# DEVELOPMENT OF AN ELISA METHOD FOR UNCOUPLING PROTEIN AND THE USE OF THIS ASSAY IN THE STUDY OF BROWN ADIPOSE TISSUE DURING PREGNANCY AND LACTATION

by

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## ARRANGEMENT OF THESIS


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## SUMMARY

Uncoupling protein (UCP) has been regarded as the regulator of thermogenesis in brown adipose tissue (BAT) by modulating the proton conductance pathway in the mitochondria of BAT.

The first aim of the present work was to develop an enzyme linked immunosorbent assay (ELISA) specific for UCP. The second aim was to make use of this ELISA method to measure UCP content as an index of BAT thermogenic capacity during the breeding cycle; pregnancy, lactation and post weaning, and to study the mechanism regulating the thermogenic function of BAT during pregnancy and lactation.

Guanosine 5'-diphosphate (GDP) binding assay is a common method of measuring the thermogenic activity of UCP. This assay has been used in the present work to indicate the purity and the native conformation of UCP during isolation and purification.

Experiments were performed using the GDP binding assay to detect changes in thermogenic activity of BAT. During cold acclimation there was 4-fold increase in GDP binding, however, noradrenaline injection did not increase GDP binding further in cold adapted rats. Noradrenaline treatment significantly increased GDP binding in warm acclimated rats. Hyperphagia is a common phenonemon accompanying cold adaptation. Since both cold stimulus and

hyperphagia enhance thermogenic activity, an experiment was designed to study the role of these two stimuli during cold adaptation. Feeding the same amount of food to the warm adapted rats as to the cold adapted rats could only slightly suppress the GDP binding activity. The cold stimulus seems to contribute more than hyperphagia to the enhancement of thermogenic function of BAT.

The method of Lin and Klingenberg (1980) was used to isolate and purify UCP from BAT mitochondria. Lubrol PX and TritonX-100 were used to dissolve mitochondrial proteins. UCP was separated from mitochondrial proteins by hydroxylapatite column chromatography. TritonX-100 was removed by Bio-Beads and the purified UCP was concentrated.

The purity of UCP was demonstrated by SDS-PAGE and GDP binding. The results of SDS-PAGE showed that the UCP solution contained one major protein band at 32 kDa. A faint band was also observed at 64 kDa corresponding to an aggregate of two molecules of UCP. The purification factor in present studies was about 15-fold as measured by GDP binding activity and the percentage recovery was 65.9%. By Scatchard analysis, Bmax and the kd of GDP binding to UCP were 30,050 pmol/mg protein and 13.64 $\mu$mol/L respectively. The high Bmax indicated the high purity of UCP and the native conformation of UCP was possibly maintained. The molecular ratio of GDP binding to UCP was 0.939 mol GDP/mol of monomer confirming the hypothesis proposed by York and his coworkers that there is only one GDP binding

one GDP binding site per molecule of UCP.

Purified UCP was injected into rabbits to raise antisera against rat-UCP. The antiserum with the highest titre was chosen and characterized. Immunoblotting technique was employed to detect the specificity of the antiserum. Mitochondrial proteins obtained from liver, skeletal muscle and white fat did not show any immuno-reaction with the antiserum. The antiserum was also specific for UCP and did not cross-react with other proteins from BAT mitochondria. A dose response to purified UCP was also demonstrated in the immunoblots studies. Moreover, the antiserum cross-reacted with UCP from BAT mitochondria of other rodent species i.e. hamster, guinea pig and mouse. All these qualities show that the antiserum is a good antibody for immunoassay.

A competitive ELISA method was established and dose response curves using purified UCP and BAT mitochondrial protein were obtained. The inter-assay precision was 12.7% and 14.1% for warm (503 $\mu$g/BAT) and cold (1105 $\mu$g/BAT) adapted rats respectively. The intra-assay precision was 6% and 9.5% at concentrations of purifed UCP 312 and 20 ng/50 $\mu$l respectively. Using this method UCP content in rats after 4 days of cold acclimation was found to increase by 90%.

During pregnancy and lactation, there was an increase in food consumption. After weaning the food consumption

returned to normal. Although there was hyperphagia, there was no enhancement of thermogenic function of BAT. In mid pregnancy, there was significant increase in UCP content and BAT weight. During late pregnancy and lactation, there was a remarkable decline in thermogenic capacity and the lowest value was observed in late lactation. During lactation there was an increase in serum prolactin (PRL) concentration. The hypothesis that the suppression of thermogenic capacity may be caused by the hyperprolactinemia was tested by pharmacologic studies: metoclopramide (which enhances PRL secretion) decreased UCP content but the suppressive effect on thermogenic capacity was abolished by simultaneous injection of bromocriptine (which decreases PRL secretion). These studies clearly indicate that the alteration of PRL level could affect UCP content in BAT and that the suppression of thermogenic function during lactation is mediated by PRL.

Using $^{125}$I-labelled PRL for the receptor binding assay, BAT membrane appeared to be devoid of PRL receptors. This suggests that PRL could not act directly on BAT and may act through other regulatory mechanisms. One possible mechanism is by modulation of the sympathetic outflow to BAT. Further studies are required to elucidate the mechanism of PRL action in regulating the thermogenic capacity of BAT during lactation.

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## ABBREVIATIONS

| | |
|---|---|
| ADP | adenosine 5'-diphosphate |
| ATP | adenosine 5'-triphosphate |
| BAT | brown adipose tissue |
| Bmax | maximum binding number |
| BSA | bovine serum albumin |
| BW | body weight |
| °C | degree Celsius |
| cm | centimeter |
| CoA | coenzyme A |
| acyl-CoA | acyl coenzyme A |
| $^{14}C$ | carbon-14 |
| Cyclic AMP | adenosine 3',5',-cyclic monophosphate |
| DIT | diet-induced thermogenesis |
| DNA | deoxyribonucleic acid |
| cDNA | complimentary deoxyribonucleic acid |
| dpm | disintegrations/minute |
| EDTA | ethylenediamine tetra-acetic acid |
| ELISA | enzyme linked immunosorbent assay |
| EM | electron microscopy |
| g | gram |
| GDP | guanosine 5'-diphosphate |
| GTP | guanosine 5'-triphosphate |
| h | hour |
| $^{3}H$ | tritium |
| 5-HT | 5-hydroxytrayptamine |
| $^{125}I$ | Iodine-125 |
| i.p. | introperitoneal |
| Kd | dissociation constant |
| kDa | kilo dalton |
| L | litre |
| log | logarithm (base 10) |
| mA | milliampere |
| mg | milligram |
| min | minute |
| mmol | millimole |
| mmol/L | millimole per litre |
| ml | millilitre |
| µCi | microcurie |
| µg | microgram |
| µl | microlitre |
| mol | mole |
| MOPS | 3-[N-Morpholino]propranesulfonic acid |
| mRNA | messager ribonucleic acid |
| MW | molecular weight |
| NAD(P) | nicotinamide-adenine dinucleotide (phosphate), oxidized |
| NAD(P)H | nicotinamide-adenine dinucleotide (phosphate), reduced |
| NE | noradrenaline |
| ng | namogram |

| | |
|---|---|
| NIDDK | National Institute of Diabetes and Digestive and Kidney Diseases |
| NIH | National Institute of Health |
| NST | non-shivering thermogenesis |
| PBS | phosphate buffered saline |
| % | per cent |
| PEG | polyethylene-glycol 4000 |
| PPO | 2,5-Diphenyloxazole |
| PRL | prolactin |
| rat-PRL | rat-prolactin |
| ovine-PRL | ovine-prolactin |
| RIA | radioimmunoassay |
| rpm | round per minute |
| SDS-PAGE | sodium dodecyl sulphate-polyacrylamide gel electrophoresis |
| SEM | standard error of mean |
| $T_3$ | 1-tri-iodo-thyronine |
| $T_4$ | 1-thyroxine |
| TES | N-tris (hydroxylmethyl) methyl-2-aminoethanesulfonic acid |
| Tris | Tris(hydroxymethyl)aminomethane |
| UCP | uncoupling protein |
| xg | gravitational field, unit of (9.81 $ms^{-1}$) |
| v/v/v | volume/volume/volume |
| v/v | volume/volume |
| W | Watt |
| w/v | weight/volume |
| wt | weight |

# CHAPTER I

# LITERATURE REVIEW

## 1. History

Brown adipose tissue (BAT), also called brown fat was first described by Conrad Gesner in 1551. Afzelius (1970) described five periods in the history of BAT. They are: BAT was considered as part of the thymus (1670-1817); an endocrine gland (1817-1863); a modified form of fatty energy reserve (1863-1902); an endocrine gland again (1902-1916); finally, an effector of thermogenesis (1916 onwards). In the past BAT was considered by many to contribute very little to thermogenesis except perhaps in hibernators because of its relatively low mass compared with other tissues such as muscle, liver and even brain.

Research in the 1960s and early 1970s established the role of BAT in the hibernator and in the newborn (for reviews see Lindberg 1970 and Joel 1965; Smith and Horwitz 1969).

In 1978, Foster and Fryman demonstrated that BAT can produce heat at a rate equivalent to 500 W/kg. This is an order of magnitude greater than the aerobic power of muscle (approximately 60 W/kg during maximum exercise).

This raised BAT as one of the most important effectors of thermogenesis. In the following year, Rothwell and Stock (1979a) proposed that in addition to thermoregulatory thermogenesis, BAT is important in the regulation of energy balance, particularly during overnutrition. They called this diet-induced thermogenesis (DIT).

In mid 1970s, studies at the molecular level provided information on the nature and the mechanism of BAT thermogenesis. Nicholls (1974) proposed that purine nucleotides are involved in the regulation of proton conductance in BAT mitochondria and it was also demonstrated that mitochondrial guanosine 5'-diphosphate (GDP) binding capacity varied according to thermogenic capacity. Later, Ricquier and Kader (1976) demonstrated that BAT possesses an apparently unique mechanism for uncoupling respiration from ATP synthesis (see review Nicholls and Locke 1984; Shrago and Strieleman 1987). It has been shown that there is a unique protein related to the uncoupling mechanism located in the inner membrane of mitochondria. This protein is called uncoupling protein (UCP) or thermogenin. This unique protein has a molecular weight of 32 kDa and is found only in BAT mitochondria and it varies with thermogenic capacity. Heaton, Wagenvoord, Kemp and Nicholls (1978) subsequently found that an azido derivative of ATP has a high binding affinity to this protein, thus establishing this as the proton conductance site in the mitochondrial membrane. Using molecular

biology techniques cDNA for UCP has been isolated (Jacobsson, Stadker, Glotzer and Kozak 1985, Bouillaud, Ricquier, Thibault and Weissenbach 1985). In the last 10 years, the mechanism of the adaptive response of brown fat during development, cold adaptation, and hyperphagia has been studied extensively. Amongst the studies on the factors controlling BAT thermogenesis the sympathetic nervous system (SNS) and the hypothalamus have received most attention.

## 2. Species Difference

BAT has been identified almost exclusively in mammals (see review Afzelius 1970); Rowlatt, Mrosovsky and English (1971) claimed to have observed perinatal BAT in the majority of 285 species studied. The presence of functional BAT in adult human is still a debatable issue. However, BAT is histologically detectable in infants, and in adults at a variety of locations in which it occurs in infancy (Aherne and Hull 1966; Hassi 1977). BAT was also found in marsupials (Loudon, Rothwell and Stock 1985). Although birds are homeothermic, BAT or tissue with similar function has not been shown (Saorela, Hissa, Pyornila, Harjula, Ojanen and Orell 1989). In various species of fish, particularly in 'warm-bodied' fish such as the shark and tuna, a thermogenic tissue with numerous mitochondria and a very rich blood supply, perhaps functioning as a 'brain heater' has been described (Carey

1982). Although further biochemical and functional characterization of this tissue is required, it appears to perform a function similar to that of BAT.

