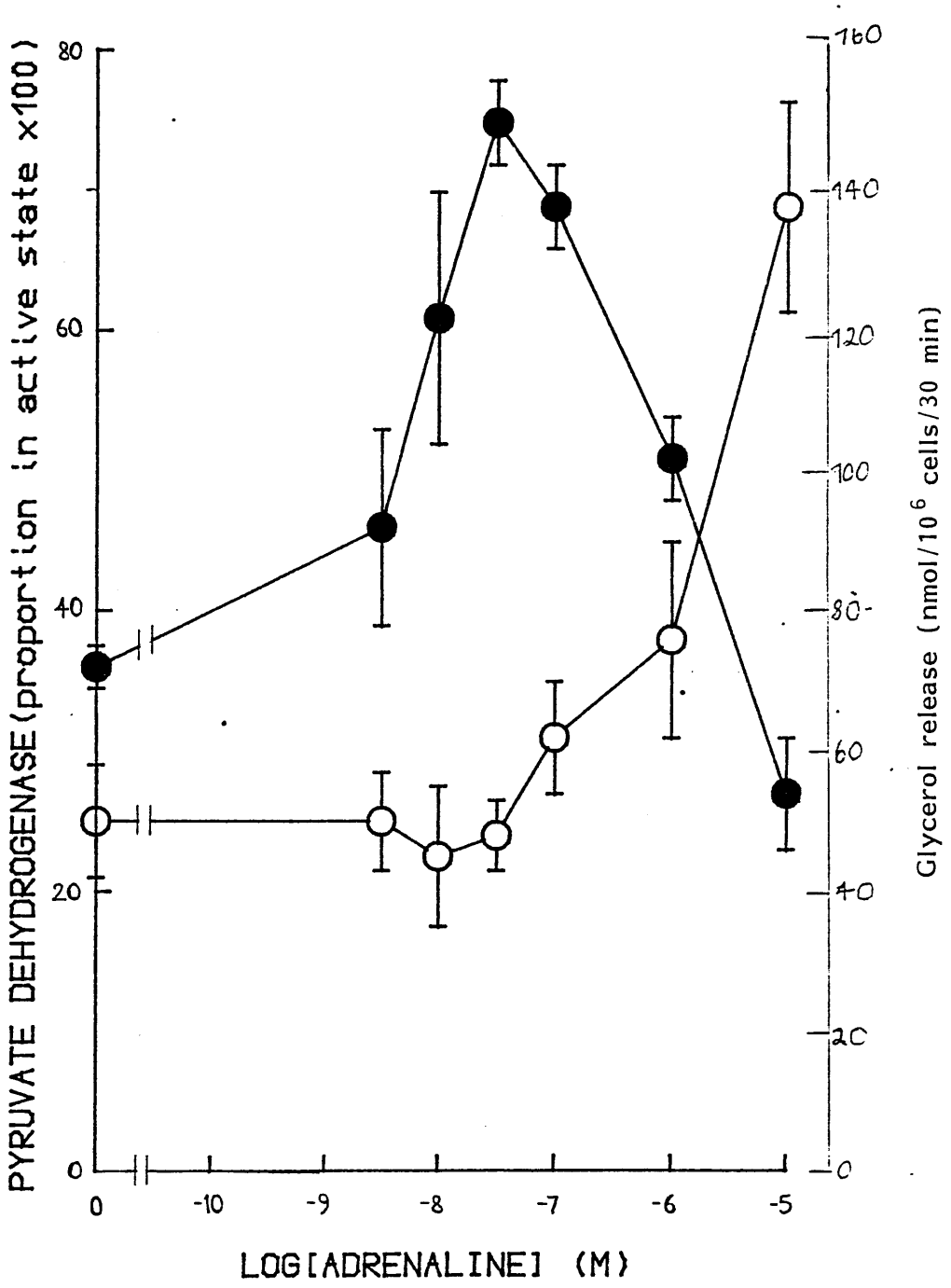

PYRUVATE DEHYDROGENASE (proportion in active state x100)
80
60
40
20
0
160
140
120
100
80
60
40
20
0
Glycerol release (nmol/10^6 cells/30 min)
0
-10
-9
-8
-7
-6
-5
LOG[ADRENALINE] (M)

Fig. 5.5. Time course for the effects of insulin and adrenaline In Vitro on pyruvate dehydrogenase activity in white adipocytes from virgin female rats

A time course is shown which was obtained when adipocytes isolated from parametrial white adipose tissue were incubated in the presence of 5mM glucose with either 50μunit/ml insulin (○), 50μunit/ml insulin plus 30nM adrenaline (●) or no additions (□) at 37°C after which PDH activity was measured. Further details of the experimental procedure are in the methods section and chapter 2. The experiment was carried out twice with similar results.

FIG.5.5.

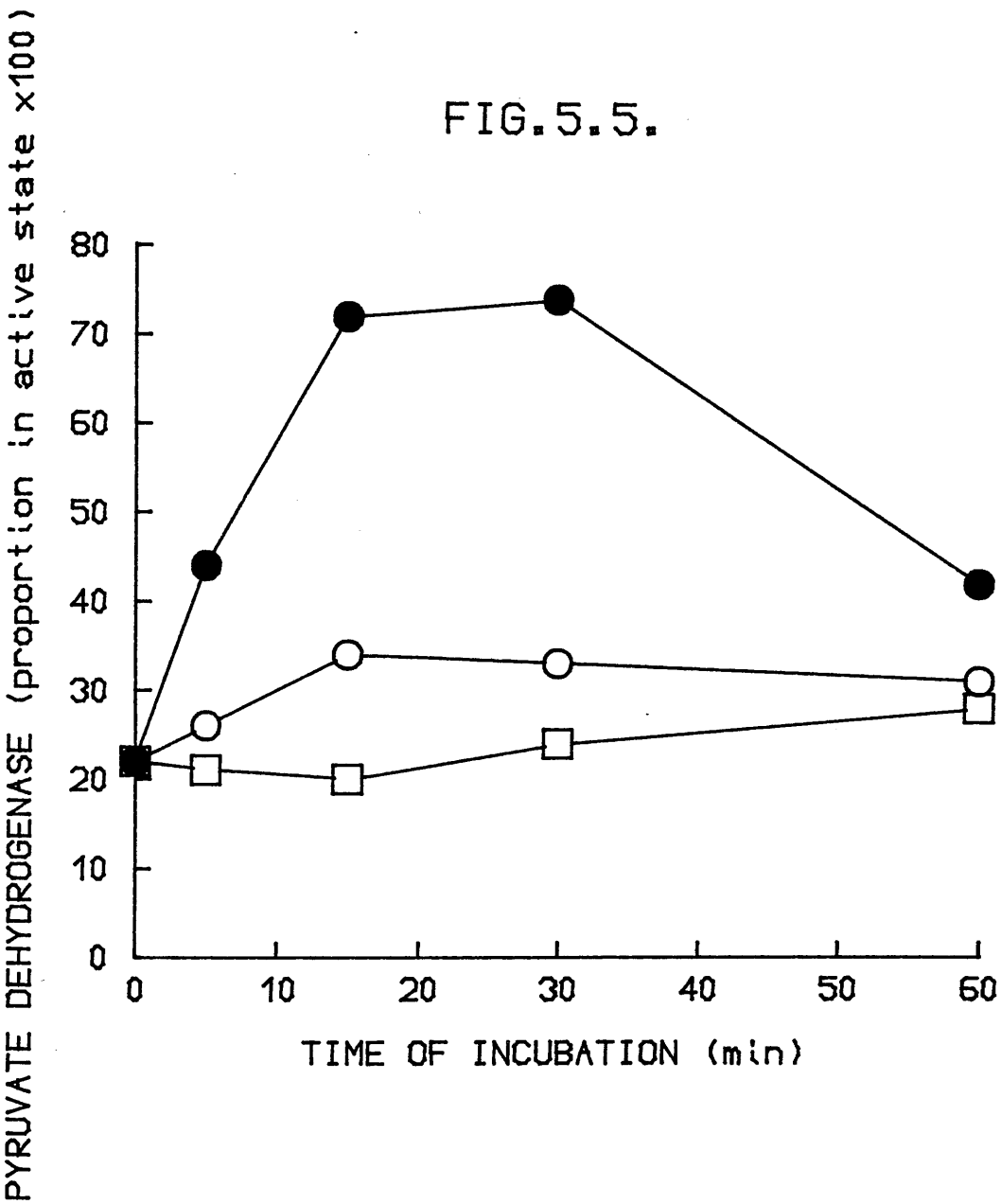

PYRUVATE DEHYDROGENASE (proportion in active state x100)
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
TIME OF INCUBATION (min)

Fig. 5.6. The effect of adrenaline In Vitro on pyruvate dehydrogenase activity of white adipocytes from lactating rats

Adipocytes isolated from 14 day lactating rats were incubated with the concentrations of adrenaline indicated in the presence of 50μunit/ml insulin and 5mM glucose at 37°C for 30 min after which PDH activity was measured. Further details of the experimental procedure are in the methods section and chapter 2. Results are means ± SEM for values from 3 separate experiments. After incubation of adipocytes for 30 min at 37°C in the absence of insulin and adrenaline 28 ± 4% of total PDH activity was in the active state.

FIG.5.6.

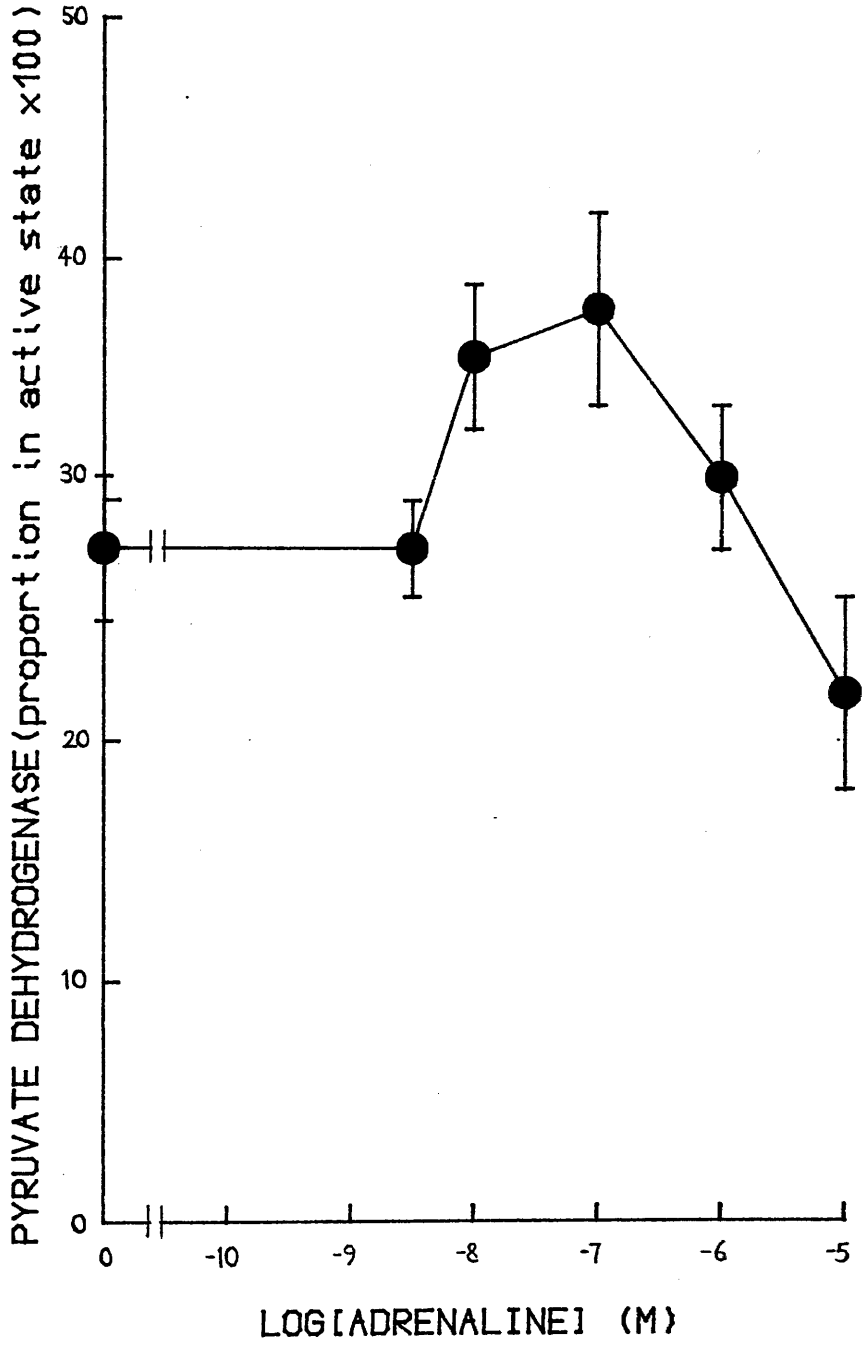

PYRUVATE DEHYDROGENASE (proportion in active state x100)
50
40
30
20
10
0
0
-10
-9
-8
-7
-6
-5
LOG [ADRENALINE] (M)

Fig. 5.7. The effect of propranolol on the stimulation by adrenaline In Vitro of pyruvate dehydrogenase activity in white adipocytes from virgin rats

Dose response relationships are shown which were obtained when adipocytes isolated from parametrial white adipose tissue were incubated with the concentrations of propranolol indicated plus 50μunit/ml insulin (○) or 50μunit/ml insulin and 30nM adrenaline (●) in the presence of 5mM glucose at 37°C for 5 min (fig. a) or 30 min (fig. b) after which PDH activity was measured. Further details are in the methods section and chapter 2. In the absence of propranolol, insulin and adrenaline 26.0% of total PDH was in the active state after incubation for 5 min and 29.5% was in the active state after 30 min.

FIG.5.7.a

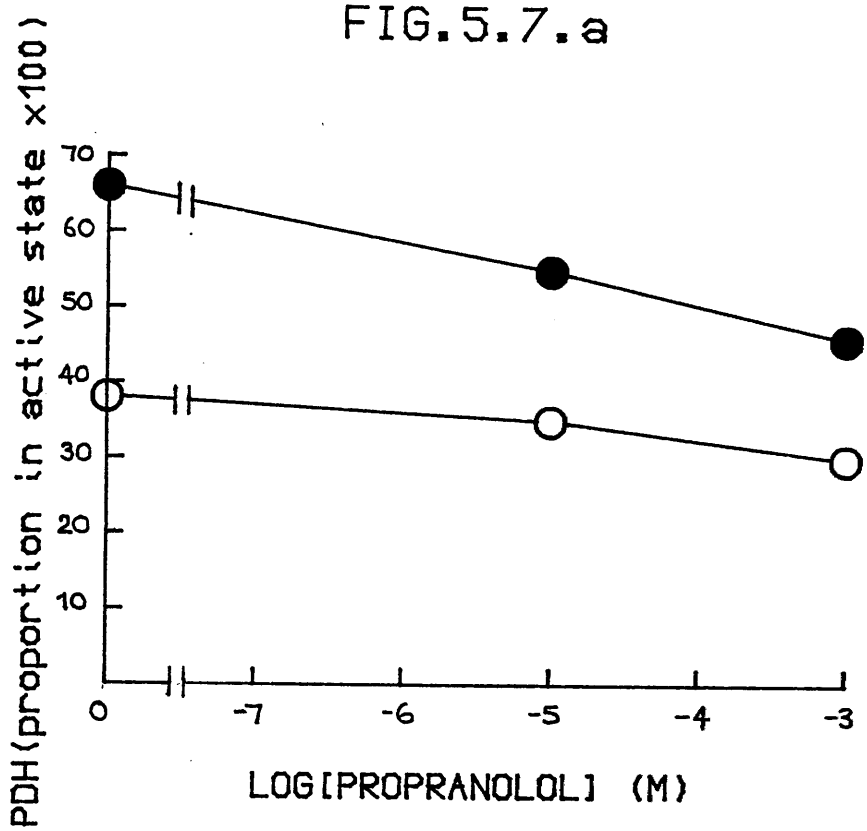


FIG.5.7.b

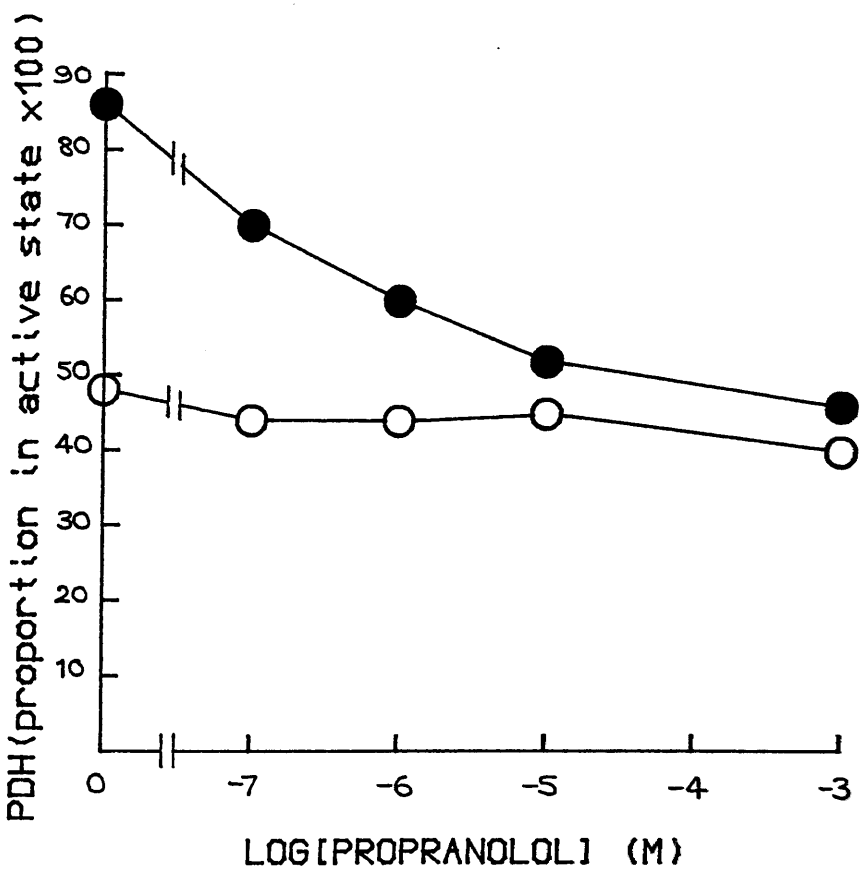


Fig. 5.8. The effect of prazosin on the stimulation by adrenaline In Vitro of pyruvate dehydrogenase activity in white adipocytes from virgin female rats

Dose response relationships are shown which were obtained when adipocytes isolated from parametrial white adipose tissue were incubated with the concentrations of prazosin indicated plus 50μunit/ml insulin (○) or 50μunit/ml insulin and 30nM adrenaline (●) in the presence of 5mM glucose at 37°C for 5 min (fig. a) or 30 min (fig. b) after which PDH activity was measured. Further details are in the methods section and chapter 2. In the absence of insulin, prazosin and adrenaline 27.0% of total PDH activity was in the active state after 5 min of incubation and 29.6% was in the active state after 30 min of incubation.