## 3. Distribution of BAT

BAT is not a single organ and is found in many sites in the body. BAT is found around the kidneys, heart and aorta, around the carotid arteries and jugular vein, along the sternum and intercostal muscles, in the subcutaneous interscapular and subscapular regions, axillary region, the nape of the neck and in small patches between the ribs (see review, Nechad 1986). The anatomical sites of BAT led Smith (1961) to suggest that the primary function of the tissue is to warm blood passing from periphery into the trunk, and to heat the major organs.

## 4. Structure of BAT

### 4.1. Macroscopic Appearance

#### 4.1.1. Innervation

Innervation of BAT has been studied mainly in the interscapular BAT, due to its accessibility. To the naked

eye, BAT is innervated by the ipsilateral intercostal nerves. It was suggested that there is extensive cross-innervation between lobes since unilateral sectioning causes 60% loss of sympathetic nerves on the ipsilateral side, and 25% loss contralaterally (Seydoux, Constantinidis, Tsacopoulos and Girstfirt 1977).

Morphological, biochemical and functional studies, clearly show that BAT possesses a very rich sympathetic innervation which is important in the short term control of its thermogenesis. Fluorescent histochemical technique shows prominant interlacing parenchymal fibres surrounding individual adipocytes and associated with arterial vessels (Cottle, Cottle, Perusse and Bukowiecki 1985). During cold exposure and overfeeding there is a significant activation of the central sympathetic outflow to brown fat (see review Barnard, Mory and Nechad 1980; Girardier and Seydoux 1986).

The mechanism of the sympathetic control of BAT is reviewed in section 13.1.

**4.1.2. Blood supply**

BAT appears as a brownish tissue and it is very distinct from white adipose tissue especially in the active state. The brownish colour of BAT is mainly due to a rich and extensive vascular supply, estimated to be 4-6

times greater than that of the white adipose tissue. Cold exposure causes a dramatic increase in blood flow to BAT (Heim and Hull 1966). The blood flow increases to 20 ml/min/g or greater in response to cold stimulation in rats (Foster and Frydman 1978 and 1979) and noradrenaline infusion increases blood flow by 7-fold in overfed rats (Rothwell and Stock 1981a). BAT depots are served by vessels derived from the major arteries and veins (Smith and Roberts 1964; Hull and Segall 1965). Hence the heat produced by the tissue is rapidly transported by the blood towards the critical structures which require warming for survival in the cold. Arteriovenous anastomoses have been demonstrated in relation to interscapular BAT (Lever, Nnodim and Symons 1985). It was suggested that while enhancing lipolysis and thermogenesis in BAT, sympathetic stimulation may increase capillary blood flow in, and heat convection from that tissue by closing regional arteriovenous anastomoses.

The relationship between brown adipocytes, its abundant blood supply and dense catecholaminergic innervation is shown in the model (Fig. 1.1) which is adapted from Nnodim (1988).

### 4.2. Microscopic Structure of BAT

BAT is composed of brown adipocytes which are the functional units. The brown adipocyte contains multiple

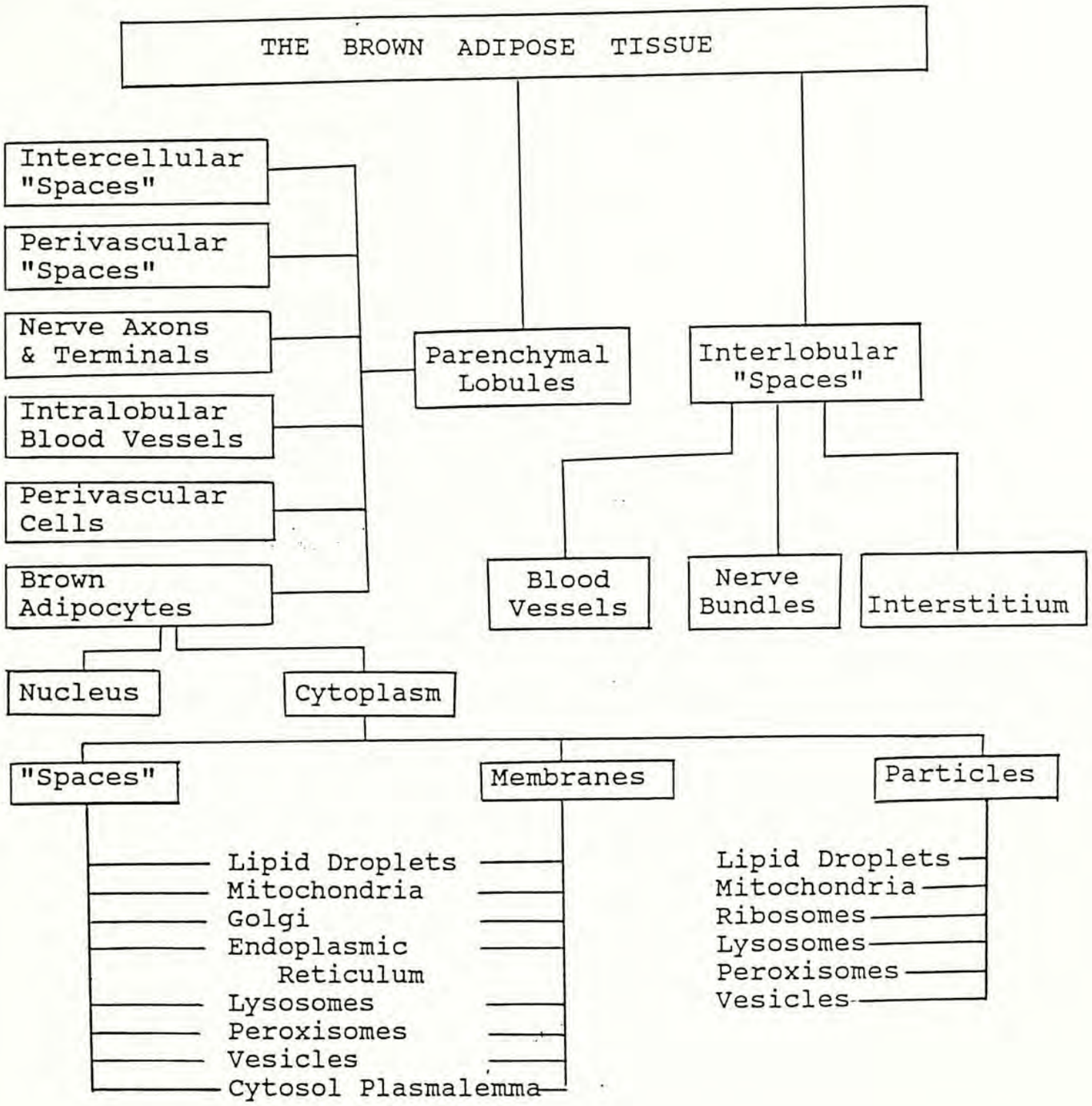


Fig. 1.1 Model to show relationships in structure of brown adipose tissue (adapted from Nnodim 1988).

lipid droplets and numerous mitochondria. The multilocular nature of lipid droplets in brown adipocyte indicates the metabolic activity of the cell. The droplets do not seem to be surrounded by a membrane but have often been observed to be apposed to cisternae of the smooth endoplasmic reticulum (Afzelius 1970; Barnard, Skala and Lindberg 1970; Ahlabo and Barnard 1974). This indicates fatty acid synthesis in this organelle. The abundance of mitochondria in the BAT cell implies a high rate of metabolism. In multilocular brown fat, numerous mitochondria are seen throughout the cytoplasm and they constitute the most characteristic structural feature of brown fat cells and also contribute to the brownish colour of the tissue. The mitochondria are often greater than 0.5 $\mu$m in diameter, compared to only about 0.3 $\mu$m in the white fat cell. BAT mitochondria have a distinctive appearance with highly packed cristae which are usually regular and traverse the whole width of the mitochondria (see review Rothwell and Stock 1984a). The endoplasmic reticulum and Golgi apparatus are relatively small in brown adipocytes and rough membranes are almost absent (see review by Afzelius 1970).

The nucleus of the brown adipocyte is spherical and contains a single nucleolus. Depending upon the presence of fat droplets in the cell, the nucleus is located centrally or at the periphery. The adipocyte is in close contact with the extracellular space, by forming numerous small invaginations and cytoplasmic vesicles, which indicate pinocytotic activity (see review Afzelius 1970; Schneider-Picard, Carpentier and Orci 1980). Intracellular communication between adjacent adipocytes is through gap junctions, which allow inorganic ions and small molecules to cross from one cell to the other. Thus the brown fat cells are electrically coupled (Revel and Sheridan 1968; Sheridan 1971). The gap junctional area increases in parallel with the thermogenic activity of BAT, which suggests that communication between brown adipocytes may be involved in the function of BAT (Schneider-Picard et al. 1980; Schneider-Picard and Girardier 1984).

### 4.3. Difference Between Brown Fat and White Fat

The morphology and biochemistry, as well as phylogeny, of these two types of tissue are distinctly different although BAT may look like white fat in older mammals.

Early observation by Hammar in 1895 demonstrated that in rats kept on a restricted diet, BAT showed no diminution in weight, whereas white fat was greatly reduced, and led him to conclude that these two adipose tissues are functionally distinct from each other (review Nechad 1986).

The colour of white fat is due to the scarcity of its blood supply and its large fat content. Sympathetic nerve fibres and vessels may be found around white adipocytes as in brown fat, but they appear to be less numerous with lower noradrenaline content than in BAT (Stock and Westermann 1963; Cottle, Cottle, Nash and Bickman 1972; Rosell and Belfrage 1979). The typical features of white adipocytes are large cell size, a dominant and large unilocular lipid droplet in the centre, fewer mitochondria with less developed cristae and a peripheral nucleus. Such characteristics indicate relative metabolic inactivity. White fat mainly consists of 80-90 per cent lipid, more than twice as much as in active BAT. The release of fatty acid is activated by sympathetic innervation in BAT but not in white fat. Although lipoprotein lipase is a key enzyme in the uptake of fatty acids in both types of tissue, it can be activated by noradrenaline and is unaffected by insulin in BAT, whereas the opposite actions are reported to occur in white fat (Wing and Robinson 1968; Ashby and Robinson 1980; Carneheim, Nedergaard and Cannon 1984).

Although BAT regresses in older mammals, and becomes indistingushable from white fat, BAT can be reactivated by sympathetic stimulation. Parallel cultures of foetal 'pre-adipocytes' from typical brown and white fat depots under identical conditions show different developmental patterns which indicates that brown and white fat cells come from different precursor cells. (Nechad, Kuusela, Carneheim, Bjorntorp, Nedergaard and Cannon 1983). The other remarkable difference in these two types of tissue is the presence of UCP in BAT mitochondria but not in white fat mitochondria (Cannon, Hedin and Nedergaard 1982).

**5. Composition of BAT**

The composition of BAT varies between species and in each individual it is affected by age, environmental temperature, and diet as well as by physiological states such as pregnancy and lactation. The composition can change rapidly so it is often misleading to infer alterations in activity of the tissue from mass alone. Brown fat is mainly composed of lipid (about 30-40 per cent of the tissue wet weight) and water (about 50-55 per cent). Triglycerides constitute 75-90 per cent of the total lipids in the tissue (see review Nechad 1986). Fatty acid composition appears to be directly influenced by the fatty acid composition of the dietary lipids. BAT also contains large amounts of phospholipids. The fatty acid profile of membrane phospholipids can have marked effects

on the activity of membrane bound enzymes involved in the activation of thermogenesis during overfeeding and cold adaptation (Thomson, Habeck, Nance and Beetham 1969; Mory, Ricquier, Pesquies and Hemon 1981; Ricquier, Mory, Nechad, Combes-George and Thibault 1983).

The protein content in BAT is about 10 per cent and increases upon cold acclimation (Thomson et al. 1969; Nedergaard, Alexson and Cannon 1980). The younger animal also has a higher protein content in its BAT than the older animal (McDonald, Horwitz and Stern 1988a) and during pregnancy and lactation a significant fall in BAT protein concentration is observed (Trayhurn, Douglas and McGuckin 1982a).