FIG.5.8.a.

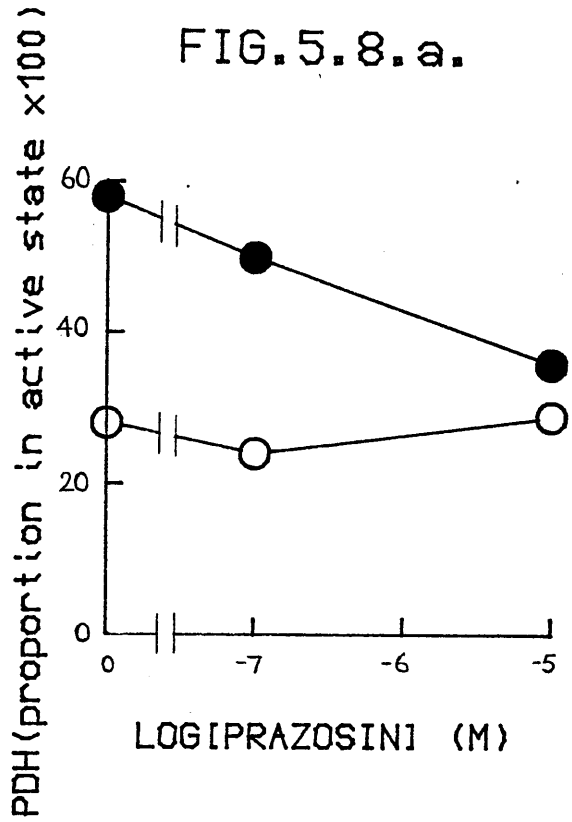


FIG.5.8.b.

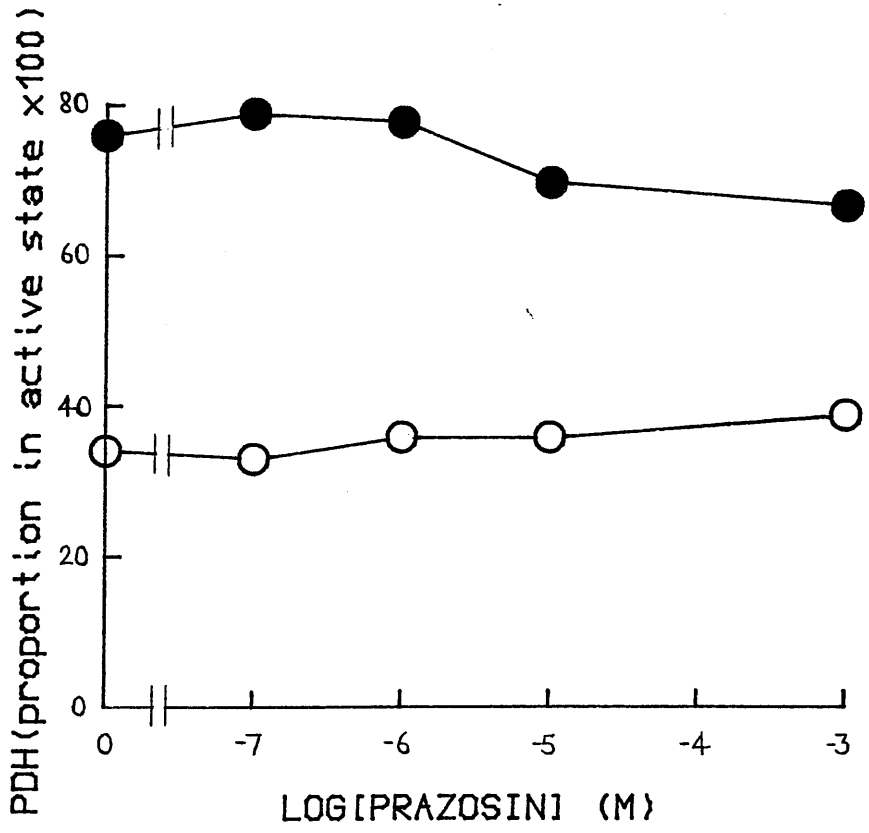


Fig. 5.9. The effect of yohimbine on the stimulation by adrenaline In Vitro of pyruvate dehydrogenase activity in white adipocytes from virgin female rats

Dose response relationships are shown which were obtained when adipocytes isolated from parametrial white adipose tissue were incubated with the concentrations of yohimbine indicated plus 50μunit/ml insulin (O) or 50μunit/ml insulin and 30nM adrenaline (●) in the presence of 5mM glucose at 37°C for 5 min (fig. a) or 30 min (fig. b) after which PDH activity was measured. Further details are in the methods section and chapter 2. In the absence of insulin, yohimbine and adrenaline 28.8% of total PDH was in the active state after 5 min of incubation and 30% of total PDH was in the active state after 30 min.

FIG.5.9.a.

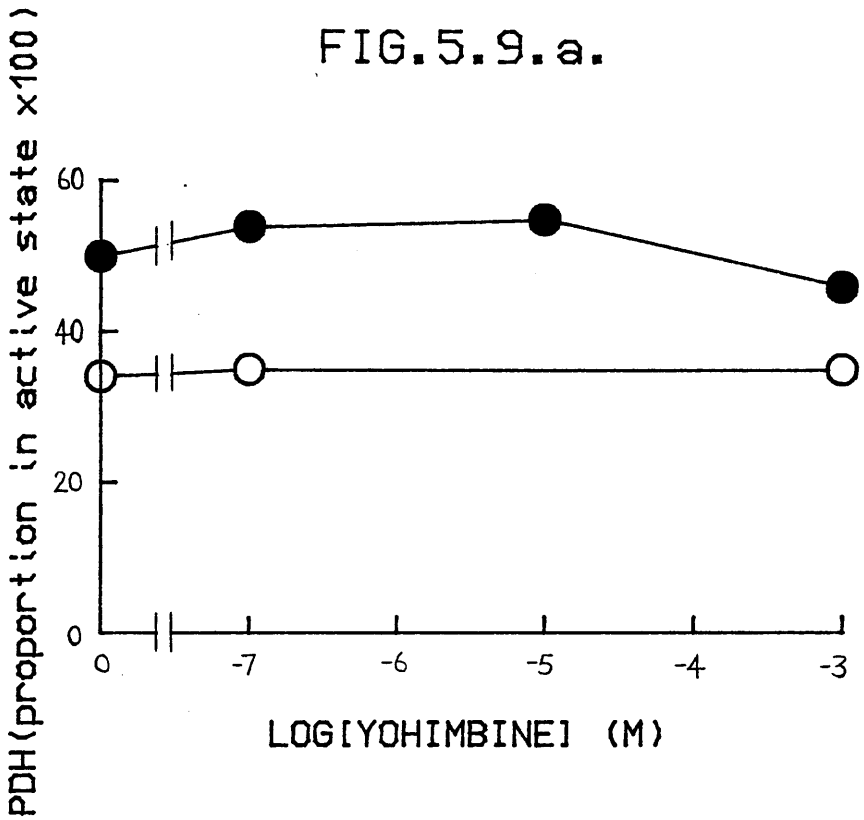


FIG.5.9.b.

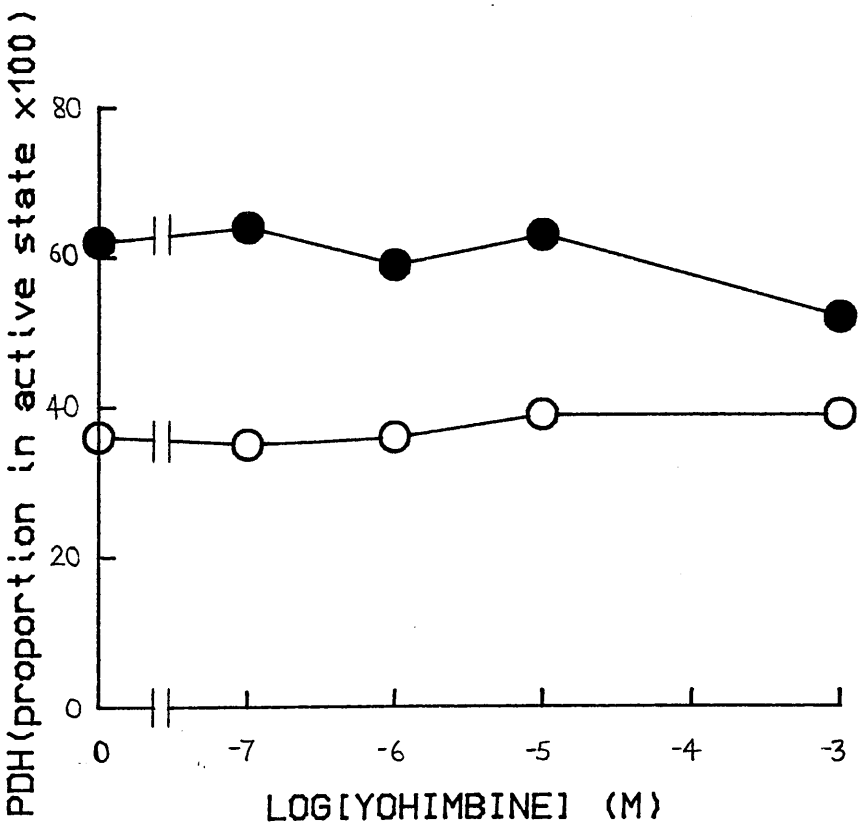


Table 5.1a. The effect of injection of noradrenaline on pyruvate dehydrogenase activity and acetyl-CoA carboxylase activity of white adipose tissue in virgin and lactating female rats and male rats

Virgin and 14 day lactating female rats were age-matched. Rats were anaesthetised throughout the experiment. Propranolol (1.5mg/kg body wt.) was injected intraperitoneally with the pentobarbital anaesthetic (60mg/kg body wt.); noradrenaline (1.25mg/kg body wt.) or 0.15M saline were injected intraperitoneally 10 min after the anaesthetic and blood and parametrial or epididymal white adipose tissue samples were removed 20 min later. The adipose tissue samples were immediately frozen in liquid N_2. Further details are in the methods section and chapter 2. Results are means ± SEM for the number of observations in parenthesis; *, **** indicate the value is significantly different from the value for rats given saline plus propranolol, $P<0.05$, <0.001 respectively.

State	Treatment	Pyruvate dehydrogenase (proportion in active state x 100)	Acetyl-CoA carboxylase (munit/10^6 cells)	
			Initial activity (assayed in absence of citrate)	Total activity (pre-incubation with citrate)
Virgin	Saline	31.4±3.5(5)	-	-
	Saline + propranolol	28.7±0.9(3)	3.0±0.8(3)	44.8±5.9(3)
	Noradrenaline	77.5±6.3(8)****	1.6±0.2(5)*	34.0±5.7(5)
	Noradrenaline + propranolol	32.9±6.3(4)	-	
Lactating	Saline + propranolol	29.0±3.0(8)	-	-
	Noradrenaline	44.7±5.8(7)*	-	-
Male	Saline + propranolol	39.6±5.7(4)	-	-
	Noradrenaline	86.6±5.4(4)****	-	-

Table 5.1b. The serum glucose and insulin concentrations after injection of virgin and lactating female rats and male rats with noradrenaline

The experimental procedure is described in the legend to table 5.1a. Results are means ± SEM for the number of observations in parenthesis; **, *** indicate the value is significantly different from the value for rats given saline plus propranolol; P<0.02; <0.01 respectively.

State	Treatment	Serum glucose (mM)	Serum insulin (μunit/ml)
Virgin	Saline	8.3±0.5(5)	55.0±10.0(5)
	Saline + propranolol	9.3±0.7(3)	52.5±10.0(3)
	Noradrenaline	14.8±1.1(8)**	87.5±15.0(8)
	Noradrenaline + propranolol	14.8±0.8(4)***	82.5±12.5(4)
Lactating	Saline + propranolol	6.7±0.6(8)	15.0± 2.5(6)
	Noradrenaline	13.3±1.6(7)***	60.0±10.0(7)***
Male	Saline + propranolol	11.7±1.9(4)	162.5 47.5(4)
	Noradrenaline	25.1±1.7(4)***	285.0±32.5(4)

Table 5.2. The effect of injection of noradrenaline on pyruvate dehydrogenase activity of white adipose tissue in streptozotocin-diabetic female rats

Streptozotocin (100mg/kg body wt) was dissolved in 1ml of 0.1M sodium citrate, pH 4.5, and injected intraperitoneally 19h prior to the experiment. Rats were anaesthetised throughout the experiment. Propanolol (1.5mg/kg body wt) was injected intraperitoneally along with the pentobarbital anaesthetic (60mg/kg body wt) while prazosin and yohimbine were injected 5 min after the anaesthetic (administration of these drugs to conscious rats caused obvious stress); adrenaline or noradrenaline (1.25mg/kg body wt), or 0.15M saline were injected 10 min after the anaesthetic and parametrial white adipose tissue was removed 20 min later and immediately frozen in liquid N_2. Further details are in the methods section and Chapter 2. Results are means ± SEM for the number of observations in parenthesis; **, *** indicate the value differs ($P<0.02$, <0.01) from that for rats given saline plus propranolol; ††, ††† indicate the value differs ($P<0.02$, <0.01) from that for rats given noradrenaline alone.

Treatment	Pyruvate dehydrogenase (proportion in active state x 100)
Saline + propranolol	38.7±2.1(3)†††
Noradrenaline	82.5±9.6(6)**
Noradrenaline + propranolol	40.3±2.8(4)†††
Noradrenaline + prazosin	34.7±2.4(3)††
Noradrenaline + yohimbine	54.3±6.3(3)
Adrenaline	74.3±5.8(2)
Adrenaline + propranolol	44.8(1)
Adrenaline + prazosin	37.6(1)

Table 5.3. The effect of cervical dislocation on pyruvate dehydrogenase activity of white adipose tissue in virgin and lactating rats

Age-matched virgin and 14 day lactating rats were used. Rats killed by cervical dislocation were conscious at the time. Propranolol (1.5mg/kg body wt.) was injected intraperitoneally at 30 min and again at 2 min prior to cervical dislocation. Parametrial white adipose tissues samples were removed immediately after death and frozen in liquid N_2. The control animals given saline and propranolol were under anaesthetic throughout the experiment and were treated as described in the legend to table 5.1. Results are means ± SEM for the number of observations in parenthesis; ***, **** indicate the value is significantly different from the value for control rats given saline and propranolol under anaesthetic, $P<0.01$, <0.001 respectively.

State	Treatment	Pyruvate dehydrogenase (proportion in active state x 100)
Virgin (anaesthetised)	Saline + propranolol	28.7±0.9(3)
Virgin (conscious)	Cervical dislocation	81.4±1.2(4)****
Virgin (conscious)	Cervical dislocation + propranolol	42.0±9.1(4)
Lactating (anaesthetised)	Saline + propranolol	29.0±3.0(8)
Lactating (conscious)	Cervical dislocation	47.8±4.9(3)***

Table 5.4. Diurnal variation in pyruvate dehydrogenase activity of white adipose tissue in virgin and lactating rats

Age-matched virgin and 14 day lactating rats were on a 12h light, dark cycle; for rats sampled in the 'light phase' the light phase began at 08.00h. Rats sampled in the 'dark phase' were on a reversed lighting regime, with the dark phase commencing at 08.00h. Rats were anaesthetised with pentobarbital (60mg/kg body wt.) 30 min before parametrial white adipose tissue was removed; propranolol (1.5mg/kg body wt.) when given, was injected intraperitoneally along with the anaesthetic. Different groups of rats were used in experiments 1 and 2. Further details are given in the methods section and chapter 2. Results are means ± SEM for the number of observations in parenthesis, * indicates the value differs significantly from the value for rats at 4h into the light phase; $P<0.05$.

Experiment	Condition	Time of day	State of rat	Pyruvate dehydrogenase (proportion in active state x 100)
1	4h into light-phase	12.00h	virgin	30.6±0.8(6)
	4h into dark-phase	12.00h	virgin	40.5±3.1(4)*
	4h into light-phase	12.00h	lactating	26.1±2.5(4)
	4h into dark-phase	12.00h	lactating	30.9±0.5(5)
2	2h into light-phase	10.00h	virgin	47.6±3.2(4)*
	2h into light-phase + propranolol	10.00h	virgin	28.7±0.9(3)
	4h into light phase	12.00h	virgin	31.4±3.5(5)

Table 5.5a. The effect of injection of noradrenaline on pyruvate dehydrogenase activity of liver, skeletal muscle and mammary gland in virgin and lactating female rats

Rats were age-matched. All rats were anaesthetised throughout the experiment. Propranolol (1.5mg/kg body wt.) was injected intraperitoneally along with pentobarbital anaesthetic (60mg/kg body wt.) 10 min prior to intraperitoneal injection of 0.15M saline or noradrenaline (1.25mg/kg body wt.) and after a further 20 min samples of skeletal muscle from the hind-limb, liver and mammary gland were removed and immediately frozen in liquid N_2. Further details are in the methods section and chapter 2. Results are mean ± SEM for the number of observations in parenthesis; **** indicates the value is significantly different from the value for rats given saline plus propranolol, $P<0.001$.

		Pyruvate Dehydrogenase (proportion in active state x 100)				
	State.....	VIRGIN			LACTATING (14 DAY)	
Tissue	Treatment..	saline + propranolol	noradrenaline	noradrenaline + propranolol	saline + propranolol	noradrenaline
Skeletal muscle		10.0±0.1(4)	10.2±0.1(5)	9.7±0.3(4)	-	-
Liver		27.4±1.6(5)	57.8±3.0(4)****	62.6±0.8(4)****	32.8±1.4(4)	76.3±5.4(6)****
Mammary gland		-	-	-	55.4±2.1(7)	74.4±2.6(7)****

Table 5.5b. The effect of injection of noradrenaline on pyruvate dehydrogenase activity of liver and skeletal muscle in male rats

For the experimental procedure see the legend to table 5.5a. Results are means ± SEM for the number of observations in parenthesis, **** indicates the value is significantly different from the value for rats given saline plus propranolol, $P<0.001$.

		Pyruvate dehydrogenase (proportion in active state x 100)	
Tissue	Treatment....	saline + propranolol	noradrenaline
Skeletal muscle		40.5±1.9(4)	40.0±1.6(4)
Liver		27.5±2.5(4)	61.5±4.6(4)****

Table 5.6. The effects of propranolol, prazosin and yohimbine on the activation of pyruvate dehydrogenase by adrenaline In Vitro in white adipocytes from virgin female rats

Adipocytes isolated from parametrial white adipose tissue were incubated at 37°C in the presence of 5mM glucose with the antagonists indicated for 2 min prior to the addition of 50μunit/ml insulin and 30nM adrenaline to the incubations which were then continued for a further 5 or 30 min following which PDH activity was measured. The concentrations of propranolol, prazosin and yohimbine in the incubations were 10μM. Further details are in the methods section and Chapter 2. Adrenaline increased the proportion of total PDH present in the active state from 33.7 ± 1.3 to 55.8 ± 2.6% (3) after 5 min of incubation and from 40.7 ± 2.2 to 75.3 ± 3.5% (3) after 30 min of incubation. Results are expressed as the percent inhibition of the increase in PDH activity induced by adrenaline and where values from more than one experiment are available are mean ± SEM for the number of observations in parentheses.

		Pyruvate dehydrogenase (% inhibition of increase in activity induced by adrenaline	
Antagonist	Incubation...	5 min	30 min
Prazosin		58.3±4.8(3)	37.2±0.9(3)
Propranolol		37.2±3.0(3)	75.6±6.4(3)
Yohimbine		0(1)	9.8(1)
Prazosin + propranolol		105.2±3.6(3)	123.4±9.2(3)
Prazosin + yohimbine		65.0(1)	37.6(1)
Propranolol + yohimbine		37.6(1)	68.8(1)
Prazosin + propranolol + yohimbine		107.5(1)	106.4(1)

Table 5.7. Activation of pyruvate dehydrogenase by the α-agonist, methoxamine, and the β-agonist, isoproterenol[1], in vitro in white adipocytes from virgin female rats

Adipocytes isolated from parametrial white adipose tissue were incubated for 5 min or 30 min at 37°C with 5mM glucose, 50μunit/ml insulin and methoxamine (10μM) or isoproterenol (1μM) following which PDH activity was measured. Further details are in the methods section and Chapter 2. Results are expressed as the proportion of PDH in the active state x 100 found in the presence of agonist minus the proportion of PDH in the active state x 100 in the absence of agonist. In the absence of agonists 37.9 ± 8.4 and 42.3 ± 8.4% of total PDH was present in the active state after 5 min and 30 min of incubation respectively. Results are mean ± SEM of 3 observations.

		Increase in proportion of PDH in active state	
Agonist	Incubation...	5 min	30 min
Methoxamine		19.8±7.7	10.4±1.3
Isoproterenol		8.3±3.5	26.8±5.8
Methoxamine + isoproterenol		27.8±9.6	36.2±4.1

CHAPTER 6

The ability of insulin to stimulate the release from adipocyte and mammary plasma membranes of a substance which activates mitochondrial pyruvate dehydrogenase

6.1. Introduction

The ability of insulin to activate PDH in white adipose tissue *in vivo* is completely lost during lactation (see chapter 4). Studies elsewhere have shown that incubation of adipocyte mitochondria with adipocyte plasma membranes results in an increase in the mitochondrial PDH activity which is markedly enhanced by the addition of insulin (see section 1.3.6.7.): such studies have suggested that insulin promotes the release of a mediator from the plasma membranes which activates PDH. Experiments are reported in this chapter which were carried out to determine if a defect exists in the ability of white adipocyte plasma membranes from lactating rats to generate this putative mediator(s) in response to insulin or in the responsiveness of adipocyte mitochondrial PDH from lactating rats to the mediator.

Mammary gland PDH activity in diabetic or 24h starved rats is stimulated by insulin *in vivo* (Field & Coore, 1976; Baxter & Coore, 1978). Experiments were therefore carried out to determine if plasma membranes prepared from mammary tissue from lactating rats also release a mediator which stimulates mitochondrial PDH activity.

6.2. Methods

Studies with whole adipocytes

Parametrial white adipose tissue was removed from age-matched virgin and 14 day lactating rats under pentobarbital anaesthesia and isolated adipocytes were prepared as described in section 2.8.1. Adipocytes were incubated in Krebs-Ringer bicarbonate buffer containing 40mg/ml BSA and 5mM glucose at 37°C with the concentration of insulin indicated in the figure legends. The duration of the incubations are also given in the legends. After the incubations cells were separated from the media and frozen in liquid N_2 for the subsequent extraction and assay of PDH activity. Further details are given in section 2.8.1.

Studies with adipose tissue pieces

Parametrial white adipose tissue was removed from young virgin female rats (150-200g) under pentobarbital anaesthesia and tissue pieces were prepared as described in section 2.8.3. The tissue pieces were incubated at 37°C for 30 min in Krebs Ringer bicarbonate buffer containing 5mM glucose and the concentrations of insulin indicated in the legend to fig. 6.3. After the incubation the tissue was blotted and immediately frozen in liquid N_2 for the subsequent determination of PDH activity. For further details see section 2.8.3.

Studies with adipocyte subcellular fractions

Parametrial white adipose tissue was removed from age-matched virgin and 14 day lactating rats under pentobarbital anaesthesia. Adipocytes were isolated as described in section 2.8.1. and plasma membrane and mitochondrial fractions were prepared as described in

section 2.8.5.1. and frozen in liquid N_2 until required. Mitochondria (about 185μg protein/ml) were pre-incubated in 50mM potassium phosphate buffer pH 7.4 with 250μM ATP, 50μM $MgCl_2$ and 50μM $CaCl_2$ for 5 min at 37°C after which plasma membranes (150μg protein/ml or a range of concentrations) and insulin (concentrations used are given in the legends to the figures and tables) were added and the incubations were continued for a further 5 min. PDH activity was then measured as the release of $^{14}CO_2$ from [1-^{14}C] pyruvate (Seals & Czech, 1981). For further details see section 2.8.5.

Studies with mammary gland plasma membranes

Plasma membrane enriched fractions were prepared from mammary glands of 14 day lactating rats and 18-21 day lactating sheep as described in 2.8.5.2. The membranes (150μg protein/ml or a range of concentrations) were tested for their ability to activate PDH in mitochondria from virgin rat adipocytes, in the presence and absence of insulin, as described in the preceding section for adipocyte plasma membranes. For further details see section 2.8.5.

6.3. Results

6.3.1. Studies with intact adipocytes and adipose tissue pieces

The values for total PDH activity in isolated adipocytes from both virgin and 14 day lactating rats (see legend to fig. 6.1.) were very similar to the values for whole adipose tissue expressed on a per 10^6 adipocytes basis (see chapter 3). This provides further evidence to show that most of the PDH activity in white adipose tissue is associated with the adipocytes (see chapter 3). Incubation of isolated adipocytes or adipose tissue pieces from parametrial white adipose tissue with insulin for up to 1h had no effect on total

PDH activity. However insulin increased the proportion of PDH present in the active state in isolated adipocytes from virgin rats (fig. 6.1.). Maximal enzyme activation was achieved with 200μunit/ml insulin which increased PDH activity by 186.1 ± 25.7% (mean ± SEM for 3 observations): concentrations of insulin greater than 500μunit/ml gave a reduced stimulation of PDH activity (fig. 6.1). This maximal stimulation of PDH activity occurred within 20 min of incubation (fig. 6.2.). The activation of PDH by insulin *in vitro* (fig. 6.2.) was therefore similar to that *in vivo* (see chapter 4) in that the proportion of PDH in the active state was maximally increased from between 20% and 30% in the absence of insulin to about 70% in the presence of insulin and this increase was achieved over a similar time course. In contrast insulin had no effect on PDH in isolated adipocytes from 14 day lactating rats (fig. 6.1.).

Insulin also increased the proportion of PDH in the active state in white adipose tissue pieces from young virgin rats (150-200g). Maximal enzyme activation was achieved with 1munit/ml insulin (fig. 6.3.) and so the sensitivity of the tissue pieces to insulin was 5 times lower than that of the isolated adipocytes. PDH activity in the tissue pieces was maximally stimulated by 127.5 ± 15.0% (mean ± SEM for 3 observations). However insulin had no effect on PDH activity in tissue pieces from older virgin rats and from 14 day lactating rats (results not shown).

6.3.2. Plasma membrane and mitochondrial fractions prepared from rat white adipocytes

Plasma membrane and mitochondria enriched fractions were prepared from white adipocytes of virgin and 14 day lactating rats as described in chapter 2. There was no significant difference in the protein recovery, 5'nucleotidase activity or succinic dehydrogenase activity between the plasma membrane or mitochondria fractions from virgin and lactating rats (table 6.1a,b). The mitochondrial fractions represented between 2% and 7% of the total protein in the adipocyte homogenates while the plasma membrane fractions contained between 2% and 3% of the total protein in the homogenates (table 6.1a). The specific activity of 5'nucleotidase was 7-fold greater in the plasma membrane fractions than in the unfractionated adipocyte homogenates while the specific activity in the mitochondrial fractions was similar to the value for the adipocyte homogenates (table 6.1a). Succinic dehydrogenase activity was similar in the plasma membrane fractions and unfractionated homogenates but was between 3 and 4 times greater in the mitochondrial fractions (table 6.1b).

Similar Scatchard plots were obtained for the binding of insulin to adipocyte plasma membranes from virgin and lactating rats (fig. 6.4.). For the purpose of analysis the plots were interpreted as comprising two components one with a K_d for insulin of about 1.1nM and a second with a K_d for insulin of about 7.5nM (table 6.2.): there was no significant difference in the K_d values and number of high and low affinity binding sites between plasma membranes from virgin and lactating rats (table 6.2.). Non-specific binding of insulin to plasma membranes from virgin rats amounted to 16.7 ± 1.3% (mean ± SEM

for 4 observations) of the total insulin bound and to plasma membranes from lactating rats was 13.6 ± 2.1% (mean ± SEM for 4 observations) of the total bound. When insulin binding to plasma membranes from either virgin or lactating rats was measured less than 5% of the insulin added per assay was degraded.

There was no significant difference in PDH activity per unit protein between mitochondrial fractions prepared from adipocytes from virgin and 14 day lactating rats (table 6.3.). Therefore, the fall in PDH activity per white adipocyte during lactation (see chapter 3) is probably due to a loss of mitochondria in white adipose tissue. PDH activity in the plasma membrane fractions was consistently less than 5% of that in the mitochondrial fractions. Storage of the mitochondria in liquid N_2 for up to 7 days had no effect on PDH activity. Incubation of mitochondrial fractions, prepared from virgin rat adipocytes, with ATP inhibited PDH activity (fig. 6.5.). The inhibition was maximal after incubation for 4 min and persisted for at least a further 6 min (fig. 6.5.). ATP had a similar effect on PDH activity in mitochondrial fractions prepared from lactating rat adipocytes (results not shown). In subsequent experiments mitochondria were routinely incubated for 5 min with ATP prior to the addition of plasma membranes and insulin (see methods section).

Co-incubation for 5 min of plasma membranes and mitochondria from virgin rats resulted in a stimulation of PDH activity which was proportional to the amount of membrane present in the concentration range tested (fig. 6.6.). The storage of plasma membranes and mitochondria in liquid N_2 for up to 7 days had no effect on the ability of the membranes to stimulate PDH activity. The addition of

insulin to co-incubations of plasma membranes and mitochondria from virgin rats resulted in a stimulation of PDH activity (fig. 6.7.): insulin had no effect on mitochondrial PDH in the absence of plasma membranes (fig. 6.7.). The effect of insulin on PDH activity was biphasic with maximal activation of PDH most commonly occurring at 100μunit/ml insulin and the enzyme activity decreasing in the presence of higher concentrations of insulin (fig. 6.7.). The average maximal stimulation of PDH activity was about 48% (table 6.3.) and occurred after 5 min of incubation then persisted for a further 25 min (fig. 6.8.). In subsequent experiments mitochondria and plasma membranes were routinely incubated with 100μunit/ml insulin for 5 min (see methods section).

In contrast to the results for virgin rats co-incubation of plasma membrane and mitochondrial fractions prepared from lactating rat adipocytes had no effect on PDH activity (fig. 6.6.) and 100μunit/ml insulin, which produced a maximal stimulation of PDH activity in co-incubations of plasma membranes and mitochondria from virgin rats, had no effect on PDH in co-incubations of plasma membranes and mitochondria of lactating rats (table 6.3.). All concentrations of insulin tested (between 10μunit/ml and 1000μunit/ml) were without effect on PDH in co-incubations of plasma membranes and mitochondria from lactating rats (results not shown). However co-incubation of plasma membranes from virgin rats with mitochondria from lactating rats produced a slight increase in PDH activity which was potentiated by insulin ($P<0.05$) but co-incubation of plasma membranes from lactating rats with mitochondria from virgin rats had no effect on PDH activity (table 6.4.). The increase in PDH

activity, due to insulin, was similar when plasma membranes from virgin rats were incubated with mitochondria from virgin (48.3%, table 6.3.) or lactating (47.8%, table 6.4.) rats. When mitochondria from virgin rats were incubated with a combination of plasma membranes from virgin and lactating rats the stimulation of PDH activity was similar to that obtained with virgin rat plasma membranes alone (fig. 6.9.).

PDH activity in mitochondrial fractions from virgin rats was stimulated by supernatant from insulin treated virgin rat plasma membranes (fig. 6.10.): maximal stimulation of PDH activity (150% stimulation) occurred with 100μunit/ml insulin. In contrast supernatant from insulin treated lactating rat plasma membranes had no effect on PDH activity in mitochondria from virgin rats (fig. 6.10).

6.3.3. Plasma membrane enriched fraction prepared from mammary gland from lactating rats

Plasma membrane fractions prepared from mammary tissue removed from 14 day lactating rats contained about 0.5% of the total protein in the unfractionated mammary homogenates (table 6.1a). The specific activity of 5'nucleotidase was about 14-fold higher in the plasma membrane fractions than in the mammary homogenates (table 6.1a) while succinic dehydrogenase activity was undectectable in the plasma membrane fractions (table 6.1b). For the purpose of analysis the Scatchard plot (fig. 6.11) for the binding of insulin to the plasma membrane fractions was resolved into two components one with a K_d of about 2.4nM and the other with a K_d of about 8.4nM (table 6.2.). Non-specific binding of insulin to the rat mammary plasma membranes

amounted to 19.2 ± 0.1% (mean ± SEM for 4 observations) of the total insulin bound and less than 5% of the insulin added per assay was degraded.

PDH activity in the rat mammary membranes was consistently less than 10% of the value for adipocyte mitochondria. Incubation of rat mammary plasma membranes with rat adipocyte mitochondria for 5 min in the absence of insulin resulted in a stimulation of PDH activity (fig. 6.12.). This stimulation of PDH activity was proportional to the amount of plasma membranes present in the incubation in the concentration range tested (fig. 6.12.). The addition of insulin to a mixture of rat mammary plasma membranes and adipocyte mitochondria produced a further stimulation of PDH activity (fig. 6.13). The stimulation of PDH activity by insulin was biphasic with the maximal effect most commonly occurring at 50μunit/ml insulin and PDH activity decreasing when higher concentrations of insulin were present (fig. 6.13.). Insulin produced a maximum 41.7 ± 9.2% (mean ± SEM of 4 observations) stimulation in PDH activity (table 6.5.).

6.3.4. Plasma membrane enriched fraction prepared from lactating sheep mammary glands

Plasma membrane fractions prepared from mammary tissue from 18-21 day lactating sheep contained about 0.8% of the total protein in the unfractionated homogenate (table 6.1a). The specific activity of 5'nucleotidase was 8-fold higher in the plasma membrane fractions than in the unfractionated homogenate (table 6.1a) and succinic dehydrogenase activity was similar in the plasma membrane fraction to that in the unfractionated homogenate (table 6.1b).

PDH activity in the sheep mammary plasma membranes was negligible. Incubation of sheep mammary plasma membranes with mitochondria from rat adipocytes produced a stimulation of PDH activity which began to plateau out when more than 40μg of the membranes were present (fig. 6.12). Insulin (fig. 6.13.; table 6.5.) had no effect on PDH activity in these incubations.

6.4. Discussion

The activation of PDH in isolated adipocytes by insulin is in agreement with other studies (Jungas, 1971; Coore et al., 1971; Weiss et al., 1974; Sooranna & Saggerson, 1979a; Jarett et al., 1985). In the present study maximal activation of PDH in adipocytes was achieved with 200μunit/ml insulin which is in agreement with the findings of Weiss et al. (1974) and Sooranna & Saggerson (1979a) although Jarett et al. (1985) achieved maximal stimulation of adipocyte PDH with 100μunit/ml insulin. Insulin also stimulated PDH activity in adipose tissue pieces which also agrees with other studies (Jungas, 1971; Coore et al., 1971; Sica & Cuatrecasas, 1973; Weiss et al., 1974; Smith & Saggerson, 1978). Weiss et al. (1974) reported that the sensitivity of isolated fat cells to insulin was 5-times higher than the sensitivity of adipose tissue pieces and this finding is confirmed in the present study.

In the present study the activation of PDH in adipose tissue pieces by insulin was only apparent when the tissue was from relatively young female rats (150-200g) and no stimulation of PDH activity was detectable in tissue from older rats that were age-matched with 14 day lactating rats. The reason for the

unresponsiveness of PDH to insulin in adipose tissue taken from older rats is not certain but it has been previously reported that the degree of stimulation of glucose metabolism in adipocytes by insulin decreases with age (Salans & Dougherty, 1971; Di Girolamo et al., 1974; Holm et al., 1975). However PDH activity in isolated adipocytes from older rats was stimulated by insulin while insulin had no effect on PDH in adipocytes from age-matched 14 day lactating rats. This is in agreement with the results in vivo (chapter 4) that the activation of adipose tissue PDH by insulin is lost during lactation and shows that this is due to an alteration at the level of the adipocyte rather than an antagonist in the circulation.