## 6. The Mechanisms of BAT Thermogenesis

The bioenergetic mechanism of BAT thermogenesis has been reviewed in detail by Nicholls and Locke (1984) and Nicholls, Cunningham and Rial (1986). The main feature of the BAT mitochondria from thermogenically active animals is the high proton permeability which can be totally inhibited by the addition of purine nucleotides to the incubation medium. The nucleotide-sensitive conductance is a unique feature of BAT mitochondria in all species studied so far including man (Ricquier, Nechad and Mory

1982; Cunningham, Leslie, Hopwood, Illingworth, Jung, Nicholls, Peden, Rafael and Rial 1985); rat (Desautels and Himms-Hagen 1981), hamster (Rial, Poustie and Nicholls 1983), guinea pig (Rial and Nicholls 1984) and rabbit (Hohorst and Rafael 1968) etc. This uncoupling system is not found in mitochondria from other tissues.

Mitochondria are the major organelles for the synthesis of ATP within a cell. The production of ATP occurs in the electron transport system of the respiratory chain located in the inner membrane of mitochondria. Brown adipocytes contain a similar respiratory chain to other types of cell (Flatmark and Pedersen 1975; Nicholls 1979).

A review of the mechanism of the electron transport system commonly occurring in mitochondria can help to understand the nature of the bioenergetic mechanism of BAT thermogenesis. According to the chemiosmotic model of Mitchell (1966), electron transfer in mitochondria by the respiratory chain is required for the oxidation of co-enzymes, and this is coupled to the extrusion of protons across the inner mitochondrial membrane. The membrane is relatively impermeable to proton and allows the build up of an electrochemical gradient across the inner membrane. This proton gradient provides the motive force for the synthesis of ATP via ATP synthetase. The permeation of protons into the mitochondrial matrix via the synthetase is the mechanism by which oxidation is coupled to

phosphorylation. Failure in the coupling of either electron transfer to proton extrusion or of proton re-entry to ATP synthesis has two important consequences : a low ATP yield accompanied by a corresponding increase in heat production, and a loss of respiratory control. Without this control, the heat production is limited by the supply of substrate and the capacity of the respiratory chain.

Studies of the bioenergetic mechanism of BAT mitochondria reveal that the mitochondria in BAT can become physiologically uncoupled. These mitochondria, unlike those in other tissues, possess a specific proton conductance regulatory mechanism which permits an increase in permeability of the inner membrane of mitochondria to the inward movement of protons and therefore destroys the proton gradient created by the electron transport system. In this situation, the phosphorylation is not coupled to ATP synthesis. When mitochondria are uncoupled, the rate of substrate oxidation and hence heat production proceeds at a maximal level.

Uncoupling of oxidative phosphorylation was observed in mitochondrial preparations of BAT. It was also found that isolated BAT mitochondria would exhibit normal respiratory control when albumin is present and fatty acids are absent in the medium. This led to the idea that free fatty acids released by lipolysis is involved in the uncoupling. However removal of fatty acid from the medium

was not entirely effective in inducing respiratory control in brown fat mitochondria (Hohorst and Rafael 1968; Rafael, Klaas and Hohorst 1968; Rafael, Ludolph and Hohorst 1969; Pedersen 1970; Sakaan, Christiansen and Grav 1972). A key finding was made by Rafael and colleagues (Hohorst and Rafael 1968; Rafael et al. 1968 and 1969) who found that the addition of millimolar concentrations of ATP to the incubation medium, together with albumin, produced immediate respiratory control in brown fat mitochondria from cold adapted guinea pigs. Subsequent work demonstrated that ATP was not unique in this respect, and several other nucleotides (ADP, GTP and GDP) are found to be equally, or more effective, GDP having the highest binding activity.

Unlike the mitochondrial adenine nucleotide translocator which is specific for ATP and ADP and which provides the only means for transporting these nucleotides into the matrix space, the proton conductance pathway can be reduced by a wide range of purine nucleotides (Heaton and Nicholls 1977), suggesting that transportation of nucleotide into the matrix space is necessary to exert the inhibitory action and this was confirmed by Nicholls (1976). Furthermore, the uncoupling mechanism is clearly insensitive to the millimolar concentration of adenine nucleotides present endogenously in the matrix. It was also found that mitochondrial GDP binding capacity varied in proportion to thermogenic capacity. Ricquier and Kader (1976) isolated a unique polypeptide (32 kDa) from BAT

mitochondrial membranes, the concentration of which varied with thermogenic status. Heaton et al. (1978) subsequently found that an azide derivative of ATP with a high affinity for the nucleotide binding site could be covalently bound to and extracted with the 32 kDa protein. This protein could also be protected by a high concentration of GDP, and thus it was identified as the nucleotide receptor at the proton conductance site in the inner membrane of mitochondria.

The 32 kDa nucleotide acceptor, now termed the uncoupling protein or thermogenin, is the central feature in the current theory of BAT thermogenesis. In response to environmental or dietary changes GDP binding capacity to UCP and the concentration of UCP show adaptive changes.

## 6.1. Factors Influencing Proton Transport by UCP

A model of the molecular mechanism by which UCP regulates proton transport in the inner membrane of brown adipocyte mitochondria has been proposed by Jezek, Houstek and Drahota (1988) (see Fig. 1.2). The model shows UCP domains as a dimeric protein (2 X 32 kDa) integral to the inner membrane in BAT mitochondria. UCP forms a gated $H^+$ channel across the inner membrane (Strieleman, Schalinske and Shrago 1985a and b) and allows for controlled uncoupling. Free energy is thus transformed into heat. In

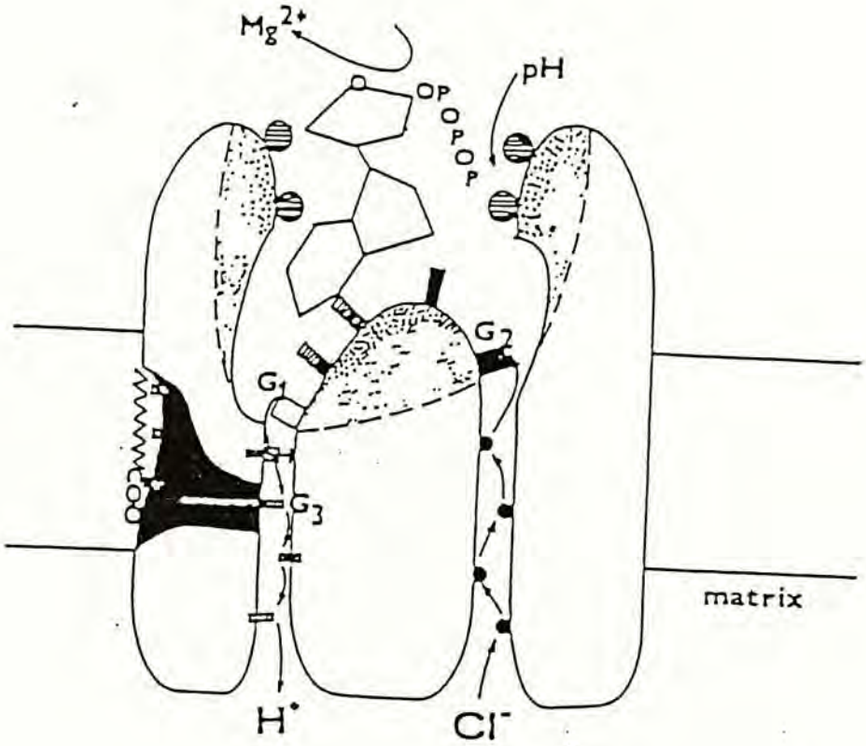


Fig. 1.2 Putative view of the UCP and location of sites of regulatory ligand binding and interaction of modulators. The model of UCP domains considers two distinct transport pathways for translocation of $H^+$ and $Cl^-$, respectively: a purine nucleotide-binding site (dotted areas) containing various amino acid residues (the rectangles and squares) interacting with nucleotide molecule. A separate gate ($G_3$) mediates the activating effect of free fatty acids (it allows $H^+$ translocation only when FFA is bound to FFA-binding site). This gate and the FFA-binding site are drawn as the black area. (Adapted from Jezek et al. 1988.)

addition to $H^+$, UCP also conducts halide anions, especially $Cl^-$ (Nicholls and Lindberg 1973), that are probably translocated by a structurally distinct pathway (Kopecky, Guerrieri, Jezek, Drahota and Houstek 1984). Both channels of UCP are closed after sufficient binding of purine nucleotides to the purine binding site located on the outer surface, (Kopecky et al. 1984; Strieleman et al. 1985a and b) each dimeric unit of UCP bearing one such site (Lin and Klingenberg 1982). The inhibitory ability of purine nucleotides was found to decrease with increasing pH (Nicholls 1974).

Using measurement of $H^+$ transport on the inner membrane of BAT mitochondria as an index of proton conductance, pH and $Mg^{2+}$ appear to be the most important modulators of purine nucleotide inhibition and membrane potential, and they act as a feedback modulator of coupling (Jezek et al. 1988). A decrease in ATP level in the presence of FFA is sufficient for opening the $H^+$ channel of UCP and consequently for the initiation of thermogenesis (Jezek et al. 1988; Jezek, Krasinskaya, Smirnova and Drahota 1989).

### 6.2. Postulated Sequence of Events during Thermogenesis

A sequence of events in the induction of thermogenesis in BAT has been proposed by Nicholls and Locke (1984), and Himms-Hagen (1984). The two schemes are rather similar and may be summarized as follows (Fig. 1.3):