It has been reported that lactation does not alter the number or affinity of insulin receptors of white adipocytes in the rat (Flint et al., 1979b). In agreement with this similar Scatchard plots were obtained in the present study for the binding of insulin to adipocyte plasma membranes from virgin and lactating rats. When the Scatchard plots were interpreted as comprising two components the values for the dissociation constants (see table 6.2.) were in good agreement with values reported by Flint et al. (1979b) for insulin binding to receptors on intact adipocytes. This suggests that the insulin receptors were not damaged during the isolation of plasma membranes. As the insulin binding capacity of adipocytes is unchanged during lactation the insulin resistance of adipocyte PDH must be due to a post-receptor defect. Studies elsewhere have suggested that insulin promotes the release of a mediator/s from plasma membranes which activates PDH in mitochondria (see section 1.3.6.7). To investigate the role of this putative mediator/s in

insulin resistance during lactation plasma membranes and mitochondria enriched fractions were prepared from adipocytes isolated from parametrial white adipose tissue of age-matched virgin and 14 day lactating rats. In previous studies by other workers subcellular fractions were prepared in either potassium phosphate buffer or Tris buffer (Seals & Jarett, 1980; Seals & Czech, 1981a): in the present study the fractions were prepared in potassium phosphate buffer as this produces plasma membranes which are more responsive to insulin (Seals & Czech, 1981a). In agreement with previous reports (Kiechle et al., 1981; Seals & Czech, 1981a) the plasma membranes and mitochondria could be stored for at least one week in liquid N_2 without any effect on the experimental results. To characterise the subcellular fractions succinic dehydrogenase activity was used as a mitochondrial marker and 5'nucleotidase was used as a plasma membrane enzyme marker. The activities of these marker enzymes in the subcellular fractions were in good agreement with values reported in other studies in which adipose tissue plasma membrane and mitochondrial fractions were prepared (McKeel & Jarett, 1970; Belsham et al., 1980; Begum et al., 1982a). There appeared to be little cross-contamination of the adipocyte plasma membrane and mitochondrial fractions and the marker enzyme activities, expressed on a protein basis, were very similar in subcellular fractions from virgin and lactating rats (see table 6.1.).

The inhibition of mitochondrial PDH activity by ATP is in agreement with previous studies (Seals & Jarett, 1980; Kiechle et al., 1981; Seals & Czech, 1981a): pre-incubation of mitochondrial fractions with ATP increases the percentage stimulation of control

PDH activity by insulin (Seals & Jarett, 1980; Seals & Czech, 1981a) and hence in all subsequent experiments the mitochondria were incubated with ATP prior to the addition of plasma membranes and insulin. In confirmation of previous reports (Seals & Jarett, 1980; Begum et al., 1982a) co-incubation of adipocyte plasma membranes and mitochondria from virgin rats resulted in a stimulation of PDH activity which was potentiated by insulin. Increasing concentrations of insulin produced corresponding increases in PDH activity until the maximum stimulation was achieved at 100μunit/ml: concentrations of insulin greater than 100μunit/ml produced a reduced stimulation of PDH activity. This biphasic response of PDH to increasing concentrations of insulin is in agreement with previous studies (Seals & Jarett, 1980; Seals & Czech, 1981a). The average maximal stimulation of PDH activity in the presence of insulin was about 48.3% (see table 6.3.) which is in good agreement with values reported by Begum et al. (1982a) but is higher than the value reported by Seals & Jarett (1980) who used Tris-HCl buffer instead of potassium phosphate buffer to prepare the subcellular fractions.

In contrast to the results for the co-incubation of subcellular fractions from virgin rat adipocytes co-incubation, either in the presence or absence of insulin, of plasma membranes and mitochondria isolated from adipocytes of 14 day lactating rats had no effect on PDH activity. To evaluate the separate contribution of mitochondria and plasma membranes to the observed difference in the effect on PDH activity of co-incubation of subcellular fractions from lactating and virgin rats mixing experiments were carried out. From table 6.4. it can be seen that the addition of plasma membranes from

virgin rats to mitochondria from lactating rats produced a stimulation of PDH activity which was potentiated by insulin but in contrast the addition of plasma membranes from lactating rats to mitochondria from virgin rats had no effect on PDH activity and no increase in PDH activity occurred after the addition of insulin to the latter incubation. There was no significant difference between the extent of the stimulation of PDH activity in mitochondrial fractions from virgin or 14 day lactating rats by plasma membranes from virgin rats either in the presence or absence of insulin. These results show that the ability of plasma membranes to stimulate PDH activity in a mitochondrial fraction in vitro is lost during lactation but suggest that the ability of mitochondrial PDH to respond to the stimulatory effects of plasma membranes is unchanged during lactation.

Supernatants obtained from insulin-treated virgin rat plasma membranes also stimulated mitochondrial PDH activity. Again increasing concentrations of insulin produced corresponding increases in PDH activity until a maximum effect was achieved with 100μunit/ml insulin and greater concentrations of the hormone produced a reduced stimulation of PDH activity. This response to insulin is in agreement with previous findings (Seals & Czech, 1981a). PDH activity was maximally stimulated by 120% by supernatant from insulin treated membranes which is in good agreement with values reported by Kiechle et al. (1981) and Begum et al. (1982a) but is greater than values found by Seals & Czech (1981a). These resuls show that plasma membranes from virgin rats release a substance or group of substances which activate mitochondrial PDH and that the production of this

substance/s is stimulated by insulin. In contrast plasma membranes from lactating rats do not release this substance/s either in the presence or absence of insulin.

It has been proposed that insulin stimulates the release of at least two different mediator substances from adipocyte plasma membranes, one which stimulates PDH activity and one which inhibits it (see section 1.3.6.7.). During lactation the production of the inhibitory substances/s could be increased and may cancel out the effects of any stimulatory substances/s released. To test this the effect of a combination of plasma membranes from virgin and lactating rats on PDH was investigated. The presence of lactating rat plasma membranes had no effect on the extent of activation of mitochondrial PDH by virgin rat plasma membranes and insulin (see fig. 6.9.) and hence it is unlikely that the lactating rat membranes produce an inhibitory substance.

In chapter 4 it was shown that the treatment of lactating rats with insulin produced a small but significant increase in the proportion of mammary gland PDH in the active state and others have reported that insulin stimulated PDH activity in mammary gland of diabetic or 24h starved rats in which the proportion of PDH in the active state was markedly decreased as compared to the value in fed lactating rats (Field & Coore, 1976; Baxter & Coore, 1978). However it is not certain that the activation of mammary gland PDH by insulin in vivo is a direct effect of the hormone upon the gland as, due to the lack of a suitable preparation of mammary tissue, it has not been possible to demonstrate such an effect of insulin in vitro (see section 1.2.3.1.4.). Insulin stimulates the release from plasma

membranes of a substance or group of substances, which stimulate mitochondrial PDH activity, in all insulin sensitive tissues that have been studied so far (see section 1.3.6.7.). The ability of plasma membranes isolated from lactating rat mammary gland to produce these putative insulin mediator/s was therefore investigated.

The activity of 5'nucleotidase in the mammary plasma membrane fraction was similar to values reported by others (Shin et al., 1975; Huggins & Carraway, 1976; Clegg, 1981). The activity of succinic dehydrogenase, a mitochondrial enzyme marker, was undetectable in the mammary plasma membrane fractions (see table 6.1.). The dissociation constants for the binding of insulin to the mammary plasma membrane fractions (see table 6.2.) were similar to those reported for insulin binding to isolated mammary acini (Flint, 1982a,b) which implies that the affinity of the receptors was not modified during the procedure for the isolation of the mammary plasma membranes. The K_d value for the binding of insulin to high affinity receptors on the mammary membranes was significantly greater than the value for high affinity receptors on adipocyte membranes ($P<0.02$; table 6.2.) but there was no significant difference between the K_d values for the binding of insulin to low affinity receptors on mammary and adipocyte membranes (Table 6.2.). The numbers of both high and low affinity receptors was about 3-fold higher in the mammary plasma membrane fraction than in the adipocyte plasma membrane fraction (table 6.2.). This does not necessarily reflect the comparative number of insulin binding sites on intact adipocytes and mammary cells as the size of the cells and efficiency of the procedures for the preparation of mammary and adipocyte plasma membranes would have to be taken into account for

such a comparison to be made from the data available. It has been reported that the number of insulin receptors on mammary epithelial cells is lower than on adipocytes in the rat (Flint, 1982a,b).

Co-incubation of the mammary plasma membranes with adipocyte mitochondria produced a stimulation of PDH activity that was potentiated by insulin. The concentration of insulin that most commonly produced a maximal stimulation of PDH was 50μunit/ml. This was lower than the concentration of insulin (100μunit/ml) that was maximally effective in incubations with adipocyte plasma membranes and so the sensitivity of the mammary membranes to insulin appears to be greater than the sensitivity of the adipocyte membranes: this may be due to the greater number of receptors present in the mammary plasma membrane fraction than in the adipocyte plasma membrane fraction (cf table 6.2.). The maximal stimulation of PDH activity in the presence of insulin was similar when mammary membranes (table 6.5.) or adipocyte plasma membranes (table 6.3.) were co-incubated with adipocyte mitochondria. These results show that insulin stimulates the release of a substance or group of substances from mammary plasma membranes which stimulate adipocyte mitochondrial PDH activity. The ability of this substance/s produced by rat mammary plasma membranes to stimulate PDH activity in intact mitochondria prepared from rat mammary tissue needs to be investigated to establish whether this substance/s could mediate the stimulation of mammary gland PDH by insulin _in vivo_.

Insulin stimulates fatty acid synthesis in the rat mammary gland _in vitro_ (Balmain & Folley, 1951; Balmain _et al._, 1954; Katz _et al._, 1974; Robinson & Williamson, 1977a,b) but has no effect on fatty

acid synthesis in sheep mammary gland slices (Balmain & Folley, 1951; Balmain et al., 1954). The ability of insulin to stimulate PDH activity in co-incubations of sheep mammary plasma membranes and rat adipocyte mitochondria was therefore tested. Addition of insulin to such incubations had no effect on PDH activity (see table 6.5.) showing that in contrast to rat mammary plasma membranes the sheep mammary plasma membranes do not release a substance which activates PDH in response to insulin.

To conclude, the present study shows that insulin resistance can be linked to impaired generation of a PDH activator substance/s from plasma membranes. Hence the loss of ability of insulin to stimulate PDH activity in white adipose tissue during lactation is accompanied by an impaired production of this putative insulin mediator/s from white adipocyte plasma membranes. Insulin stimulates the release of the PDH activator/s from plasma membranes from rat mammary gland which is an insulin sensitive tissue (see section 1.1.2.5.). In contrast insulin has no effect on the generation of such a substance from plasma membranes from sheep mammary gland and the hormone also appears to have no biological effects on this tissue.

Other physiological states which produce insulin resistance have been shown to result in a reduced ability of plasma membranes to generate the putative insulin mediator/s. The ability of insulin to stimulate fatty acid synthesis and lipogenesis in hepatocytes from fasted and non-ketotic diabetic rats is reduced (Caro & Armatruda, 1982; Armatruda & Chang, 1983) and this is accompanied by a markedly reduced ability of hepatocyte plasma membranes to generate the

putative insulin mediator/s: refeeding of fasted rats and insulin treatment of diabetic rats restores the ability of insulin to generate the mediator (Armatruda & Chang, 1983). Adapting rats to a high fat diet results in a reduced insulin responsiveness of adipocytes (Ip et al., 1976; Begum et al., 1982a) and hepatocytes (Tepperman et al., 1978) and again this is accompanied by a reduced ability of insulin to stimulate the generation of the putative mediator substance/s from the respective plasma membranes (Begum et al., 1982a; 1983). Very recently it has also been shown that an increased insulin responsiveness of PDH in white adipocytes from rats subjected to acute exercise is accompanied by enhanced insulin stimulated production of the PDH activator substance/s from the adipocyte plasma membranes (Begum et al., 1986).

The loss of generation of the insulin mediator/s from adipocyte plasma membranes during lactation is not due to a change in the insulin binding capacity of the tissue but involves a post-receptor defect. The exact nature of the putative insulin mediator/s is uncertain (see section 1.3.6.7.). However very recently two novel mediator substances which are produced by bovine liver plasma membranes (Saltiel & Cuatrecasas, 1986) and cultured murine myocytes (Saltiel et al., 1986) after exposure to insulin have been partially purified and characterised. Studies suggest that insulin stimulates a selective phospholipase C in the plasma membranes which hydrolyses a novel glycolipid present in the membranes resulting in the generation of the putative mediators along with diacylglcyerol (see section 1.3.6.7.). It is possible that the PDH activator/s generated by adipocyte plasma membranes in response

to insulin are produced in a similar manner. Therefore the loss of the ability of insulin to generate these putative mediators from adipocyte plasma membranes during lactation could be due to a reduced availability of the glycolipid precursor in the membranes, a reduced amount of the selective phospholipase C present in the membranes, a reduced responsiveness of the enzyme or an impairment in the coupling of the enzyme to the insulin receptor. Elucidation of the nature of the adaptation which prevents the generation of the putative insulin mediator/s during lactation will depend on further characterisation of the mediators and their production.

Although during lactation there appears to be a defect in the response of white adipocytes to insulin at the level of the plasma membrane the possibility that other post-receptor defects exist, for example at the level of the mitochondria, cannot be excluded. It is thought that the putative insulin mediators enters mitochondria and interacts directly with PDH-phosphatase resulting in a stimulation of phosphatase activity which leads to an increase in the amount of PDH present in the active dephosphorylated form (see section 1.2.3.1.1.). The mitochondria used in the present study were stored frozen in liquid N_2 and so were unlikely to be intact. Therefore the response of PDH activity to the mediator/s in these mitochondrial fractions does not necessarily reflect the ability of PDH to respond to the insulin mediator in situ. It cannot be ruled out that during lactation the ability of the mediator/s to enter mitochondria and gain access to PDH-phosphatase is also impaired or that the environment of the phosphatase is modified in such a way that the stimulation of the enzyme by the mediator/s is reduced.

Fig. 6.1. The effect of insulin on pyruvate dehydrogenase activity in adipocytes from virgin and lactating rats

Adipocytes, isolated from parametrial white adipose tissue of virgin (●) or 14 day lactating rats (○), were incubated with the concentrations of insulin indicated in the presence of 5mM glucose at 37°C for 20 min after which PDH activity was measured. Further details are in the methods section and chapter 2. Results are means ± SEM for 3 observations. Total PDH activity in the virgin rat adipocytes was 45.3 ± 4.8munit/10^6 adipocytes and in the lactating rat adipocytes was 22.2 ± 5.9munit/10^6 adipocytes.

FIG.6.1.

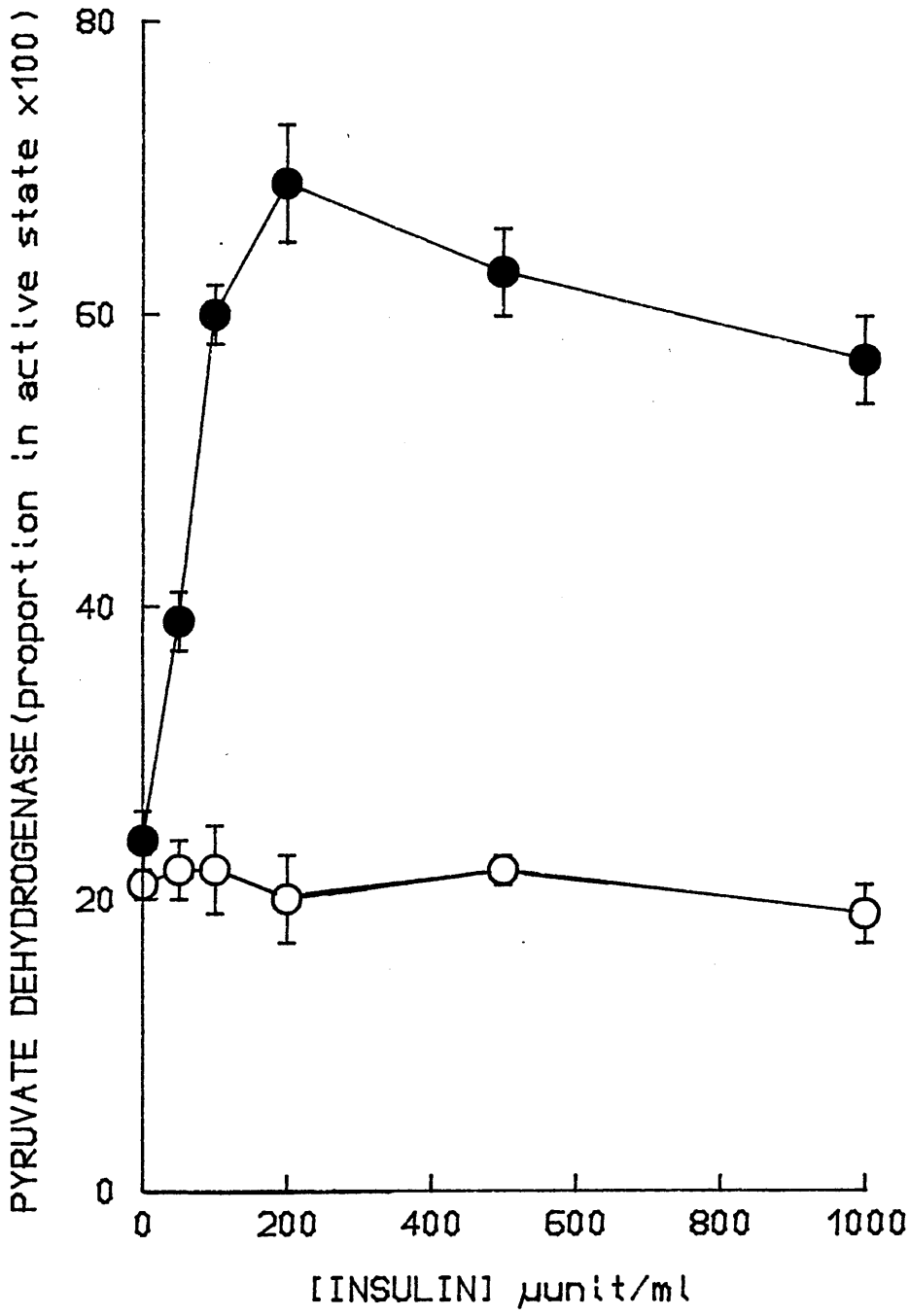

PYRUVATE DEHYDROGENASE(proportion in active state x100)
80
60
40
20
0
0
200
400
600
800
1000
[INSULIN] μunit/ml

Fig. 6.2. Time course for the stimulation of adipocyte pyruvate dehydrogenase activity by insulin

A time course is shown for the activation of PDH by insulin. Adipocytes, isolated from parametrial white adipose tissue of virgin rats, were incubated with (●) or without (○) 200μunit/ml insulin in the presence of 5mM glucose at 37°C for the times indicated after which PDH activity was measured. Further details are in the methods section and chapter 2. The experiment was carried out three times with similar results.

fig.6.2.

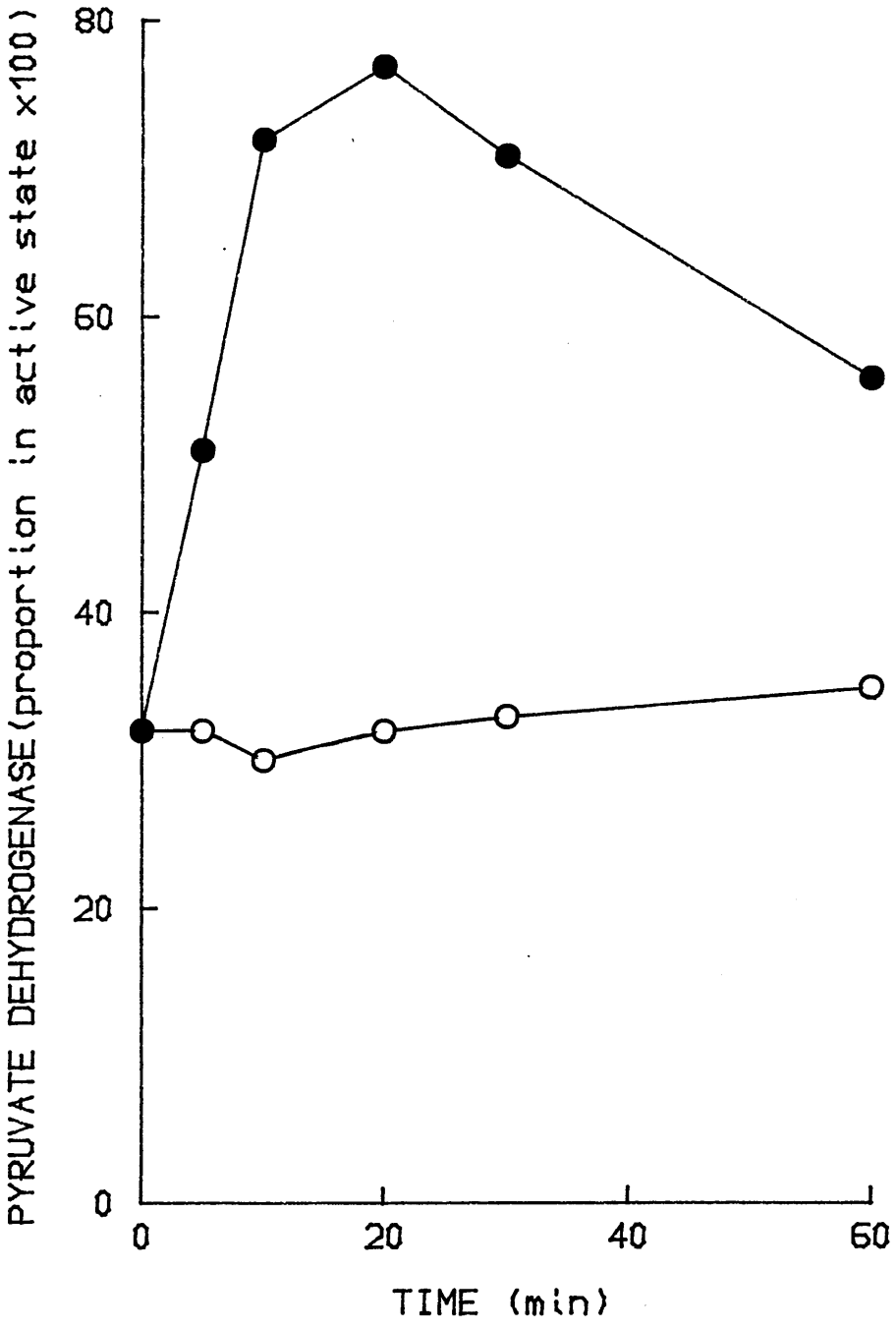

PYRUVATE DEHYDROGENASE(proportion in active state x100)
80
60
40
20
0
0
20
40
60
TIME (min)

Fig. 6.3. The effect of insulin In Vitro on pyruvate dehydrogenase activity in white adipose tissue pieces from virgin rats

Tissue pieces were prepared from parametrial white adipose tissue of virgin female rats and were incubated with the indicated concentrations of insulin in the presence of 5mM glucose at 37°C for 30 min after which pyruvate dehydrogenase activity was measured. Further details are in the methods section and chapter 2. Results are means ± SEM for 3 experiments. Total PDH activity was 220.5 ± 12.9munit/g tissue.

FIG.6.3.

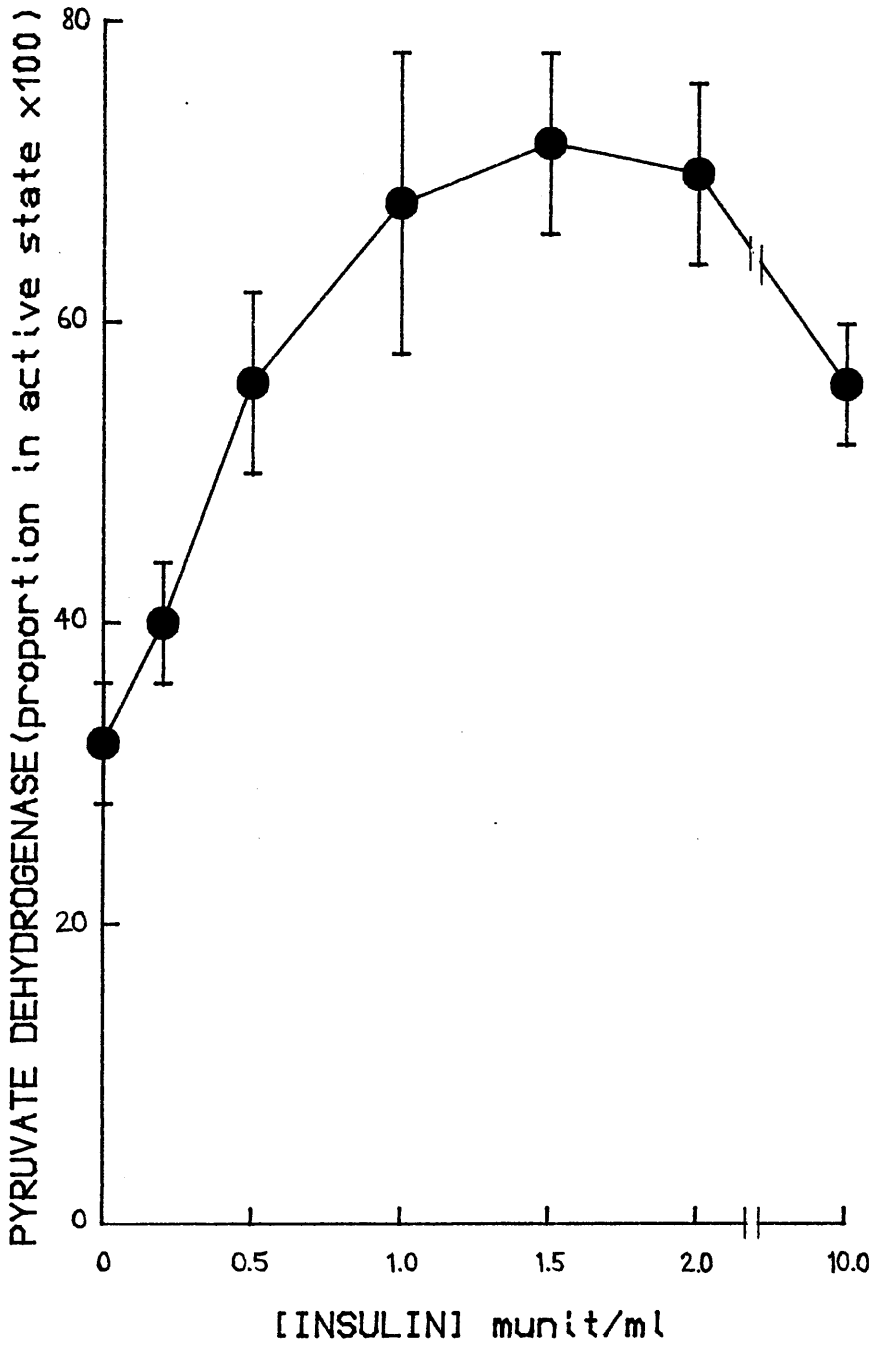

PYRUVATE DEHYDROGENASE (proportion in active state x100)
80
60
40
20
0
0
0.5
1.0
1.5
2.0
10.0
[INSULIN] munit/ml

Fig. 6.4. Scatchard plot of the insulin binding to plasma membranes prepared from white adipocytes from virgin and lactating rats

^{125}I-labelled insulin binding to plasma membranes from virgin (○) or lactating (●) rats was measured and corrected for non-specific binding as described in chapter 2. Each point is the mean value obtained from separate determinations with plasma membranes prepared from adipocytes of four rats.

FIG.6.4.

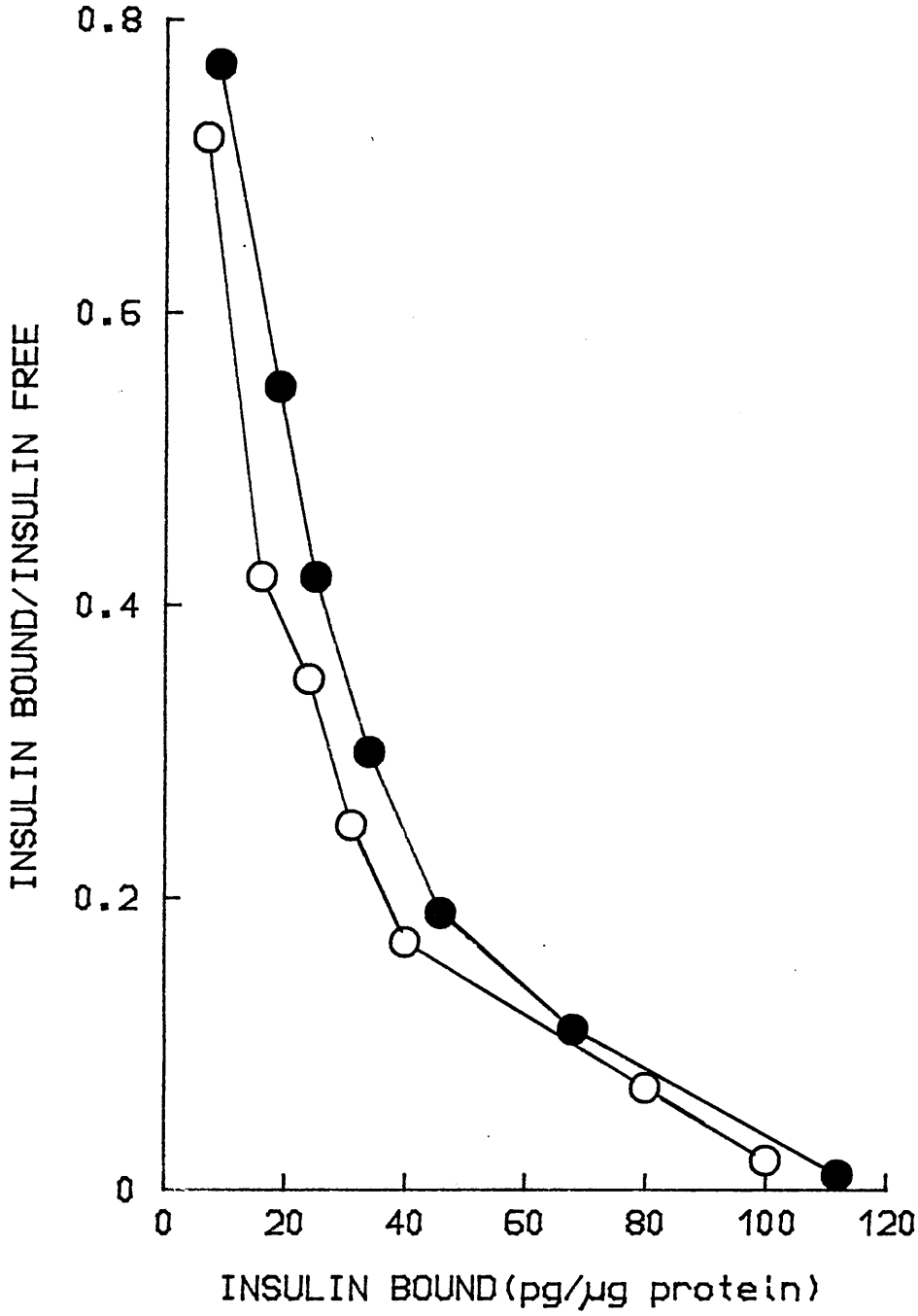

0.8
0.6
0.4
0.2
0
INSULIN BOUND/INSULIN FREE
0
20
40
60
80
100
120
INSULIN BOUND(pg/μg protein)

Fig. 6.5. Inhibition of pyruvate dehydrogenase activity by ATP

A time course for the inhibition of mitochondrial PDH activity by ATP is shown. Adipocyte mitochondria (180μg protein/ml) from virgin rats were incubated in the presence (O) or absence (●) of 250μM ATP for the times indicated after which PDH activity was measured. For further details see the methods section and chapter 2. The experiment was carried out three times with similar results.

FIG.6.5.

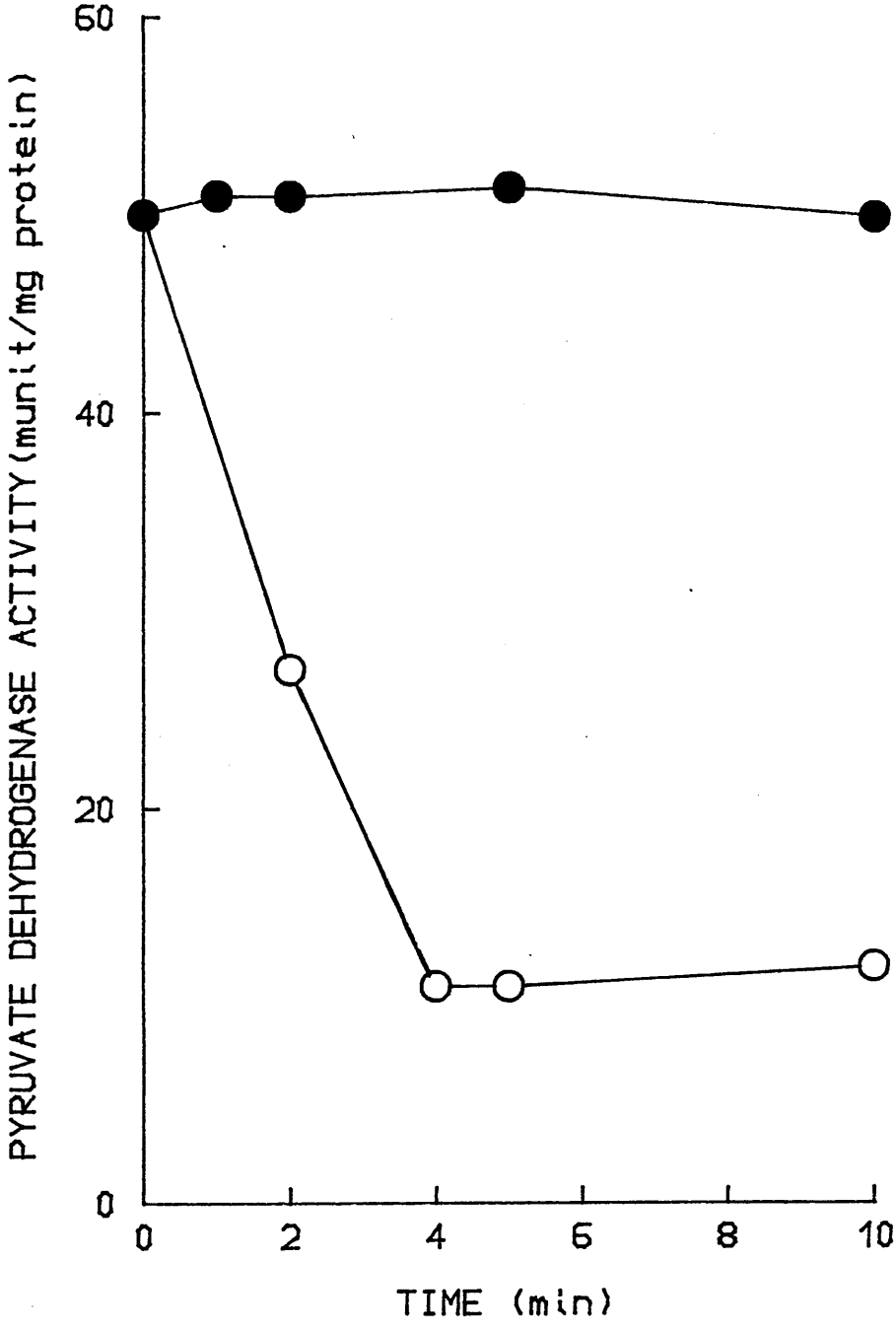

PYRUVATE DEHYDROGENASE ACTIVITY(munit/mg protein)
60
40
20
0
0
2
4
6
8
10
TIME (min)

Fig. 6.6. The effect of adipocyte plasma membranes from virgin and lactating rats on pyruvate dehydrogenase activity

Dose response relationships are shown for the ability of adipocyte plasma membranes to stimulate mitochondrial PDH activity. Adipocyte mitochondria (180μg protein/ml) from virgin (●) or 14 day lactating (○) rats, were pre-incubated with 250μM ATP at pH 7.4 and 37°C for 5 min after which the incubations were continued for a further 5 min in the presence of the indicated concentrations of adipocyte plasma membranes from virgin (●) or 14 day lactating (○) rats. PDH activity was then measured. For further details see the methods section and Chapter 2. The experiments were carried out three times with similar results.

FIG.6.6.