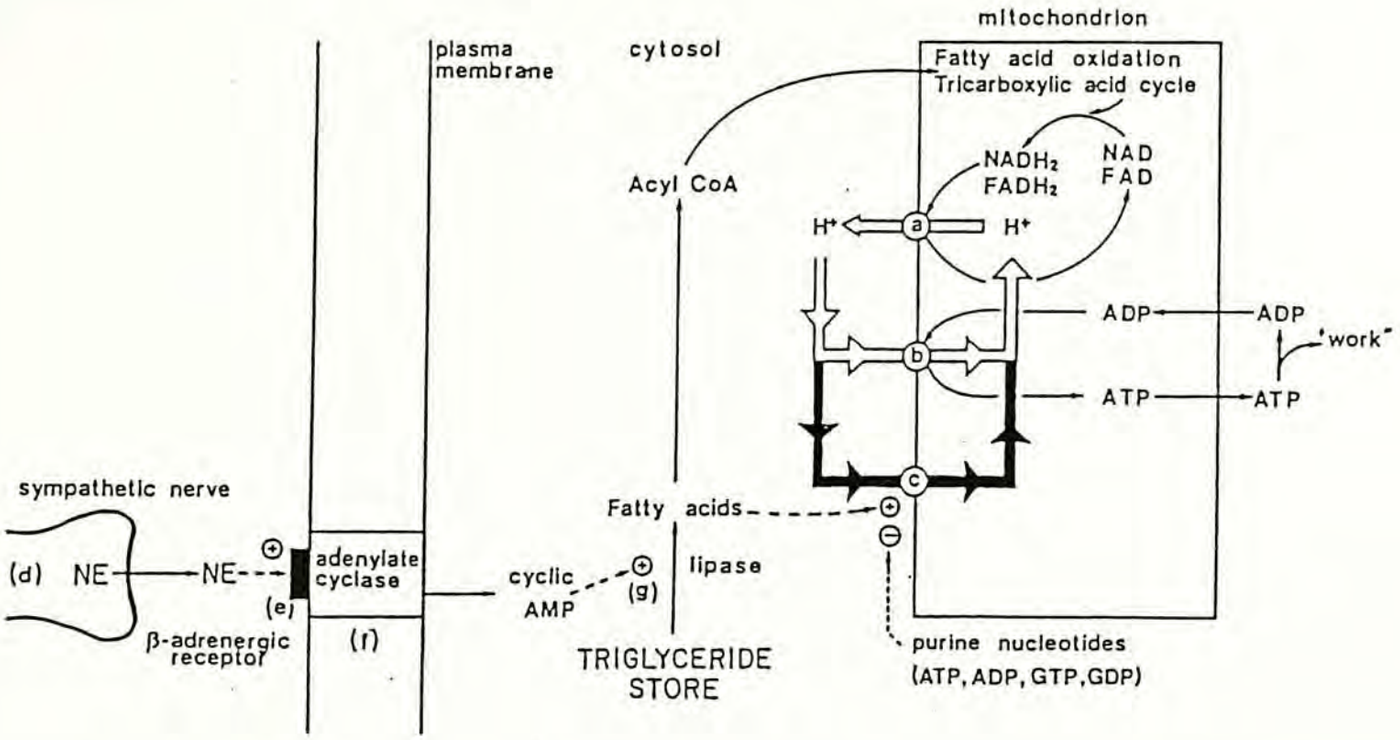


Fig. 1.3 Heat Production in Brown Adipose Tissue. The mitochondrial inner membrane contains the electron-transport system (a), which is reponsible for creating the proton gradient (open cycle) and for the oxidative phosphorylation (b), which is driven by the proton gradient. A proton-conductance mechanism (c) dissipates the proton gradient (closed cycle) and thus uncouples the mitochondria. The proton-conductance mechanism is associated with a specific component of the inner membrane, a 32 kDa polypeptide, to which purine nucleotides bind and thereby inhibit uncoupling. Thermogenesis is triggered by noradrenaline, which is released from sympathetic-nerve endings (d) and interacts with a beta-adrenergic receptor (e) to stimulate adenylate cyclase (f). The resulting increase in cyclic-AMP production causes kinase-dependent activation of a triglyceride lipase (g) and thus accelerates lipolysis. Fatty acids produced during lipolysis serve as the intracellular signal for the operation of the proton-conductance mechanism, as well as the fuel for increased thermogenesis. Other events (not shown) that occur during stimulated thermogenesis and as yet have no known specific role include the interaction of noradrenaline with alpha-adrenergic receptors, stimulation of the activity of sodium-potassium-ATPase, and modification of cytosolic levels of purine nucleotides. ATP denotes adenosine triphosphate, ADP adenosine diphosphate, GTP guanosine triphosphate, GDP guanosine diphosphate, NE noradrenaline, NAD nicotinamide adenine dinucleotide-oxidized, FAD flavinadenine dinucleotide-oxidized, $NADH_2$ nicotinamide adenine dinucleotide-reduced, $FADH_2$ flavin adenine dinucleotide-reduced, $H^+$ hydrogen ion, and CoA coenzyme A. (Adapted from Himms-Hagen 1984.)



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(1) Noradrenaline binds to the $\beta$-receptors in the plasma membrane of the brown fat cell. (The $\beta$-receptor is increasingly considered to be of a novel subtype $\beta_3$(Arch, Ainsworth, Cawthorne, Piercy, Sennitt, Thody, Wilson and Wilson 1984; Arch 1989)).

(2) Stimulates adenyl cyclase and Cyclic AMP in the cytosol rises.

(3) A protein kinase is activated by cAMP.

(4) Lipase is phosphorylated and activated by the protein kinase.

(5) Lipolysis is accelerated and fatty acids are liberated and activated to acylcarnitine.

(6) Respiratory control inhibits the oxidation of acylcarnitine, and therefore the acylcarnitine and acyl-CoA pools fill up.

(7) Free fatty acids serve as the intracellular signal for reversing the purine nucleotide inhibition of UCP allowing the operation of the proton conductance mechanism.

(8) The increase in proton conductance allows acylcarnitine to be oxidized, and a steady state concentration of fatty acid is reached with a balance

between lipolysis and oxidation. On termination of lipolysis all these events would occur in reverse.

## 7. Measurements of Thermogenic Capacity of BAT

There are many methods to determine the thermogenic activity of BAT and the most appropriate method to assess and interpret the thermogenic status of BAT is an important consideration in all studies on thermogenesis (see review Trayhurn 1989). The commonly used methods to measure thermogenic activity of BAT are summarized in Table 1.1.

The most direct measure of thermogenesis is the rate of $O_2$ consumption <u>in vivo</u>. However this indicates the general $O_2$ consumption of the whole body and it may not reflect the contribution of BAT. Some researchers have used noradrenaline induced increase in $O_2$ consumption as a measure of thermogenic activity of BAT. However, this method has not been widely employed because of the technical difficulty to obtain reliable and precise values of $O_2$ consumption in the free-living animal without using anaesthesia or surgery.

Measurement of BAT weight is the least reliable index of the thermogenesis of BAT. Although there is a significant hypertrophy of BAT in cold acclimated rodents and 'cafeteria'-fed animals, tissue weight of BAT is also

Table 1.1 Measurements on BAT (modification of Trayhurn 1989)

(1) Weight

(2) Total protein content

(3) Mitochondrial protein content

(4) Cytochrome C oxidase (EC 1.9.3.1) activity
(as an index of mitochondrial content)

(5) Mitochondrial GDP binding

(6) Mitochondrial swelling; proton conductance

(7) UCP

(8) mRNA for UCP

(9) Norepinephrine-induced increase in $O_2$ consumption

high in obese animals. The increased tissue weight of BAT in obese animals is due to a high fat content in this tissue rather than an increased thermogenic function. The total protein content and mitochondrial protein content of BAT provide a crude marker of metabolically active tissue mass but give no direct indication of thermogenic function of BAT.

The most widely used method to determine the thermogenic activity of BAT is GDP binding to BAT mitochondria. In many studies the binding values are expressed in relation to mitochondrial protein mass commonly based on single incubation of BAT mitochondria with a single concentration of GDP binding containing isotopically labelled GDP. The Bmax value of GDP obtained from the Scatchard analysis is more reliable and reflects the maximum GDP binding activity of BAT. However a large amount of mitochondria are required to do a Scatchard plot and this may not be feasible in many studies. Therefore it is not common to perform a Scatchard analysis to obtain the Bmax of GDP binding.

The interpretation of GDP binding data has been a matter of some debate and is discussed in section 9.1.2. Some authors argue that GDP binding is an index of the amount of UCP (Nedergaard and Cannon 1985; Sundin, Moore, Nedergaard and Cannon 1987), while others consider it to be an indicator of thermogenic activity (Himms-Hagen 1986; Tryhurn 1986). Concomitant measurements of GDP binding and

UCP content reveal that there is dissociation of these two parameters in some situations (Gribskov, Henningfield, Swick and Swick 1986; Trayhurn and Wusteman 1987a; Peachey, French and York 1988). Hence GDP binding is regarded as an index of thermogenic activity and not a measure of UCP. Moreover it has been reported that GDP binding can be altered *in vitro* e.g. washing with albumin (Gribskov *et al*. 1986), a low pH medium (Stribling 1983), or a low osmolarity medium (Nedergaard, Raasmaja and Cannon 1984). Therefore the value of GDP binding can be altered during the isolation of mitochondria.

Mitochondrial swelling is another measure of mitochondrial proton conductance. Nicholls and Lock (1984) have reviewed various methods of determining mitochondrial swelling and they found that there is a high correlation between the mitochondrial swelling and GDP binding (Rial and Nicholls 1984; Peachey *et al*. 1988).

Recently, with rapid development of molecular biology, cloning of UCP has been achieved. Full-length cDNA of rat UCP (Bouillaud *et al*. 1985; Ridley, Patel, Gerber, Morton and Freeman 1986) and partial cDNA of mouse UCP (Jacobsson *et al*. 1985) were achieved. Using differential hybridization, hybrid-selected translation of mRNA and immunoprecipitation, rat UCP cDNAs have been isolated (Bouillaud *et al*. 1985). The identity of UCP cDNA was also further confirmed by DNA sequencing (Bouillaud, Weissenbach and Ricquier 1986). Rat UCP cDNA was then used

as a tool to screen rat, human, bovine and rabbit genomic libraries (Bouillaud, Raimbault, Casteilla, Cassard and Ricquier 1988a; Bouillaud, Villarroya, Hentz, Raimbault, Cassard and Ricquier 1988b; Casteilla, Bouillaud, Forest and Ricquier 1989; Balogh, Ridley, Patel and Freeman 1989).

Recently UCP mRNA, UCP gene expression and UCP sequence have been determined in several species. Using UCP mRNA as a probe, many unique characteristics of BAT have been confirmed. Some of these profound findings in molecular biology of UCP are summarized in Table 1.2 (adapted from Ricquier 1989). UCP mRNA is unique to BAT and is not detected in non-brown-adipocytes (Bouillaud et al. 1985; Jacobsson et al. 1985; Jacobsson, Nedergaard and Cannon 1986). UCP mRNA is rapidly inducible by cold exposure (Ricquier, Mory, Bouillaud, Thibault and Weissenbach 1984 and Ricquier, Bouillaud, Toumelin, Mory, Bazin, Arch and Penicaud 1986; Jacobsson et al. 1985; Jacobsson et al. 1986) and by injection of $\beta$-adrenoceptor agonist (Jacobsson et al. 1986; Ricquier et al. 1986) but is decreased by returning to a thermoneutral environment (Reichling, Ridley, Patel, Harley and Freeman 1987). Although molecular techniques appear to have important potential in future research on UCP, however, they are limited to few laboratories at the present time.

Trayhurn (1989) has suggested that the concentration of UCP in mitochondria is regarded as an index of the

Table 1.2 A summary of findings in molecular studies of UCP (adapted from Ricquier 1989)

| Strategy | Results |
|---|---|
| Northern analysis | -UCP mRNA is specific for brown fat<br>1.5 kb mRNA in rodents, 1.8 kb mRNA in cattle, sheep and man<br>-UCP mRNA level is increased in BAT activated by cold exposure or refeeding |
| Run-on Transcription | -UCP gene is regulated at the transcriptional level<br>-Transcription can be activated by $\beta$-agonist<br>-Transcription is impaired in obese (fa/fa) rats |
| DNA sequencing | -Determination of amino acid sequence of UCP in rat, calf and man<br>-UCP is homologous to other mitochondrial carriers |
| Gene isolation | -Rat and human UCP gene have been isolated<br>-UCP gene transcription site has been identified<br>-Rat UCP gene has been entirely sequenced<br>-UCP has one gene |

capacity of the proton conductance pathway, while the total amount in a BAT depot sets the thermogenic capacity of that particular depot. The UCP content of BAT varies greatly with different conditions such as cold acclimation, overfeeding or fasting and it is discussed later.

Before the successful purification of UCP, UCP is detected by polyacrylamide gel electrophoresis and this method is rather non-specific and non-quantitative. UCP can be purified by the method of Lin and Klingenberg (1980) and using the purified UCP, specific antiserum against UCP can be raised. Therefore immunological assay specific for UCP can be developed. Several immunological methods to assay the concentration of UCP have been described i.e. radioimmunoassay (RIA) (Lean, Branch, James, Jennings and Ashwell 1983; Peachey et al. 1988), enzyme linked immunosorbent assay (ELISA) (Desautels 1985; Nedergaard and Cannon 1985; Sundin et al. 1987) or immunoblotting (Henningfield and Swick 1987). ELISA technique has become popular method in the area of immunoassay because of its advantages i.e. long shelf live of the reagents, no radiation hazard, no problem of waste disposal and ease of handling large number of samples.

## 8. Age-related Differences in BAT

In most mammals investigated, BAT has been found to be prominent in the newborn in which non-shivering thermogenesis (NST) represents a major means of compensation for heat loss once they leave the warm maternal environment (see review Nedergaard, Connolly and Cannon 1986). The fresh weight of BAT is around one to two per cent of the body weight during the perinatal period (reviewed by Barnard and Skala 1970; Alexander 1979). The weight of BAT after the perinatal period varies with age and physiological state. In older animals BAT appears to be paler in colour, accumulates fat, and becomes indistinguishable from white fat.