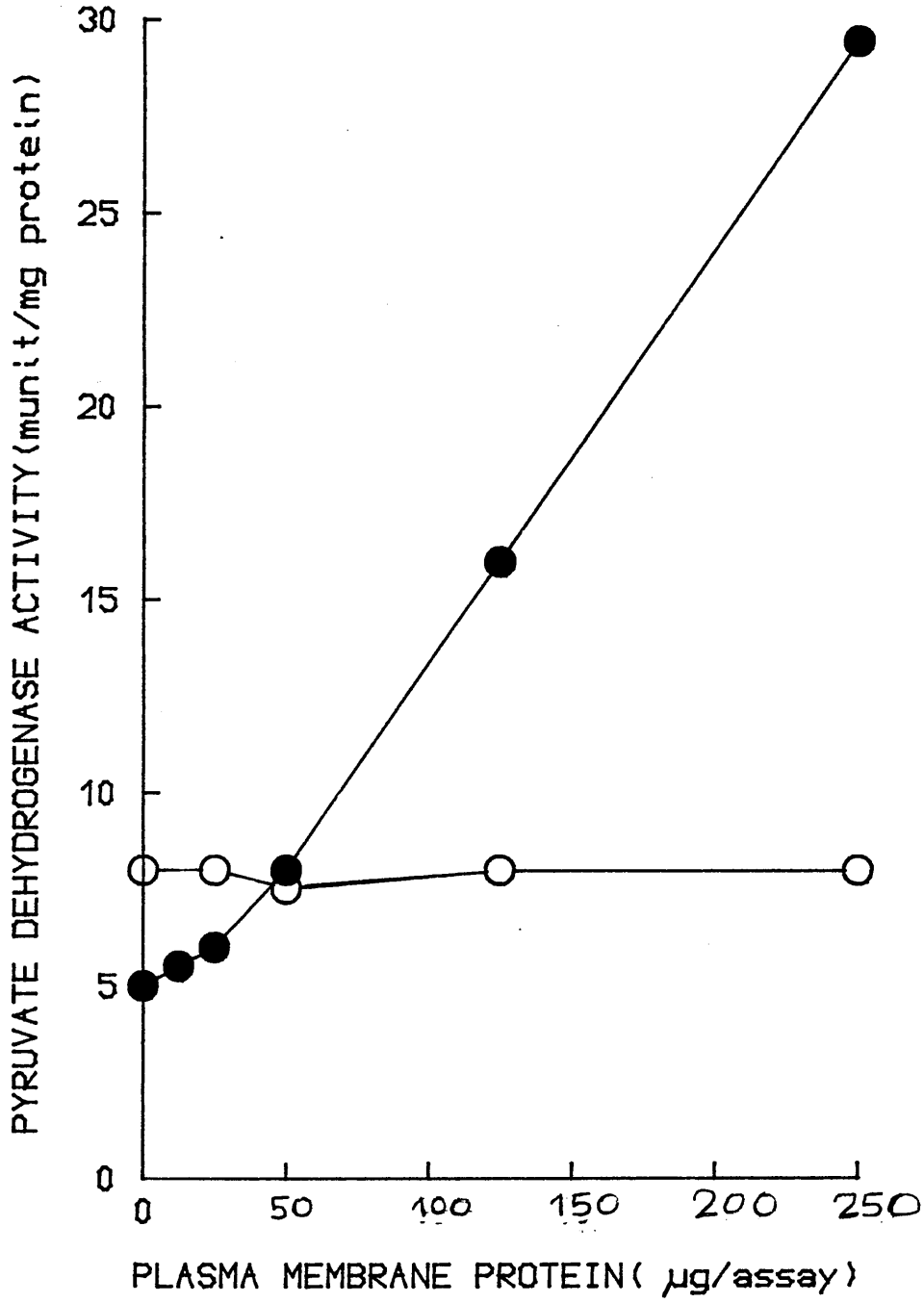

PYRUVATE DEHYDROGENASE ACTIVITY(munit/mg protein)
30
25
20
15
10
5
0
0
50
100
150
200
250
PLASMA MEMBRANE PROTEIN(μg/assay)

Fig. 6.7. Stimulation of pyruvate dehydrogenase activity by addition of insulin to a mixture of mitochondria and plasma membranes isolated from adipocytes of virgin rats

A dose response relationship for the activation of PDH by insulin is shown. Adipocyte mitochondria (180μg protein/ml) were pre-incubated with 250μM ATP at pH 7.4 and 37°C for 5 min after which the incubation was continued for a further 5 min in the presence (●) or absence (O) of adipocyte plasma membranes (150μg protein/ml) and with the concentrations of insulin indicated. PDH activity was then measured. For further details see the methods section and chapter 2. The experiment was carried out three times with similar results.

FIG.6.7.

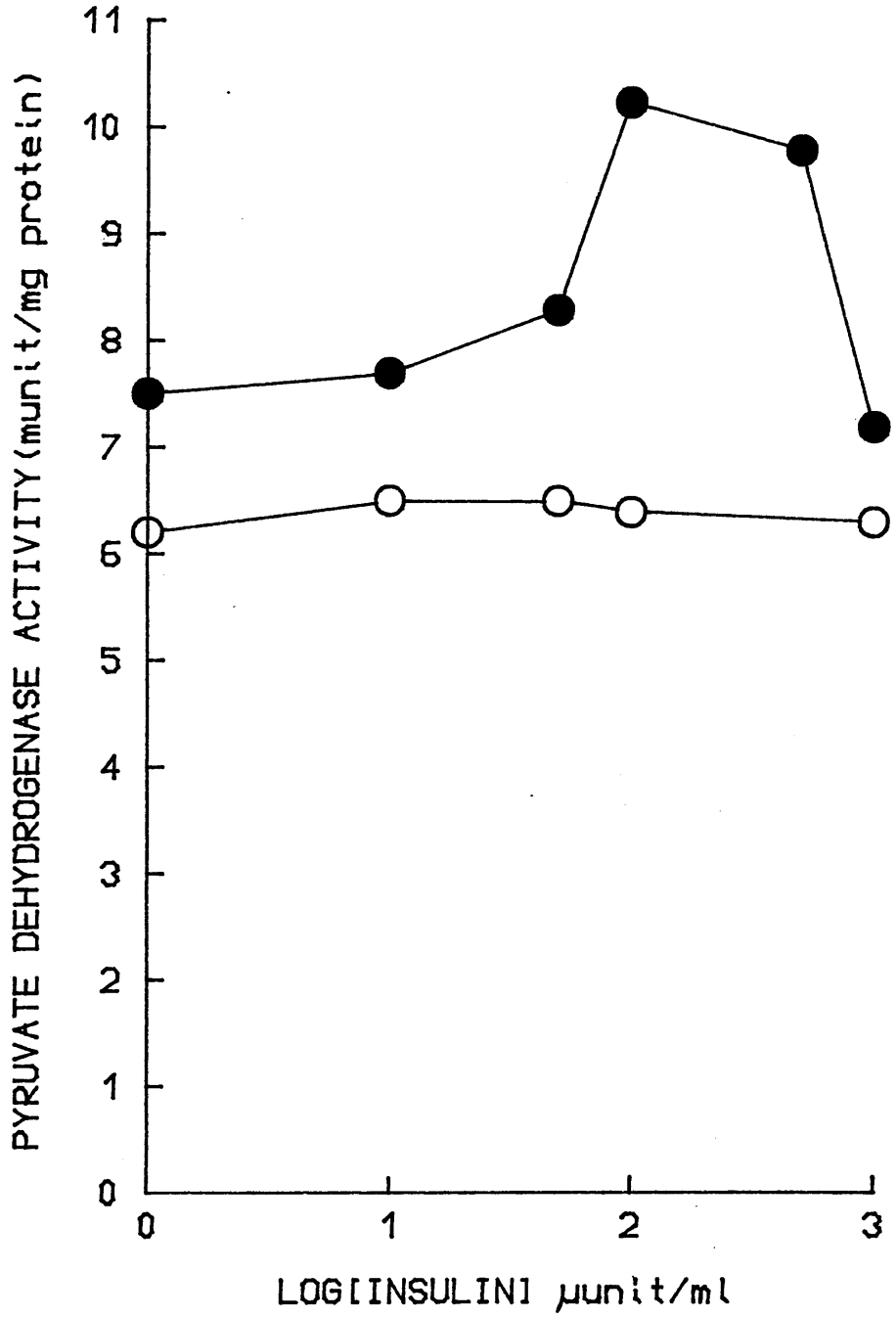

PYRUVATE DEHYDROGENASE ACTIVITY(munit/mg protein)
11
10
9
8
7
6
5
4
3
2
1
0
0
1
2
3
LOG[INSULIN] μunit/ml

Fig. 6.8. Time course for the stimulation of pyruvate dehydrogenase activity by the addition of insulin to a mixture of plasma membranes and mitochondria from adipocytes of virgin rats

A time course for the activation of mitochondrial PDH by insulin is shown. Adipocyte mitochondria (180μg protein/ml) were pre-incubated with 250μM ATP at pH 7.4 and 37°C for 5 min after which the incubation was continued in the presence of insulin (100μunit/ml) with (●) or without (O) plasma membranes (150μg protein/ml) for the times indicated. For further details see the methods section and chapter 2. The experiment was carried out twice with similar results.

FIG.6.8.

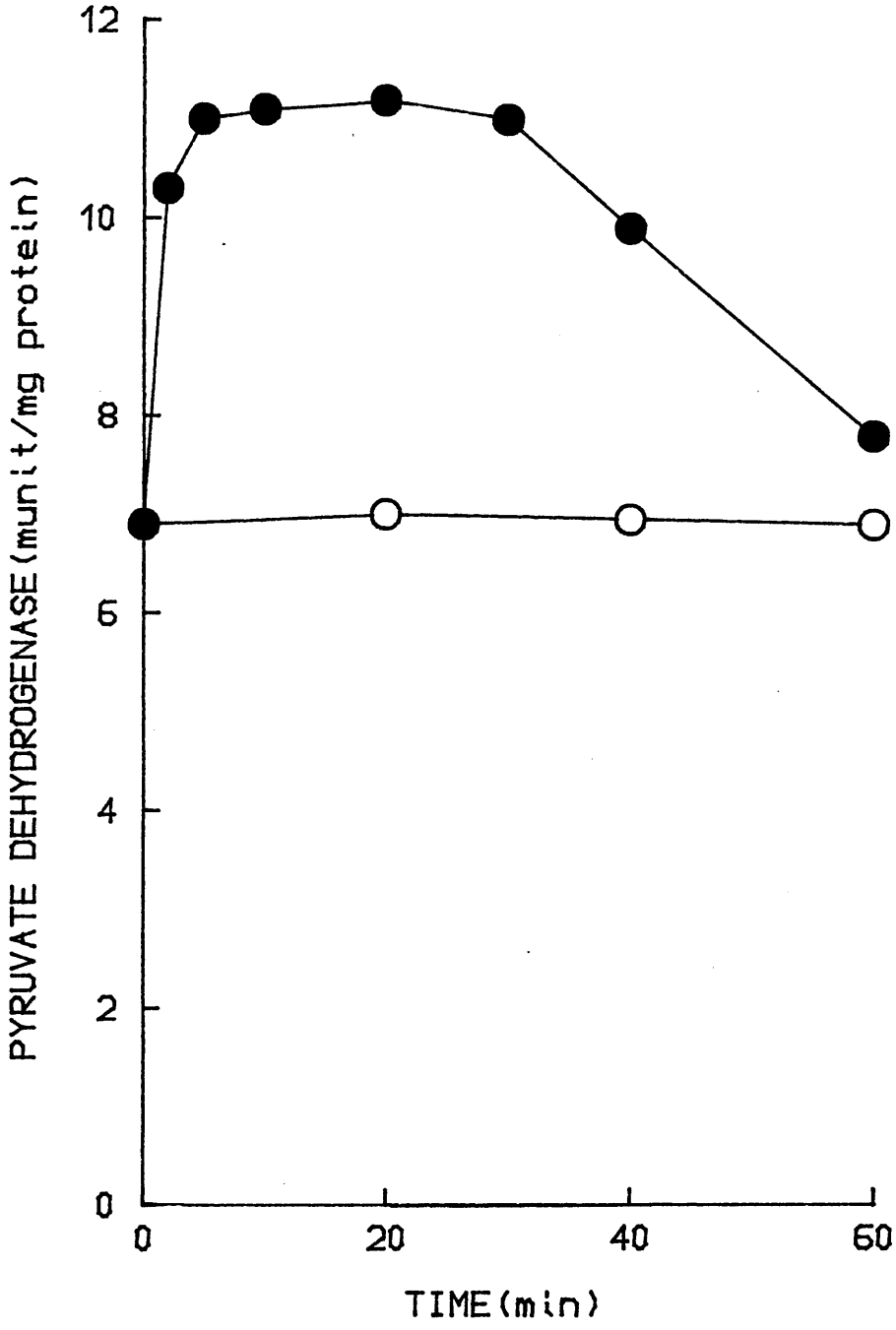
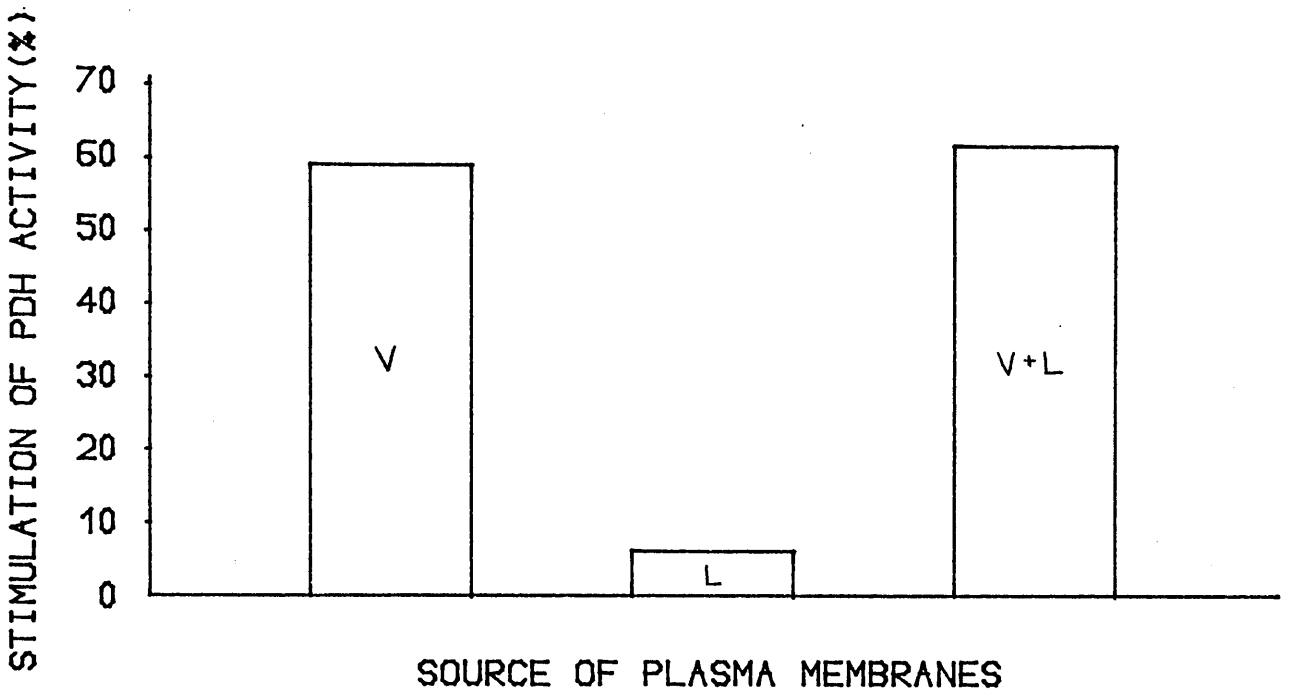

12
10
8
6
4
2
0
0
20
40
60
PYRUVATE DEHYDROGENASE(munit/mg protein)
TIME(min)

Fig. 6.9. The effect of lactation on the ability of adipocyte plasma membranes to stimulate and inhibit pyruvate dehydrogenase activity

Mitochondria were from adipocytes from virgin rats and plasma membrane fractions were prepared from adipocytes of virgin and 14 day lactating rats. Mitochondria (180μg protein/ml) were pre-incubated with 250μM ATP at pH 7.4 and 37°C for 5 min then the incubations were continued for a further 5 min in the presence of insulin (100μunit/ml) plus plasma membranes (150μg protein/ml) from virgin (V) or 14 day lactating (L) rats or a combination of plasma membranes (150μg protein/ml of each type) from both virgin and 14 day lactating rats (V + L). PDH activity was then measured. For further details see methods section and chapter 2. Results are for one experiment only.

FIG.6.9.


STIMULATION OF PDH ACTIVITY(%)
70
60
50
40
30
20
10
0
V
L
V+L
SOURCE OF PLASMA MEMBRANES

6.10. The effect of supernatant from insulin-treated adipocyte plasma membranes on pyruvate dehydrogenase activity

Dose response relationships are shown for the stimulation of PDH by insulin. Plasma membranes (150μg protein/ml), isolated from adipocytes of virgin (●) or 14 day lactating (O) rats, were incubated with the indicated concentrations of insulin at 37°C and pH 7.4 for 5 min and were then pelleted by microcentrifugation. The supernatants from each plasma membrane sample were added to mitochondria (180μg protein/ml), from adipocytes of virgin rats, which had been pre-incubated with 250μM ATP for 5 min. The mitochondria and supernatants were incubated together for 5 min prior to the measurement of PDH activity. Further details are in chapter 2. The experiment was carried out three times with similar results.

FIG.6.10.

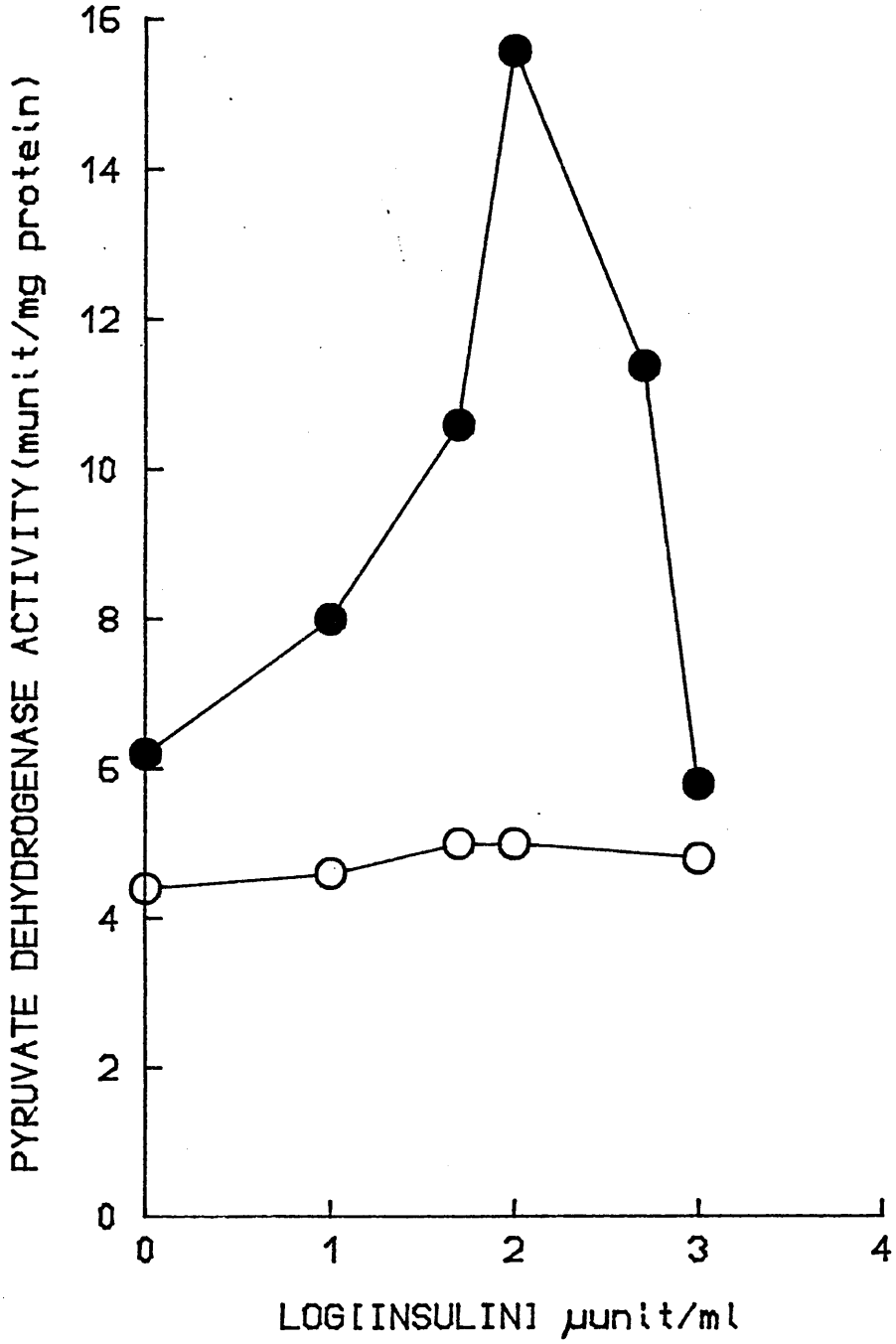
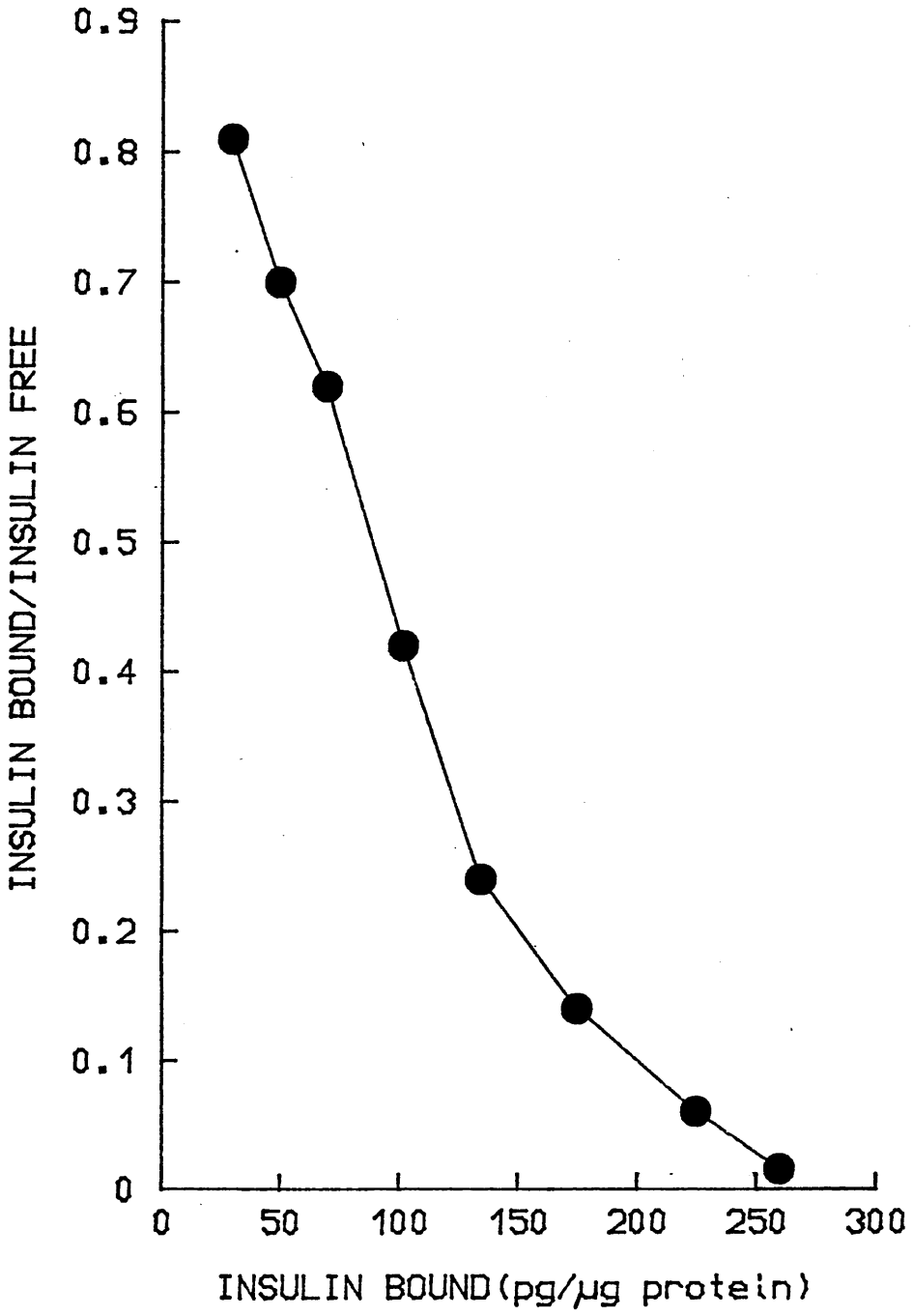

PYRUVATE DEHYDROGENASE ACTIVITY(munit/mg protein)
16
14
12
10
8
6
4
2
0
0
1
2
3
4
LOG[INSULIN] μunit/ml

Fig. 6.11. Scatchard plot of the insulin binding to plasma membranes prepared from mammary tissue from 14 day lactating rats

^{125}I-labelled insulin binding was measured and corrected for non-specific binding as described in chapter 2. Each point is the mean value obtained from separate determinations with plasma membranes prepared from mammary tissue of four rats.

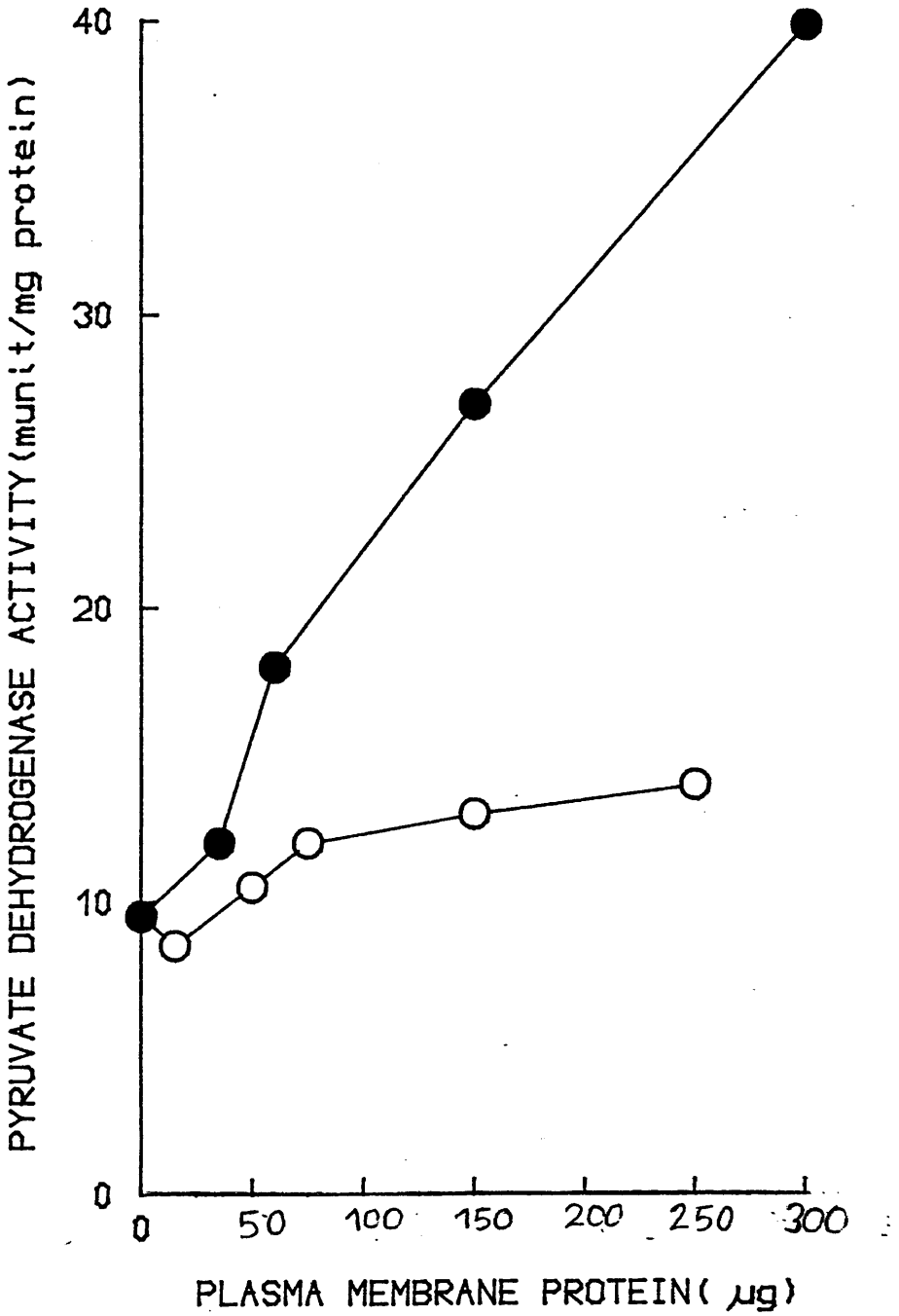
FIG. 6. 11.


0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
INSULIN BOUND/INSULIN FREE
0
50
100
150
200
250
300
INSULIN BOUND (pg/µg protein)

6.12. Stimulation of pyruvate dehydrogenase activity by mammary plasma membranes from lactating rats or sheep

Dose response relationships for the stimulation of mitochondrial PDH activity by mammary plasma membranes from 14 day lactating rats or 18-21 day lactating sheep are shown. Rat adipocyte mitochondria (180μg protein/ml) were pre-incubated for 5 min at pH 7.4 and 37°C with 250μM ATP after which the incubation was continued for a further 5 min in the presence of rat mammary (●) or sheep mammary (○) plasma membranes. PDH activity was then measured. For further details see the methods section and chapter 2. The results for sheep mammary plasma membranes are from one experiment only and three experiments were carried out using rat mammary plasma membranes, each time with similar results.

FIG. 6. 12.


40
30
20
10
0
0
50
100
150
200
250
300
PYRUVATE DEHYDROGENASE ACTIVITY(munit/mg protein)
PLASMA MEMBRANE PROTEIN(μg)

6.13. The activation of pyruvate dehydrogenase by the addition of insulin to a mixture of plasma membranes from mammary tissue and adipocyte mitochondria

Dose response relationships for the activation of PDH by insulin are shown. Mitochondria were prepared from adipocytes from virgin rats and plasma membrane fractions were from mammary tissue from 14 day lactating rats or 18-21 day lactating sheep. Mitochondria (180μg protein/ml) were pre-incubated for 5 min with 250μM ATP at pH 7.4 and 37°C after which the incubations were continued for a further 5 min in the presence of 150μg/ml rat plasma membranes (●) or 150μg/ml sheep plasma membranes (○) along with the indicated concentrations of insulin. PDH activity was then measured. For further details see the methods section and chapter 2. The experiment was carried out three times with similar results.

FIG. 6. 13.

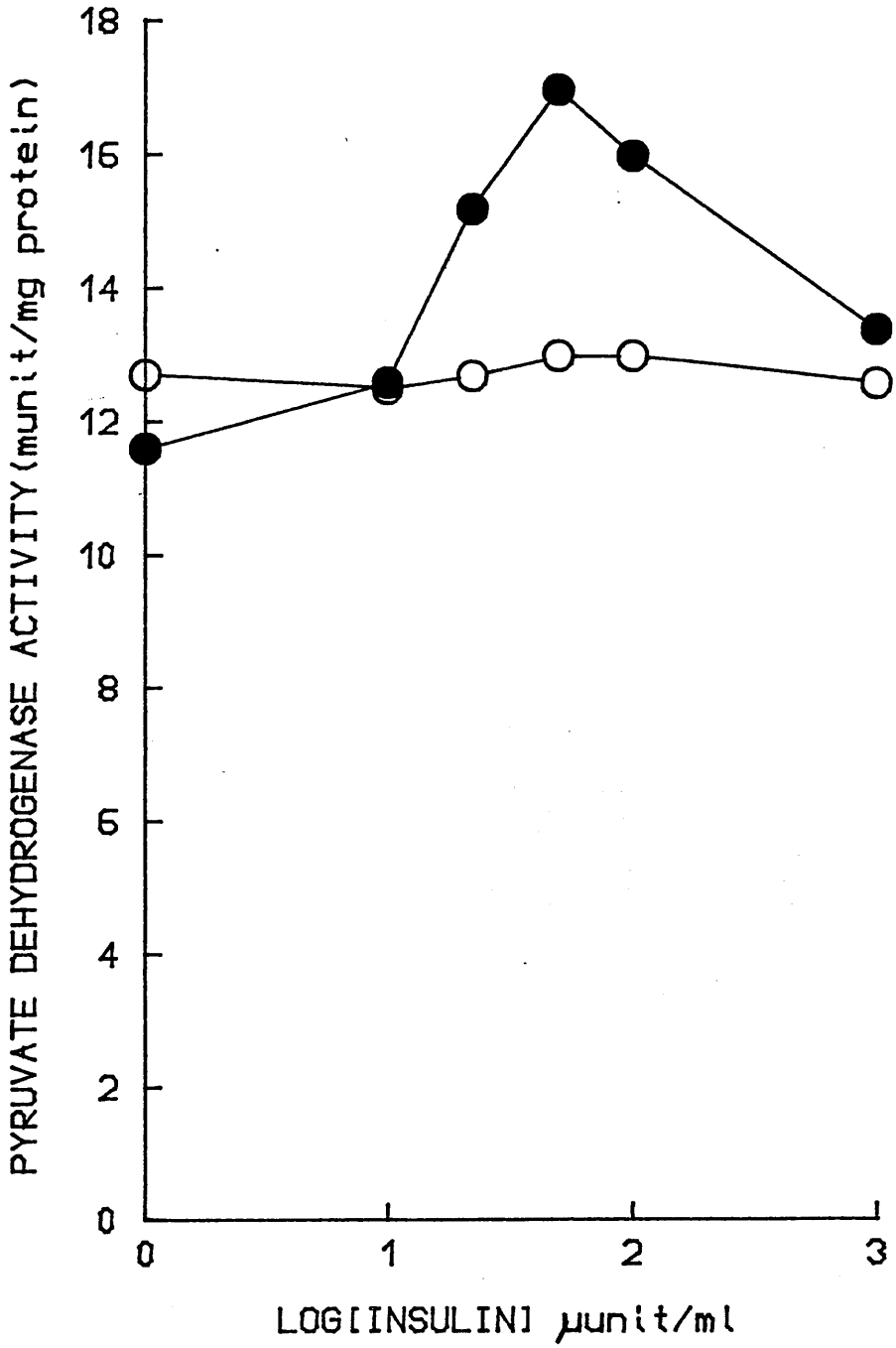

PYRUVATE DEHYDROGENASE ACTIVITY(munit/mg protein)
18
16
14
12
10
8
6
4
2
0
0
1
2
3
LOG[INSULIN] μunit/ml

Table 6.1a. Protein recovery and 5'nucleotidase activity in mitochondrial fractions prepared from adipocytes and plasma membrane fractions prepared from adipocytes and mammary tissue

Mitochondria and plasma membrane fractions were prepared as described in chapter 2. Details of the procedures used to measure protein concentrations and 5'nucleotidase activity are in chapter 2. Results are means ± SEM with the number of observations in parenthesis.

				5'nucleotidase		
					S. A. Frac.*	
State	Tissue	Fraction	Protein recovery (% of homogenate)	Specific activity (munit/mg protein)	specific activity homogenate	Recovery (% of homogenate)
Virgin	rat adipose	mitochondria	5.4±1.2(5)	2.8±0.4(5)	0.8±0.1(5)	3.9±1.0(5)
Virgin	rat adipose	plasma membrane	2.6±0.2(5)	17.4±1.7(5)	7.0±1.1(5)	18.8±3.9(5)
Lactating (14 day)	rat adipose	mitochondria	3.1±0.8(4)	2.3±0.6(4)	1.0±0.2(4)	3.2±1.0(4)
Lactating (14 day)	rat adipose	plasma membrane	2.1±0.3(4)	15.2±1.6(4)	7.0±0.8(4)	14.4±2.2(4)
Lactating (14 day)	rat mammary	plasma membrane	0.5±0.1(7)	571.4±66.6(7)	14.3±1.9(7)	6.7±1.3(7)
Lactating (18-21 day)	sheep mammary	plasma membrane	0.8±0.3(4)	273.2±47.4(4)	8.0 1.7(4)	6.6±1.1(4)

*Specific Activity Fraction

Table 6.1b. Succinic dehydrogenase activity in mitochondrial fractions prepared from adipocytes and plasma membrane fractions prepared from adipocytes and mammary tissue

Mitochondria and plasma membrane fractions were prepared as described in chapter 2. Details of the succinic dehydrogenase assay are also in chapter 2. The protein recovery and 5'nucleotidase activities in the fractions are given in table 6.1a. Results are means ± SEM with the number of observations in parenthesis.