Detailed study of the changes during development and ageing in interscapular BAT in rats has been reported (Nnodim and Lever 1985; Nnodim 1988). BAT can be identified on the 17th day of gestation. Brown adipocytes generally retain a multilocular lipid distribution into old age, and although the catecholaminergic supply is reduced, a widespread distribution of noradrenergic vasomotor and parenchymal nerves between brown adipocytes are still found in BAT of senile rats (Nnodim and Lever 1985). The surface area and vacuolar volume of lipid droplets are high in the early postnatal period, increase slightly in adulthood and fall only slightly in old age (Nnodim 1988). The relative constancy of the surface area

of lipid droplets and vacuolar volume in adulthood indicates a persistent of the ability of BAT to respond to appropriate stimuli.

Unlike rats and other animals including hibernators who retain the multidroplet storage format throughout life (Afzelius 1970), rabbits show a transformation in the pattern of lipid storage in brown adipocytes. The large interscapular BAT of the adult animal consists of unilocular cells (Dawkins and Hull 1964), in parallel with a decline in thermogenic function of the tissue.

Although there is much evidence to suggest an age dependent decrease in the ability of BAT to maintain a constant body temperature when exposed to cold, other reports show no attenuation in GDP binding of BAT mitochondria in old rats (McDonald, Stern and Horwitz 1987). These authors suggested that the thermogenic capacity of BAT does not decrease in old age but that the metabolic capacity of the body is attenuated by the fall in lean body mass.

In rats during the first 20 h of life, there is a marked but gradual increase in the level of mRNA coding for UCP (Obregon, Jacobsson, Kirchgessner, Schotz, Cannon and Nedergaard 1989). However if pups are exposed to a thermoneutral temperature (35°C) for the first 12 h after birth, no increase in UCP mRNA is observed (Obregon et al. 1989). This indicates that post-natal recruitment in BAT

is a consequence of cold stress rather than a pre-programmed process. The post-natal involution of BAT as the animal grows can be delayed or reversed by external stimuli such as cold, overfeeding or repeated sympathetic stimulation (see review Himms-Hagen 1986; Nicholls and Locke 1984; Holloway 1989).

In the human newborn BAT is known to be relatively abundant (Aherne and Hull 1966; Cannon and Johansson 1980): it has also been found in some adults (Hassi 1977). The characteristic multilocular cells have been demonstrated in adults of all ages (Himms-Hagen 1979). BAT begins to regress after the first year and in the adult human very little is left. Recently UCP has been found in BAT mitochondria from adults (Bouillaud, Combes-George and Ricquier 1983). Moreover, active BAT has been demonstrated in patients with phaeochromocytoma (Ricquier et al. 1982), and significant amounts of BAT have been found around the neck arteries of outdoor workers in Finland (Huttunen, Hirvonen and Kinula 1981). Histochemical studies and measurement of mitochondrial enzyme activities suggest that working in the cold halts the regression of the thermogenic activity of BAT in man. BAT has also been identified in perinephric adipose tissue of adults but is less frequently found above the age of 50 (Cunningham et al. 1985). However the evidence in adult man for transformation into active BAT in response to cold and overfeeding is not convincing (reviewed by Trayhurn and James 1983; Rothwell and Stock 1984a).

Since NST of BAT is stimulated by catecholamines which activate adenylate cyclase through the β-adrenergic receptor, decrease in sympathetic activity to BAT may be the primary reason for the declining activity of the tissue in maturing mammals. In certain tissues, β-adrenergic receptor function declines with age (Scarpace and Abrass 1986). Similar declines in β-adrenergic receptor density and adrenergic stimulated adenylate cyclase activity have been reported in the BAT of the old rats (Scarpace, Mooradian and Morley 1988b). These biochemical alterations in the effector unit, together with the functional unit of thermogenesis, may contribute to the inability of older animals to thermoregulate when exposed to cold.

## 9. Non-shivering Thermogenesis and BAT

In small animals and young animals the first response to a low environmental temperature is shivering. This type of thermogenesis is succeeded by another type of thermogenesis, so called non-shivering thermogenesis or cold-induced thermogenesis. NST is shown to occur in BAT and shivering decreases as the capacity of BAT for NST increases.

The magnitude of NST varies with the physiological state of the animal and with environmental factors. It is generally greater in hibernators than in euthermic species and reflects the greater requirement for rapid rewarming during arousal from hibernation. The duration of cold adaptation or a pre-adaptation to cold also alters the thermogenic capacity in BAT. Apart from hibernators and cold-adapted animals, the neonates of nearly all mammals exhibit NST. In neonates capacity for NST depends on such factors as size of the animal, body insulation, nesting habits and the rate of development of behavioural and motor mechanisms.

Since NST is very important in newborn mammals, Cannon and Nedergaard (1986) classified newborn mammals according to their NST capacity of BAT (total amount of UCP as index): well-developed 'precocial' newborns (e.g. guinea pig), less developed 'altricial' newborns (e.g.

rats) and very 'immature' newborns (e.g. hamsters). The 'immature' group has virtually no thermogenic capacity at birth (e.g. the hamster has none until 2 weeks after birth). In the 'altricial' newborns, the development occurs after birth and in the 'precocial' group, BAT is fully developed at birth. In the guinea pig, active BAT mitochondria are found from the 15-16th day of gestation and the amounts increase until birth. UCP could be identified in BAT from the embryo of mouse and rat 2 days before birth, and UCP rapidly increases thereafter (Houstek, Kopeck, Rychter and Soukup 1988). In these 'altricial' animals BAT mitochondria lack UCP and are non-thermogenic *in utero* and become thermogenic 2 days before birth.

A low environmental temperature normally produces hypertrophy of BAT in mice, guinea pigs, rats, and hamsters (Girardier 1983, Barnard *et al*. 1980; Cannon and Nedergaard 1983; Flatmark and Pedersen 1975; Nicholls and Locke 1984; Trayhurn, Richard, Jennings and Ashwell 1983; Rafael and Vsiansky 1983; Rabi and Cassuto 1976; Bukowiecki and Collet 1983) and possibly even in humans (Huttunen *et al*. 1981). However in some rodent species, an absence of cold-induced hypertrophy of BAT has also been reported e.g. antelope ground squirrel (Balcer and Chaffee 1981) and the Monogolian gerbil (Trayhurn and Douglas 1984; Steffen and Roberts 1977).

It has been demonstrated that NST can be modified by

photoperiod in some animals e.g. Syrian hamster and Siberian hamster. A short photoperiod produces hypertrophy of BAT in the Siberian hamster (Rafael, Vsiansky and Heldmaier 1981) and the Syrian hamster (Wade 1983; Bartness and Wade 1984). In the natural environment, a short photoperiod is linked to a cold environmental temperature. A long photoperiod prevents cold-induced growth of BAT in hamsters (Trayhurn and Douglas 1984; Balcer and Chaffee 1981; Feist 1983). The seasonal acclimation in autumn and winter also enhances thermogenic capacity in BAT for wild bank voles and wood mice (Klaus, Heldmaier and Ricquier 1988). In hibernators, the thermogenic activity increased during the early stage of arousal from hibernation (Rafael et al. 1981; Feist, Florant, Greenwood and Feist 1986; Horwitz, Hamilton and Kott 1985). Despite the coincidence of changes in ambient temperature and in brown fat thermogenic properties, it remains unclear as to which environmental cues are primarily responsible for the seasonal arousal of thermogenesis in these animals.

During cold adaptation there is significant increase in mitotic rate in BAT; it is maximal during the first week of cold exposure and declines thereafter (Bukowiecki, Collet, Follea, Guay and Jabjah 1982; Cameron and Smith 1964; Hunt and Hunt 1967). Significant increase in tissue DNA content is detected only after 1-2 days of exposure to cold (Bukowiecki, Follea, Vallieres and Le Blanc 1978; Thomson et al. 1969). However, mitotic activity is

stimulated mainly in capillary endothelial cells, adipose tissue interstitial cells and preadipocytes rather than in mature brown adipocytes (Bukowiecki et al. 1982). Recent studies of the sequence of events in cellular differentiation in BAT suggest that the proliferation of brown adipocytes and the differentiation of interstitial cells to brown adipocytes represent the fundamental processes which explain the enhanced capacity of cold acclimated rats to respond to catecholamines (Bukowiecki, Geloen and Collet 1986).

## 9.1. Changes In BAT During Cold Acclimation

### 9.1.1. GDP Binding

The capacity of BAT mitochondria to bind GDP, a direct method for assessing the thermogenic activity, increases acutely after exposure to cold. The GDP binding capacity does not always parallel the changes in the concentration of UCP. Increased GDP binding can be elicited within 20 min of cold exposure at 4°C (Swick and Swick 1986) or after acute administration of a sympathomimetic substance (Milner, Wilson, Arch and Trayhurn 1988). This increase in NST does not require protein synthesis (Desautels and Himms-Hagen 1979; Gribskov et al. 1986), is rapidly reversible (Himms-Hagen 1983) and is associated with an ultrastructural change in the isolated mitochondria (Desautels & Himms-Hagen 1980). The increase in UCP content is a slower process than GDP

binding (Peachey et al. 1988; Trayhurn, Ashwell, Jennings, Richard and Stirling 1987). The rapid increase in GDP binding capacity of BAT without the corresponding change in UCP concentration is explained as unmasking of binding sites on already existing UCP molecules (Trayhurn et al. 1983; Ashwell, Jennings, Richard, Stirling and Trayhurn 1983a; Girbskov et al. 1986; Peachey et al. 1988; Trayhurn et al. 1987). Unmasking can be achieved in vitro by preincubating the mitochondria at a lower pH (Stribling 1983) or measuring the binding capacity in an incubation medium of low osmolarity (Nedergaard and Cannon 1984). Henningfield and Swick (1989) reported that the concentration of UCP is correlated with GDP binding to $Mg^{2+}$ activated mitochondria but not with GDP binding to untreated mitochondria. Therefore, they suggested that the binding of GDP to untreated mitochondria is a reflection of thermogenic activity at the time of sacrifice of animals, whereas GDP binding after $Mg^{2+}$ treatment reflects total thermogenic capacity of the mitochondrion.

Contrary to the 'unmasking' theory, other studies report a parallel alteration in UCP content and GDP binding during cold adaptation in hamsters (Sundin et al. 1987), rats (Nedergaard and Cannon 1985) and guinea pig (Rial and Nicholls 1984). Nedergaard and Cannon (1987) suggested that the 'unmasking' of GDP binding sites is due to mitochondrial swelling. On the contrary, Milner and Trayhurn (1988) could not observe any effect of mitochondrial swelling induced by KCl on GDP binding and

they suggested that the acute unmasking of GDP binding sites in BAT mitochondria is not simply due to mitochondrial swelling but due to conformational changes or alterations in its oligomeric structure of UCP (Milner et al. 1988; Peachey et al. 1988).

When a cold acclimated animal is placed in a warm temperature, a rapid drop in GDP binding occurs without a simultaneous change in UCP content. (Peachey et al. 1988; Trayhurn et al. 1987) and this is suggested to be due to remasking of the UCP. The unmasking and remasking process appears to be rapid and reversible in response to changes in environmental temperature.

In general, increased demand for NST in the rat involves a two-phase response in the activity of BAT. The first phase is a rapid activation of the tissue by increasing GDP binding and it is likely to be due to unmasking of binding sites. The second phase requires a longer duration of cold exposure and it is a trophic response involving hypertrophy of BAT, increased number of mitochondria (Himms-Hagen 1986) and increased de novo synthesis of UCP (Peachey et al. 1988).