			Succinic dehydrogenase		
State	Tissue	Fraction	Specific activity (munit/mg protein)	Specific activity fraction: Specific activity / Specific activity homogenate	Recovery (% of homogenate
Virgin	rat adipose	mitochondria	16.9±6.5(5)	3.2±0.5(5)	16.2±3.0(5)
Virgin	rat adipose	plasma membrane	3.7±1.4(5)	0.7±0.2(5)	1.3±0.5(5)
Lactating (14 day)	rat adipose	mitochondria	19.9±1.9(4)	3.7±0.4(4)	12.5±2.2(4)
Lactating (14 day)	rat adipose	plasma membrane	5.3±0.4(4)	1.0±0.1(4)	2.1±0.3(4)
Lactating (14 day)	rat mammary	plasma membrane	ND*	—	—
Lactating (18-21 day)	sheep mammary	plasma membrane	4.0±1.6(4)	1.1 ±0.2(4)	0.6±0.3(4)

*ND - activity not detectable

Table 6.2. Apparent dissociation constant and binding capacity for insulin binding to plasma membranes from rat adipocytes and mammary tissue

Plasma membrane fractions were prepared from adipocytes from virgin and 14 day lactating rats and from mammary tissue from 14 day lactating rats. For the purpose of analysis Scatchard plots (figs. 6.4., 6.11.) for ^{125}I-labelled insulin binding to the plasma membranes were interpreted as comprising of two components and the dissociation constants (kd) and number of insulin binding sites (n) were evaluated. For further details see chapter 2. Results are means ± SEM for 4 observations; ** indicates that the value is significantly different ($P<0.02$) from the value for plasma membranes prepared from virgin rat adipocytes. 1nM insulin is equal to 150μunits/ml.

Source of plasma membrane	Kd (nM)		Binding capacity (10^{-9} x sites/μg protein)	
	High affinity sites	Low affinity sites	High affinity sites	Low affinity sites
Virgin rat adipose tissue	1.1±0.06	7.2±1.5	4.9±0.5	10.5±0.5
Lactating rat adipose tissue	1.1±0.07	7.7±0.5	4.8±0.4	10.7±0.6
Lactating rat mammary gland	2.4±0.4**	8.4±2.4	15.8±2.1	28.5±1.0

Table 6.3. The effect of lactation on the activation of adipocyte mitochondrial pyruvate dehydrogenase by adipocyte plasma membranes and insulin

Mitochondria and plasma membrane fractions were prepared from adipocytes of virgin and 14 day lactating rats. Mitochondria (180μg protein/ml) were preincubated for 5 min with 250μM ATP at pH 7.4 and 37°C after which the incubation was continued for a further 5 min with or without plasma membranes (150μg protein/ml) and insulin (100μunit/ml) as indicated in the table. PDH activity was then measured. Further details are in the methods section and chapter 2. The results are means ± SEM for 4 observations; * indicates that the value is significantly different ($P<0.05$) from the value for co-incubation of plasma membranes and mitochondria in the absence of insulin.

Incubation components			PDH activity	Insulin effect	
Mitochondria	Plasma membranes	Insulin	(munit/mg protein)	Absolute change	% stimulation
Virgin rat	-	-	8.9±1.4		
Virgin rat	Virgin rat	-	12.1±1.7		
Virgin rat	Virgin rat	+	17.6±1.1*	5.5±1.0	48.3±10.1
Lactating rat	-	-	9.4±1.4		
Lactating rat	Lactating rat	-	9.4±1.5		
Lactating rat	Lactating rat	+	9.2±1.3	-0.2 0.3	-

Table 6.4. The effect of lactation on the ability of adipocyte plasma membranes to activate mitochondrial pyruvate dehydrogenase in the presence and absence of insulin

Plasma membrane and mitochondrial fractions were prepared from adipocytes of virgin and 14 day lactating rats. For the experimental procedure see the legend to table 6.3. Results are means ± SEM for 4 observations; * indicates the value is significantly different ($P<0.05$) from the value for the co-incubation of plasma membranes and mitochondria in the absence of insulin.

Incubation components				Insulin effect	
Mitochondria	Plasma Membranes	Insulin	PDH activity munit/mg protein	Absolute change	% stimulation
Virgin rat	-	-	8.9±1.4		
Virgin rat	Lactating rat	-	8.9±1.5		
Virgin rat	Lactating rat	+	8.7±1.1	-0.1±0.5	-
Lactating rat	-	-	9.4±1.4		
Lactating rat	Virgin rat	-	10.5±1.6		
Lactating rat	Virgin rat	+	15.2±0.9*	4.7±1.3	47.8±18.0

Table 6.5. The effect of mammary plasma membranes from lactating rats and lactating sheep and insulin on pyruvate dehydrogenase activity in adipocyte mitochondria

Mitochondria (180μg protein/ml), isolated from adipocytes of virgin rats, were pre-incubated for 5 min with 250μM ATP at 37°C and pH 7.4 after which the incubations were continued for a further 5 min following the addition of 14 day lactating rat mammary plasma membranes (150μg protein/ml), 18-21 day lactating sheep mammary plasma membranes (150μg protein/ml) and insulin (50μunit/ml) as indicated in the table. PDH activity was then measured. Further details are in the methods section and chapter 2. Results are means ± SEM for 4 observations; *** indicates the value is significantly different ($P<0.001$) from the value for the co-incubation of plasma membranes with mitochondria in the absence of insulin.

Incubation components				Insulin effect	
Mitochondria	Plasma Membranes	Insulin	PDH activity (munit/mg protein)	Absolute change	% stimulation
Rat adipocyte	-	-	16.7±0.9(4)		
Rat adipocyte	Rat mammary	-	18.6±0.2(4)		
Rat adipocyte	Rat mammary	+	26.3±1.5(4)***	7.7±1.6(4)	41.7±9.2(4)
Rat adipocyte	-	-	14.2±2.7(4)		
Rat adipocyte	Sheep mammary	-	17.4±2.7(4)		
Rat adipocyte	Sheep mammary	+	17.2±3.1(4)	-0.2±0.5(4)	-

CHAPTER 7

Final Discussion

This study is an investigation of changes in PDH activity and in the control of PDH by insulin and catecholamines in the tissues of the rat during the reproductive cycle.

Tissue specific changes occurred in PDH activity during pregnancy and lactation in the rat (chapter 3 and chapter 4). Between parturition and peak lactation total PDH activity in white adipose tissue and the proportion of PDH in the active state in skeletal muscle (see chapter 3 and chapter 4) declined while both total PDH present and the proportion of the enzyme present in the active state in the mammary gland increased substantially. These changes in PDH activity were accompanied by tissue specific changes in the responsiveness of PDH to insulin (chapter 4). The ability of insulin to stimulate PDH activity in white adipose tissue was completely lost during lactation whereas the ability of the hormone to stimulate the enzyme activity in skeletal muscle and liver was retained. This loss of ability of insulin to stimulate PDH activity in white adipose tissue appeared to be due to an impaired generation from adipocyte plasma membranes of an unidentified insulin mediator substance/s (chapter 6).

Initially as an aside, the effects of catecholamines on PDH activity in rat white adipose tissue were investigated. This was prompted by the potent stimulatory effect of stress on the enzyme

activity in this tissue along with the conflicting reports which existed in the scientific literature concerning the effects of adrenaline and noradrenaline on white adipose tissue PDH (see chapter 5). Results of the present study showed that catecholamines stimulated PDH activity in white adipose tissue in vivo and in isolated adipocytes in vitro (chapter 5). This observation is physiologically relevant as it aids the understanding of the response of the rat to eating. Feeding results in an increase in sympathetic activity in the rat (see Le Magnen, 1984; Landsberg & Young, 1985) which by increasing PDH activity in white adipose tissue will promote glucose utilisation in the tissue and therefore help diminish the hyperglycemia that follows a meal. Catecholamines appear to promote glucose oxidation but not fatty acid synthesis in white adipose tissue (see chapter 5) therefore the stimulation of PDH activity by catecholamines will not serve to increase lipid stores in the tissue but may contribute to increased heat production found on eating in rats (see section 5.4.3.). The ability of noradrenaline to activate white adipose tissue PDH was reduced in lactating rats (see chapter 5).

The reduced PDH activity in skeletal muscle and white adipose tissue during lactation will limit the irreversible use of circulating glucose by these tissues. Furthermore the resistance of white adipose tissue PDH to stimulation by insulin and catecholamines will mean that the increase in serum insulin concentration and sympathetic nervous activity that occurs in rats on feeding (see Le Magnen, 1984; Landsberg & Young, 1985) will not result in an increased flux of glucose to acetyl-CoA in white adipose tissue.

These changes in PDH activity in white adipose tissue and skeletal muscle in the lactating rat along with the impaired activation of PDH in white adipose tissue by insulin and catecholamines and the increase in PDH activity in the mammary gland will all facilitate the preferential use of circulating lactate and glucose carbon for milk production.

The reasons for many of the changes in PDH activity and its endocrine control during lactation can only be speculated upon. The fall in the serum insulin concentration during lactation can explain the decline in active PDH in skeletal muscle (see chapter 3). Prolactin may be involved in the increase in the proportion of PDH in the active state in mammary gland during lactation as withdrawal of this hormone for 24h results in a decrease in active PDH in the gland while having little effect on the total amount of enzyme present (Field & Coore, 1975; 1976). However the reasons for the fall in total PDH in white adipose tissue and the reciprocal rise in mammary gland during lactation are not certain. Removal of pups for 48h produces a fall in total PDH in mammary gland (Coore & Field, 1974; this study chapter 4) and a rise in total PDH in white adipose tissue (see chapter 4). Prolactin has been implicated in the reciprocal changes in lipoprotein lipase activity, insulin receptor numbers and the rate of lipogenesis in white adipose tissue and mammary gland in the lactating rat (Flint et al., 1981; Flint et al., 1984; Vernon & Flint, 1984) and it is therefore tempting to speculate that prolactin also induces reciprocal changes in the total amount of PDH present in these tissues. It appears that prolactin primes mammary PDH to respond to insulin (Field & Coore, 1976) and perhaps it has a

reciprocal effect on white adipose tissue causing the loss of ability of insulin to activate PDH. It is interesting that the proportion of PDH in the active state in white adipose tissue is increased in 48h weaned rats to values greater than those in virgin rats (see chapter 4). This is probably caused by the increase in the serum insulin concentration which occurs on weaning (see chapter 4) and this suggests that the refactoriness of white adipose tissue to insulin is lost within 48h of the removal of pups. If prolactin is responsible for the insulin resistance of white adipose tissue during lactation this effect of weaning might be prevented by the concurrent administration of prolactin and mimicked by administration of bromocriptine to lactating rats. However any effects of prolactin on white adipose tissue must be indirect as the hormone does not appear to bind to white adipocytes (see section 1.1.2.1.).

The role of growth hormone in the metabolic adaptations which occur during lactation has received little attention. However, it was shown recently that an antiserum to rat growth hormone acted synergistically with bromocriptine (which lowers serum prolactin) to diminish milk yield in rats (Madon et al., 1986). Also removal of the litter from lactating rats results in a fall in the rate of noradrenaline-stimulated lipolysis of adipocytes which can be prevented by administration of growth hormone to such rats and mimicked by injecting lactating rats with an antiserum to growth hormone (Vernon et al., 1987). However serum growth hormone levels in the rat show little change during lactation (Madon et al., 1986) or after litter removal (Vernon et al., 1987). In contrast to prolactin, growth hormone binds directly to rat adipocytes (Fagin et

al., 1980; Eden et al., 1982). The decrease in lipolytic response on litter removal could be due to rat adipocytes becoming less sensitive to rat growth hormone, although they do respond to ovine growth hormone (Vernon et al., 1987). Endogenous growth hormone, which is biologically active during lactation, may become less effective on litter removal, possibly due to a change in growth hormone-binding proteins in blood or to a change in the physical state of the hormone itself (see Vernon et al., 1987). It is established that growth hormone antagonises insulin action in adipocytes (Schwartz, 1980; Schwartz & Eden, 1985) and the hormone could be involved in the loss of ability of insulin to activate PDH in white adipose tissue during lactation. If growth hormone becomes less effective after litter removal this could explain the increase in PDH activity in white adipose tissue which occurs in 48h weaned rats probably in response to the increase in the serum insulin concentration (see chapter 4).

Both prolactin and growth hormone may therefore be involved in impaired generation of an insulin mediator/s from adipocyte plasma membranes during lactation (see chapter 6). Other insulin resistant states are accompanied by reduced production of the insulin mediator/s from plasma membranes; fasting and diabetes mellitus reduce hepatic mediator production (Armatruda & Chang, 1983) and feeding rats a high fat diet reduces mediator production from white adipocyte and hepatocyte plasma membranes (Begum et al., 1982a; 1983). However it is unlikely that prolactin or growth hormone are involved in insulin resistance induced by diabetes, fasting or feeding a high fat diet. It remains unknown whether a common defect in the insulin-transducing system is responsible for impaired

generation of the insulin mediator/s in all of these insulin resistant states.

Recent studies elsewhere suggest that the putative insulin mediators are carbohydrate-phosphate substances containing inositol and glucosamine and are hydrolysed, from a glycolipid present in plasma membranes, by an insulin-stimulated selective phospholipase C (see section 1.3.6.7.). Depletion of the putative insulin mediators from rat adipocyte plasma membranes, either by repeated centrifugation of membranes (see section 1.3.6.7.) or by treatment of the membranes with a phosphatidylinositol-specific phospholipase C from S. aureus (Saltiel & Cuatrecasas, 1986; Saltiel et al., 1986) would reveal whether or not the amount of glycolipid precursor present in plasma membranes is lower in adipocytes from lactating rats than virgin rats. Alternatively the loss of insulin stimulated mediator production from lactating rat plasma membranes could be due to a reduced amount or responsiveness of the endogenous insulin-sensitive phospholipase C or to a defect in the coupling of the insulin-receptor to this enzyme. Further elucidation of the role the endogenous phospholipase C has in promoting insulin resistance in lactating rat adipocytes will require the purification and further characterisation of this enzyme. It is also possible that lactating rat adipocyte plasma membranes release the putative insulin mediator/s but in a modified form which does not activate PDH. Labelled inositol and glucosamine can be incorporated into the mediators produced by cultured murine myocytes (Saltiel et al., 1986) and the culture of white adipocytes from lactating rats in the presence of [^{3}H]-inositol and [^{3}H]-glucosamine might allow the

detection of any mediator produced by the cells even if it were in an inactive form. In addition the culture of rat adipocytes along with agents such as prolactin and growth hormone, which may influence white adipose tissue metabolism in the lactating rat, could reveal the effects of these hormones on the ability of adipocyte plasma membranes to generate the putative insulin mediator(s).

The mechanism of the activation of white adipose tissue PDH by catecholamines is unknown. In liver and heart adrenaline activates PDH via an increase in cytoplasmic Ca^{++} which is relayed into mitochondria (see section 1.2.3.2.). In the present study the stimulation of white adipose tissue PDH activity by adrenergic agents occurred via both α_1 and β-receptors (see chapter 5). In many tissues the stimulation of α_1-receptors leads to an increase in the intracellular phosphoinositide turnover ultimately resulting in the stimulation of membrane bound protein kinase C and an increase in intracellular Ca^{++} concentration (see section 1.4.1.3.). Cheng & Larner (1985) who also observed an α_1-adrenergic stimulation of white adipose tissue PDH activity, showed that this was secondary to an increase in intracellular Ca^{++} levels and concluded that this was probably due to the mobilisation of Ca^{++} from intracellular stores. However the mechanism of the activation of white adipose tissue PDH by β-agonists is less clear. Stimulation of β-adrenergic receptors results in the activation of adenylate cyclase, via coupling of the receptor to a guanine nucleotide regulatory protein, and this leads to an increase in the intracellular cAMP concentration (see section 1.4.1.1.). Although an involvement of cAMP in mediating the stimulation of white adipose tissue PDH activity by β-agonists can

not be ruled out studies on mitochondria isolated from white adipose tissue have failed to detect any effect of cAMP on PDH (Coore et al., 1971). Recently it has been shown that insulin enhances the α_1-adrenergic stimulation of phosphoinositide turnover in white adipocytes and that although β-agonists by themselves have no effect on phosphoinositde metabolism in white adipocytes they potentiate the insulin stimulated formation of phosphoinositides (Pennington & Martin, 1985). It is therefore tempting to speculate that the synergistic effect of insulin and catecholamines on white adipose tissue PDH activity (see chapter 5) is due to effects of these hormones on phosphoinositide metabolism. However it is unlikely that all of these hormonal effects on PDH activity are mediated by increases in intracellular Ca^{++} levels: the stimulation of PDH activity in adipose tissue by insulin does not involve increases in the intramitochondrial Ca^{++} concentration (see section 1.2.3.1.). Clearly further studies are necessary to elucidate the mechanisms whereby catecholamines stimulate PDH activity in adipose tissue.

The reasons for the reduced responsiveness of white adipose tissue to catecholamines during lactation are not certain. It is not known whether the catecholamine binding capacity of white adipocytes is reduced during lactation. However in the absence of adenosine the ability of catecholamines in vitro to stimulate lipolysis in white adipose tissue is unchanged during lactation suggesting that there is no change in the number of β-receptors on fat cells (see section 1.1.2.1.). The response of adipocytes to the antilipolytic effect of adenosine is increased during lactation. As a result the lipolytic effect of catecholamines on adipocytes in vitro is reduced when

adenosine is allowed to accumulate in the incubations (see section 1.1.2.1.). PDH activity in white adipocytes is stimulated by adenosine (Wong et al., 1984) and therefore an increase in the response to adenosine during lactation is unlikely to account for the reduced stimulation of PDH by catecholamines.

During lactation the tissue specific changes in PDH activity and its endocrine control will help the rat meet the substrate and energy demands of milk production. At present the understanding of the mechanisms responsible for these changes is limited. Further knowledge of these mechanisms could eventually allow the manipulation of the metabolism of selective tissues and of the integration of metabolism between tissues; this ability could be used in the control of obesity and metabolic disorders. Lactation also provides a system for the study of the mechanism of action of hormones such as insulin and catecholamines. Identification of the defects responsible for the loss of responsiveness of white adipose tissue to insulin and catecholamines during lactation may provide some insight into the mechanism of action of these hormones. Therefore the study of the control of metabolism during lactation is not only important in itself but is also relevant to the study of obesity and hormone action.

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