### 9.1.2. Concentration of UCP

The concentration of UCP is at a minimum at thermoneutrality and increases more or less linearly with decreasing temperature as illustrated in Fig. 1.4 (adapted from Himms-Hagen 1986). The magnitude of its increase

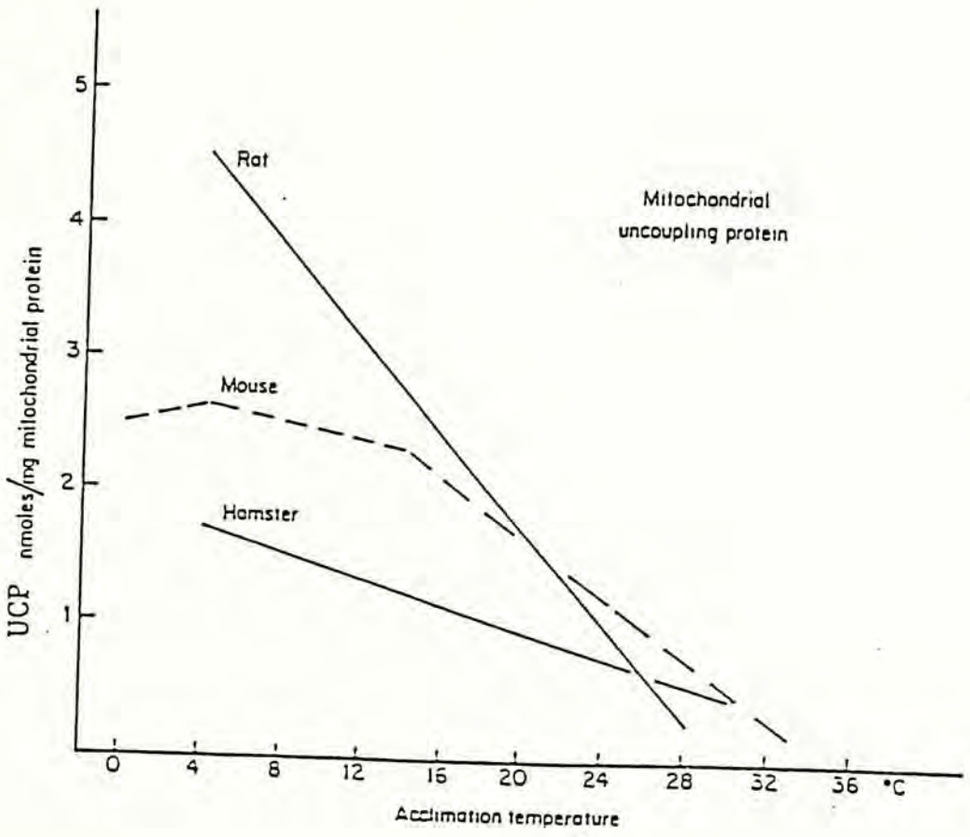


Fig. 1.4 Relation of concentration of mitochondrial UCP to acclimation temperature in rats, mice, and hamsters. The concentration of UCP is measured by an immunological assay and expressed in terms of mitochondrial protein. The total amount in the tissue will change even more markedly because of increase in total mitochondrial mass. (Adapted from Himms-Hagen 1986.)

varies with the type of animal. Rats have greater response than mice or hamsters. The period of cold exposure required to induce a significant change varies from less than 1 h to 12-24 h or more (Nedergaard and Cannon 1985 and 1987; Peachey et al. 1988; Trayhurn et al. 1987).

The effect of temperature on the amount of UCP can also be shown by the measurement of mRNA levels which increase with decrease in temperature (Freeman, Heffernan, Dhalla and Patel 1989). Upon deacclimation, there is a marked loss of UCP mRNA within 24 h, and this precedes the loss of UCP from mitochondria (Patel and Freeman 1987). Loss of UCP mRNA is selective, since there is no change in the relative proportion of cytochome C oxidase subunit IV mRNA or poly(A)$^+$RNA to total RNA (Patel and Freeman 1987).

### 9.1.3. Metabolic changes in BAT during Cold Acclimation

The major energy source for NST is fatty acids (Lafrance, Lagace and Routhier 1980) which come from triglycerides by the action of lipoprotein lipase in BAT. Potentially active hormone-sensitive lipase has been demonstrated in BAT even in animals living in a thermoneutral environment (Holm, Fredrikson, Cannon and Belfrage 1987). This enables the tissue to respond immediately to a sudden cold stress via catecholamine induced phosphorylation of hormone-sensitive lipase and thereby to mobilize the stored triacylglycerol for thermogenesis. This enzyme activity increases during cold adapation (Carneheim et al. 1984; Deshaies, Arnold

and Richard 1988) through a β-adrenergic pathway (Carneheim et al. 1984). A positive correlation between lipoprotein lipase activity and mitochondrial GDP binding has been found in cold acclimated rats (Desharies et al. 1986; Richard, Arnold and Le Blanc 1986). The activation of BAT lipoprotein lipase is not depended on thyroid hormone (Hemon, Ricquier and Mory 1975) or pituitary trophic hormones (Goubern, Laury, Zizine and Portet 1985). In addition to the supply of fatty acid from triacylglycerol, fatty acids can also be obtained from de novo synthesis (Trayhurn 1979; Agius and Williamson 1980 and 1981).

In addition to fatty acids, recent studies have indicated that glucose may also be an important fuel after a carbohydrate load, especially in cold acclimated rats (McCormack and Denton 1977). Several-fold increases in some key enzyme activities of glycolysis (e.g. fructose 2,6-biphosphate, phosphofructokinase-2 and pyruvate kinase) have been reported in BAT of cold adapted rats (Sorbino, Gualberto and Pintado 1988). Cold exposure results in an enhancement of amino acid utilization and of glucose uptake, with high lactate efflux (Lopez-Soriano, Fernandez-Lopez, Mampel, Villarroya, Iglesias and Alemany 1988), and with significant depletion of glycogen and triglyceride (Kuroshima and Ohno 1988). Lopez-Soriano et al. (1988) have also found an increase in higher branched chain amino acid utilization, a general decrease in most amino acids and considerable accumulation of glutamate in BAT (Lopez-Soriano and Alemany 1987a and b).

## 10. Diet-induced Thermogenesis and BAT

For an organism to be in energy balance, the energy intake should equal energy expenditure. When energy intake is greater than expenditure, energy may accumulate as fat resulting in obesity.

The possibility that mammals, including man, are able to maintain energy balance during hyperphagia by increasing heat production (i.e. DIT) has been a controversial subject for many years. This has been largely because of the difficulties in inducing voluntary hyperphagia in laboratory animals. In 1976, Sclafani and Springer described a dietary regimen that is successful in producing obesity in laboratory rats. This 'cafeteria' or 'supermarket diet' comprised a large variety of palatable foods from which rats were allowed to select. These food items include chocolate chip cookies, salami, cheese, banana, marsh mallows, milk chocolate and peanut butter.

In 1979, Rothwell and Stock reported that rats given the 'cafeteria' diet consumed 80% more energy than the chow-fed controls. The hyperphagia is due to a combination of a greater weight of food eaten and the higher energy density of the 'cafeteria' foods. The weight gain of 'cafeteria'-fed rats, however, is only 27% greater than that of controls. The energy cost of weight gain (g gain

per calorie eaten) is significantly lower in 'cafeteria'-fed rats than controls and body energy gain per unit intake is similarly reduced in 'cafeteria'-fed rats. Rothwell and Stock concluded that part of the 'missing' energy could be attributed to the greater energy needed to digest, absorb and utilize the additional food consumed, and the remaining missing energy had been dissipated as heat, in a process called DIT.

Other terms used in the past to describe the stimulatory effects of food on metabolic rate are specific dynamic action and 'luxuskonsumption'. DIT is now the most commonly used term.

### 10.1. Mechanism of DIT

There are many similarities between NST and DIT. In both there is an increase in BAT mass and activity (Rothwell and Stock 1979b and 1980). In the BAT of 'cafeteria'-fed rats, there are increases in the size of brown adipocytes, wet weight, DNA, total protein, and also proliferation of mitochondria in parallel with the growth of the tissue (Tulp, Frink and Danforth 1982a; Himms-Hagen, Triandafillou and Gwilliam 1981).

An increase in proton conductance in 'cafeteria'-fed rodents assayed by GDP binding has been demonstrated

(Himms-Hagen et al. 1981; Brooks, Rothwell, Stock, Goodbody and Trayhurn 1980; Ashwell, Rothwell, Stirling, Stock and Winter 1984; Nedergaard et al. 1984; Rothwell and Stock 1984b). The GDP binding to BAT mitochondria is significantly elevated as early as 3 h after a single meal (Lupien, Glick, Saito and Bray 1985) and it remained high at 10 h.

However, reports on the effects of 'cafeteria'-feeding on UCP level are conflicting. Himms-Hagen et al. (1981) using polyacrylamide gel electrophoresis showed no change in UCP whereas Falcou, Bouilland, Mory, Apflelbaum and Ricquier (1985) found an increase. Nedergaard et al. (1984) and Ashwell et al. (1984), using a more sensitive immunological assay detected a significant increase in UCP. An increase in mRNA of UCP has been reported using a cDNA probe (Falou et al. 1985). The elevation of mRNA in 'cafeteria'-fed rats is weaker than that in cold adapted rats but the results clearly indicated that 'cafeteria' feeding activates UCP biosynthesis in BAT.

Rothwell, Stock and Tedstone (1986) reported that the acute thermic effect of a meal also depended on anticipatory, oro-nasal and gastrointestinal stimuli. The size of the response depended not only on the energy and nutrient content but also on taste, smell, bulk and the previous feeding experience of the animal. Inhibition of the thermogenic response to food by $\beta$-adrenergic blocker indicated that a major part of the response is

sympathetically mediated. 'Cafeteria'-fed rats exhibit increased sensitivity to noradrenaline (Rothwell, Stock and Sudera 1986a) due to an increase in the total number of BAT $\beta$-adrenoreceptors (Rothwell and Stock and Sudera (1986b). Furthermore, Young and Landsberg (1979) have reported enhanced noradrenaline turnover in overfed rats, which is of similar magnitude to that seen in cold-adapted animals.

## 10.2. Controversies in DIT

In the studies of Rothwell and Stock high energy intake was considered as the only cause of enhancement in DIT. However, the composition of 'cafeteria' diet varies very much in each 'cafeteria'-fed rat, because the animals are allowed to select the food items in the 'cafeteria' diet which consists of several items of foods with varible composition; usually high in carbohydrate and fat, and low in protein. There is evidence to show that the composition of food has an effect on DIT and this is discussed in the next section.

Dietary factors causing hyperphagia have been reviewed by Ramirez, Tordoff and Friedman (1989). There are many types of diet which can induce obesity and DIT. Ideally studies of dietary obesity should make it possible to identify the components of the diet that are responsible for the obesity (e.g. chemical components such

as fat, protein and carbohydrate, and physical properties such as texture and bulk). However a change of one factor in food inevitably leads to a change in others, increased fat content will also change taste and texture. Such changes not only affect the macronutrient composition but also the micronutrient, vitamins and minerals. Influence of these on thermogenesis is usually neglected. For the above reasons 'cafeteria' diet as a cause of DIT and the role of BAT in DIT are debatable issues and many workers question whether this diet is an appropriate tool for the study of thermogenesis (Maxwell, Nobbs and Bates 1987; Crist and Romsos 1987; reviews : Hervey and Tobin 1981 and 1983; Mansell, McDonald and Fellows 1987; Moore 1987).

Nevertheless there is strong evidence to support the presence of DIT during 'cafeteria' feeding.

## 10.3. Nutritional Factors Inducing DIT

Nutritional factors such as the amount and type of fat, carbohydrate and protein may affect thermogenesis. A marked reduction in energy efficiency and increase in BAT activity are found in animals tube-fed with medium-chain triglycerides (Gurr, Rothwell and Stock 1979). Medium-chain triglycerides and unsaturated fat diets produce a slightly greater effect on resting $O_2$ consumption than a long-chain saturated fat diet (Rothwell and Stock 1986a). Ide and Sugano (1988) reported that the in vitro activation of respiratory rate by noradrenaline is higher in rats fed polyunsaturated fats than those fed saturated fats.

Sucrose feeding increases caloric intake, but the increased carcase lipid accounted for only half the excess energy intake. These animals have more interscapular BAT and a higher GDP binding activity than chow fed rats (Kanarek and Orthen-Gambill 1982; Kanarek, Aprille, Hirsch, Gualiere and Brown 1987). Sucrose feeding also stimulates lipogenesis in BAT and elevates BAT fatty acid synthetase activity but not lipoprotein lipase activity (Granneman and Wade 1983). The relative intake of sucrose solution is an indirect index of the protein content in the diet. Animals with a higher protein content in the diet have a low consumption of sucrose solution and a low GDP binding to BAT mitochondria. In contrast a low protein diet enhances thermogenic response in rats (Swick,

Gribskov and Swick 1985; Rothwell and Stock 1987a).

### 10.4. DIT in Man

There is some evidence for the presence of adaptive DIT in man (Welle and Campbell 1983; King, McMahon and Almond 1986; Sim and Danforth 1987; Welle, Nair and Campbell 1989). During intravenous infusion with hypertonic glucose (with a fixed and sufficient nitrogen intake as amino acid), there is a progressive rise in $O_2$ consumption and $CO_2$ production with increasing glucose infusion (King et al. 1986). The synthesis of fat from glucose also increases and reaches a plateau, but the glucose oxidation continues to increase, corresponding to the glucose supply, indicating that there is adaptive DIT. King et al. (1986) suggested that BAT may be involved in DIT.

Another study showed that overfeeding with carbohydrates for 6 and 10 days increased the resting metabolic rate in man (Welle et al. 1989). However propranolol does not prevent the increase in resting metabolic rate of the overfed subjects indicating that the effect of overfeeding in man is not mediated by increased $\beta$-adrenergic activity (Welle et al. 1989). They suggested that an increase in lipogenesis from carbohydrate and an increase in Na-K-ATPase activity could contribute to the

increase in resting metabolic rate rather than an increase in DIT in BAT.

Although there is debate about the presence of functional BAT in the adult human, some research workers (Cunningham et al. 1985) have reported that the perinephric BAT has functionally active UCP as indicated by nucleotide sensitive proton conductance and uncoupling response to fatty acids. However the respiratory capacity measurements indicated that total perinephric BAT in adult man can only account for one-fivehundredth of the whole body response to infused noradrenaline (Cunningham et al. 1985). Thus considerable caution must be exercised in extrapolating the results of animal experiments to the adult human.

## 10.5. Neuroendocrine Control of BAT in DIT

The most obvious hormonal change found in overfeeding is a rise in serum $T_3$ (Rothwell and Stock 1979a; Tulp, Gregory and Danforth 1982b), but the metabolic significance of this is largely unknown, and thyroid hormones seem to exert only a permissive role in DIT. Sundin (1981), and Rothwell and Stock (1984c) reported that high doses of $T_3$ do not stimulate mitochondrial purine nucleotide binding of BAT. Adrenal steroids, especially corticosterone, are the most active hormones in

the control of BAT in DIT. It has been known for some time that bilateral adrenalectomy arrests the development of obesity in genetically obese rodents (Yukimura and Bray 1978) and stimulates BAT thermogenesis (Holt and York 1982; Marchington, Rothwell, Stock and York 1983). Rothwell, Stock and York (1984) have shown that adrenalectomy can prevent the development of diet-induced obesity in old 'cafeteria'-fed rats by restoring BAT activity to the level of young animals. For detailed discussion of the role of adrenal hormone in BAT thermogenesis, refer to section 13.2.4.

Prolactin (PRL), growth hormone, TSH, glucagon and melatonin have been suggested as modulators of BAT thermogenesis but no direct connection with the diet-induced activation of BAT has been described.

Some anorectic agents such as fenfluramine (a serotonergic drug), have been assumed to act via their effects on central 5-hydroxytryptamine (5-HT), but recently a thermogenic action of fenfluramine has also been suggested (Blundell 1977) since it caused a sustained weight loss even after food intake had returned to normal (Levitsky, Strup and Lupoli 1981). Lupien and Bray (1985) have also reported an increase in GDP binding activity of BAT after fenfluramine injection in rats. Treatment with fenfluramine or 5-HT also stimulates resting $O_2$ consumption and these effects are partially suppressed by pretreatment with a ganglionic blocker (Rothwell and Stock

1987a). Thermogenic activity induced by low protein diet is also abolished by prior treatment with serotonergic blocker (Rothwell and Stock 1987a). All these findings indicate the possible involvement of the serotonergic pathway in DIT.

Bilateral functional decortication impairs the increase of $O_2$ consumption in response to noradrenaline to the same extent as administration of propranolol indicating that the brain cortex is involved in the thermogenesis (De Luca, Mona, Pellicano and Zena 1987). Bilateral functional decortication also causes a significant decrease of BAT temperature and $O_2$ consumption in 'cafeteria'-fed rats but not in controls. The cerebral cortex could therefore be involved in the control of BAT thermogenesis (De Luca et al. 1987).

In summary, thyroid hormones and corticosterone are the most important hormonal factors in modifying DIT. Serotonergic pathways in the central nervous system and the cerebral cortex are also probably involved in regulating DIT.

## 10.6. Effects of Fasting in BAT

A reduction in caloric expenditure during a prolonged fast is associated with a reduction in plasma $T_3$ (Rothwell, Saville and Stock 1982), a reduction in sympathetic activity in BAT (Young, Saville, Rothwell, Stock and Landsberg 1982), marked atrophy of BAT, and decrease in total protein content of BAT (Rothwell, Saville and Stock 1984; Lopez-Soriano and Alemany 1987a and b; Trayhurn and Jennings 1986 and 1988; Levin and Trayhurn 1987; Desautels and Dulos 1988). As early as after 24 h of starvation the BAT wet weight is significantly lower than in control rats (Desautels 1985; Trayhurn and Jennings 1986; Lopez-Soriano and Alemany 1987a). BAT mitochondrial protein and cytochrome oxidase activity also decrease after fasting (Levin and Trayhurn 1987; Trayhurn and Jennings 1986 and 1988) indicating a major decrease in mitochondrial mass. Mitochondrial GDP binding is also reduced (Desautels 1985; Trayhurn and Jennings 1986 and 1988), suggesting that the activity of the proton conductance pathway is suppressed. Noradrenaline stimulated $O_2$ consumption is reduced in fasting mice (Desautels and Dulos 1988) and golden hamsters (Levin and Trayhurn 1987). However the effect of fasting on UCP is controversial. A selective loss of UCP in BAT has been reported in mice by Trayhurn and Jennings (1986 and 1988) whereas Desautels (1985) could not find any change in mice and Levin and Trayhurn (1987) could not find a change in golden hamsters.

Chronic food restriction also suppresses thermogenic capacity (Rothwell and Stock 1982) which recovers to normal after refeeding (Levin and Trayhurn 1987; Trayhurn and Jennings 1986 and 1988; Desautels and Dulos 1988). The time required for complete recovery is longer than that required for suppression of thermogenic capacity during fasting.

In summary, overfeeding causes DIT and there are increases in basal $O_2$ consumption, mitochondrial mass, cytochrome C oxidase activity, GDP binding and UCP in BAT. The dominant role of the SNS in activating BAT thermogenesis during overfeeding has also been established. In almost all respects, the mechanisms of DIT and NST are identical but the afferent signals and central mechanisms involved in the control of DIT remain unclear and further research is required.

## 11. Obesity and BAT

Obesity is generally regarded as a major nutritional problem, especially in affluent western societies, both as a risk factor to health and a cause of increased mortality. It is linked to an increased incidence of several diseases, including coronary artery disease, hypertension and diabetes.

There is considerable difficulty in conducting accurate and long term energy balance studies in human subjects. Long term energy balance studies can be made with a high degree of precision in laboratory animals. Energy balance studies have been performed in rodents and understanding of obesity in rodents may give some insight into the cause of obesity in man. This section focuses on the thermogenic function of BAT in relation to obesity in animals and genetically obese mice and rats are generally used as examples in this review.

Either excessive food intake or lower energy expenditure due to a defect in the thermogenic function of BAT may cause obesity. Many studies have shown that the thermogenic function of BAT is defective in some obese animals especially in the genetically obese rodents e.g. ob/ob mice and Zucker (fa/fa) rats.

The propensity to become obese is inherited as an

autosomal recessive trait in ob/ob mice and fatty Zucker (fa/fa) rats. These mutants, when allowed free access to food, spontaneously overeat and become obese (Mercer and Trayhurn 1987; Harris, Tobin and Hervey 1987; Smith 1989). However if these animals are pair-fed the same amount and type of food as normal animals, they still gain excessive weight (Thurlby and Trayhurn 1979). Therefore obesity in these mutants is partly caused by an excessive consumption of food and partly due to a defect in energy expenditure.

Moreover defective BAT has been shown in several types of obese animals (for reviews see Himms-Hagen 1984; Trayhurn 1986) including obese (ob/ob) mouse, the fatty (fa/fa) rat, the diabetic (db/db) mouse, the gold thioglucose mouse, mice treated with corticosterone, and rats with a lesion in the ventromedial hypothalamus.

Obese mice also show defective thermoregulation. At thermoneutrality (33°C) the resting metabolic rate of adult ob/ob and lean mice is similar, whereas at lower temperatures the metabolic rate of the obese is 20% below that of the lean sibling (Trayhurn and James 1978) indicating that at normal environmental temperature (20-25 °C) ob/ob mice expend less energy than lean animals on the thermoregulatory thermogenesis. In lean mice, the gain in carcase energy rises with increasing environmental temperature (Thurlby and Trayhurn 1979). Therefore the environmental temperature plays a major role in determining the excess energy gain of ob/ob mice (Thurlby

and Trayhurn 1979). Obese mice also deposit considerably less protein than their lean controls (Thurlby and Trayhurn 1979). Genetically obese Zucker (fa/fa) rats maintain a normal lean body mass but deposit an excess amount of body fat (Harris et al. 1987).

Ultrastructural abnormalities in BAT mitochondria have been found in ob/ob mice and these include the irregular appearance of the cristae which are less well developed (Hogan and Himms-Hagen 1980). Thurlby and Trayburn (1980) measured the blood flow to BAT using radioactively labelled microspheres and found it to be reduced. From tissue blood flow and the arteriovenous difference in $O_2$ tension across the interscapular site it is estimated that almost all of the reduction in NST in the ob/ob mouse could be attributed to BAT (Thurlby and Trayhurn 1980).

The mass of BAT in ob/ob mice is several-fold higher than in lean mice. This difference is not seen before 14 days of age (Goodbody and Trayhurn 1982). The expansion of BAT mass begins at around weaning (Mercer and Trayhurn 1983a) and it is several-fold greater than their lean siblings (Himms-Hagen and Desautels 1978; Trayhurn, Jones, MaGuckin and Goodbody 1982b; Himms-Hagen, Hogan and Zaror-Behrens 1986; Tulp, Stevens and Barbie 1989). The increase in mass is due to accumulation of fat in BAT, in parallel with the increase of body fat (Himms-Hagen et al. 1986).

Cytochrome oxidase activity, protein content (Trayhurn, Jones, McGuckin and Goodbody 1982b) and DNA content of BAT are lower in ob/ob mice (Goodbody 1982) and obese diabetic mice (Ohta, Kitazaki and Tsuda 1988).

The thermogenic potential of BAT measured by GDP binding activity is low in ob/ob mice (Himms-Hagen and Desautels 1978; Hogan and Himms-Hagen 1980) and Zucker rats (Holt, York and Fitzsimons 1983). However in some studies GDP binding activity is not found to be significantly lower (Trayhurn et al. 1982b; Himms-Hagen et al. 1986; Ohta et al. 1988). Scatchard analysis shows that affinity of GDP to BAT mitochondria in the adult ob/ob mice is not different from that of lean mice. The number of binding sites however, is reduced (Goodbody 1982).

In genetically obese animals such as ob/ob mice, obesity is not visually detected until the animals are about 4 weeks old when the total body lipid content of the mutant animal is several-fold higher than their lean siblings (Thurlby and Trayhurn 1979). Thermogenic function of BAT assayed by GDP binding is lower in animals older than 4 weeks (Himms-Hagen and Desautels 1978; Hogan and Himms-Hagen 1980; Thurlby and Trayhurn 1980). Thus the decrease in thermogenic function of BAT may be secondary to the obese state. In order to verify this, measurements were made at different ages (Goodbody and Trayhurn 1982). At 14 days of age there are no differences between the

ob/ob and lean mice in the total amount of BAT, DNA content, protein content, and cytochrome oxidase activity but respiratory rates of BAT mitochondria and number of GDP binding sites are lower in ob/ob mice. The UCP concentration, on the other hand, is similar to that of lean controls at 14 days but lower at 10 weeks. In Zucker rats, GDP binding is low as early as 10 days and this remains low throughout the life of the animal. The UCP content in Zucker rats is low only after 12 weeks of age. This dissociation between the level of GDP binding and the amount of UCP in young ob/ob mice and obese Zucker rats is suggested to be due to masking of UCP. In obese Zucker rats the response to refeeding at 2 days of age is similar to that in lean controls (Planche and Joliff 1986), but at 7 days, the response is lower in obese rats (Planche and Joliff 1986). Thus it is clear that a decrease in BAT thermogenesis is a very early defect in these obese mutants, and develops prior to obesity.

The suppression of mitochondrial respiration rate, and membrane potential (Goodbody and Trayhurn 1982) and mitochondrial Ca transport (Trayhurn and Fraser 1983) support the conclusion that the activity of the proton conductance pathway is reduced in the pre-obese animals.

## 11.1. NST and DIT in Obese Animals

The ob/ob mouse is very sensitive to cold and at temperatures around 4°C it is unable to survive for more than a few hours (Trayhurn and James 1978). In response to acute cold exposure adult ob/ob mice fail to show increase in GDP binding (Himms-Hagen and Desautels 1978; Mercer and Trayhurn 1986). However the response to cold stress as shown by an increase in GDP binding is normal in the very young of the genetic mutants (Goodbody and Trayhurn 1982). Young (5-week-old) obese Zucker rats show a normal increase in BAT mitochondrial GDP binding in response to cold but this response is attenuated in 10-week-old obese rats (Holt et al. 1983). Other studies have shown that the increase in GDP binding in response to cold exposure is normal up to around 4 weeks of age but not in older ob/ob mice (Mercer and Trayhurn 1984). In 4 weeks old Zucker (fa/fa) rats there is an increase in both GDP binding and UCP concentration in response to cold acclimation (Ashwell, Holt, Jennings, Stirling, Trayhurn and York 1985). These studies show that there is impaired response to cold in genetically obese animals and this impairment develops at a later age.

In obese mice voluntary overfeeding causes significantly higher energy gain and lower increases in cytochrome oxidase activity and GDP binding compared to lean controls (Trayhurn et al. 1982b). Similarly sucrose

feeding to obese mice (KKA$^{y}$) show a poor response (Ohta et al. 1988; Holt et al. 1983). Therefore, obese mice show a poorer response to overfeeding. A high fat diet, however, produces a similar increase in GDP binding in obese mice (Mercer and Trayhurn 1987). A diet rich in polyunsaturated fatty acids appears to result in preferential stimulation of the thermogenic activity of BAT in ob/ob mice (Mercer and Trayhurn 1987).

In general, obese animals show reduced NST and when they become hyperphagic, there is a reduced DIT response resulting in energy gain.

## 11.2. Regulation of BAT in Obese Animals

### 11.2.1. SNS in Obese Animals

The importance of SNS in the regulation of thermogenesis in BAT is discussed in section 13.1. In genetically obese animals a reduction in the SNS activity may be responsible for the suppression of thermogenic function and a reduced level of BAT noradrenaline turnover has been demonstrated in obese animals (Holt and York 1989; Marchington et al. 1983; Young and Landsberg 1983; Triandafillou and Himms-Hagen 1983; York, Marchington, Holt and Allars 1985). The increase in noradrenaline turnover seen in BAT during cold exposure in normal mice is less than in obese (Young and Landsberg 1983).

The spontaneous activity of the sympathetic nerve of the interscapular BAT measured by electrophysiological techniques is reduced in the obese (fa/fa) rat compared with the lean control and it was concluded that the sympathetic tone is suppressed in the genetically obese rat (Holt and York 1989). Furthermore there is an attenuated response in nerve firing rate to electrical stimulation of the ventromedial nucleus (VMN) in the obese rat suggesting that maximal increase in nerve firing rate is lower in the obese rat due to a defect at the level of the VMN (Holt and York 1989).

The mechanism of this defect in the obese (fa/fa) rat is unknown. Hyperinsulinaemia may be a possible factor for the suppression of sympathetic firing. It has been reported that serum and cerebral spinal fluid insulin levels are high in the obese rat (Stein, Dorsa, Baskin, Figlewicz, Ikeda, Frankmann, Greenwood, Porte and Woods 1983). Mercer and Trayhurn (1986) showed that treatment with hypoglycaemic drugs can normalize NST in ob/ob mice and intrahypothalamic injection of insulin suppresses the firing rate of sympathetic nerve (Sakaguchi and Bray 1989) and brown fat thermogenesis (Amir, Lagiorgia and Pollock 1989). However, the number of insulin receptors in various brain regions of the obese rat is severely depressed and in some areas insulin receptors are undetectable (Figlewiez, Dorsa, Stein, Baskin, Paguette, Greenwood, Woods and Porte 1985). Therefore the role of hyperinsulinaemia as a suppressor of SNS activity in obese rats remains unclear.

### 11.2.2. Corticosteriods in Obese Animals

Adrenalectomy is found to be an effective treatment in preventing further development of genetic, dietary or hypothalamic obesity (Bruce, King, Phelps and Veita 1982; Rothwell et al. 1984). Energy efficiency in obese Zucker rats fed with both high carbohydrate and high fat diets is reduced by adrenalectomy to value close to those found in lean rats (Allars, Holt and York 1987). Adrenalectomy has no effect on BAT in lean animals but normalizes cytochrome oxidase activity and mitochondrial GDP binding in obese animals (Holt and York 1984; Trayhurn and Mercer 1984). Romsos, Vander Tuig, Kerner and Grogan (1987) have confirmed that adrenalectomy decreases energy intake and increases thermogenic activity of BAT. Although adrenalectomy prevents obesity in obese (ob\ob) mice fed a high carbohydrate non purified diet, it does not prevent the development of obesity in normal mice fed a high-fat diet or high glucose diet (Grogan, Kim and Romsos 1987; Kim and Romsos 1987). The interaction between the composition of diet and neuroendocrine regulation in ob/ob mice requires further elucidation.

Corticosteroid-treated mice develop obesity even when they are pair-fed to prevent hyperphagia (Galpin, Henderson, James and Trayhurn 1983a), and BAT cytochrome oxidase and GDP binding are both decreased (Galpin, Hendersen, James and Trayhurn 1983b). It is therefore suggested that corticosteroids suppress the activation of DIT (Galpin et al. 1983b).

Although adrenalectomy can normalize the derangement of thermogenesis of BAT in obese Zucker rats, plasma concentrations and diurnal rhythm of plasma corticosterone and plasma ACTH have been reported to be normal (Yukimura, Bray, Wolfsen 1978). However total excretion of corticosterone is found to be significantly greater in obese than lean Zucker rats (Cunningham, Calles-Escandon, Garrido, Carr and Bode 1986). The existence of chronic hypercorticosteronaemia in obese Zucker rats is further supported by the impaired response to CRF in this genetic form of obesity (Cunningham et al. 1986).

Recently, the hypothalamus and pituitary have been shown to be involved in the regulation of thermogenesis in rats and studies have revealed that hypophysectomy exerts more potent effects than adrenalectomy (Rothwell and Stock 1985). In obese rats with hypothalamic lesions, there is suppression of the redox state of BAT (the ratio of NAD(P)H to NADP) in response to electrical nerve stimulation and noradrenaline, together with an involution of BAT (Seydoux, Rohner-Jeanrenaud, Assimacopoulos, Jeanrenaud and Girardier 1981). Hypophysectomy in genetically obese Zucker rats significantly reduces body weight and stimulates energy expenditure, increases the thermic response to food, and increases BAT mitochondrial GDP binding activity (Holt, Rothwell, Stock and York 1988). All these changes can be reversed by corticosterone replacement, but the increased BAT activity is only partially restored (Holt et al. 1988). Hypophysectomy has

a small effect on energy balance in lean Zucker rats but increases the acute thermic response to food and BAT mitochondrial GDP binding capacity; these effects are inhibited by replacement of corticosterone (Holt et al. 1988). The above data indicate that the hypothalamic-pituitary-adrenal axis plays a role in the development and maintenance of genetic obesity. The effect of hypophysectomy may be related to an increase in CRF levels. The observation that ventricular injection of CRF stimulates heat production in BAT (LeFeuvre, Rothwell and Stock 1987) supports this hypothesis.

In summary, the increased sensitivity to the inhibitory effects of glucocorticoids possibly at the hypothalamic levels may be a primary cause of defective thermogenesis in genetically obese animals (LeFeuvre et al. 1987; reviews: Rothwell and Stock 1986b; York 1989).

### 11.2.3. Adrenergic Receptors in Obese Animals

It has been known for a long time that catecholamines and sympathomimetic agents stimulate metabolic rate in mammals primarily due to their action on BAT (Foster and Frydman 1978; Rothwell and Stock 1984b). In obese Zucker rats, but not in lean controls clenbuterol (a $\beta_2$-adrenergic agonist) significantly depresses energy efficiency and increases the thermogenic response to food and BAT activity (Rothwell and Stock 1987b).



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However, the density of $\alpha_1$-adrenergic receptors but not $\beta_1$-adrenergic receptors in BAT has been shown to be lower in obese Zucker rats than in lean rats (Raasmaja and York 1988). Adrenalectomy restores the density of $\alpha_1$-adrenergic receptors in BAT of obese Zucker rats to the value observed in lean rats (Raasmaja and York 1988). Therefore the low density of adrenergic receptors may play a role in animal obesity.

### 11.2.4. Insulin in Obese Animals

In addition to the possible effects on the SNS, insulin may directly affect the thermogenic function of BAT (Rothwell and Stock 1981b; Rothwell, Saville and Stock 1983; Seydoux, Trimble, Bouillaud, Assimacopoulos-Jeannet, Bas, Ricquier, Giacobino and Girardier 1984). Hyperinsulinaemia is found in obese ob/ob mice (Bray and York 1979). BAT has a high capacity for lipogenesis which is stimulated by insulin (Agius and Williamson 1980). Lipogenesis in BAT is elevated in ob/ob mice presumbably by a consequence of hyperinsulinaemia which is, however, transient and falls substantially by the age of 35 days (Mercer and Trayhurn 1983a). Mercer and Trayhurn (1984) showed that marked insulin resistance causes a fall in lipogenesis in BAT. The peripheral insulin sensitivity in ob/ob mice can be restored by ciglitazone, an oral hypoglycaemic drug (Chang, Wyse, Gilchrist, Peterson and Diani 1983b; Change, Wyse and Gilchrist 1983a). Insulin

sensitivity in BAT and the acute response to cold are also normalized (Mercer and Trayhurn 1986) by this drug.

The mechanism by which the development of insulin resistance in BAT leads to an impairment in thermogenic responsiveness is not clear. Insulin possibly acts by modifying the SNS activity which in turn alters the BAT thermogenic function. This is discussed in more detail in section 13.2.2. The routes of action of insulin and corticosteroids on SNS and BAT are schematically shown in Fig. 1.5 (adapted from Trayhurn 1986).

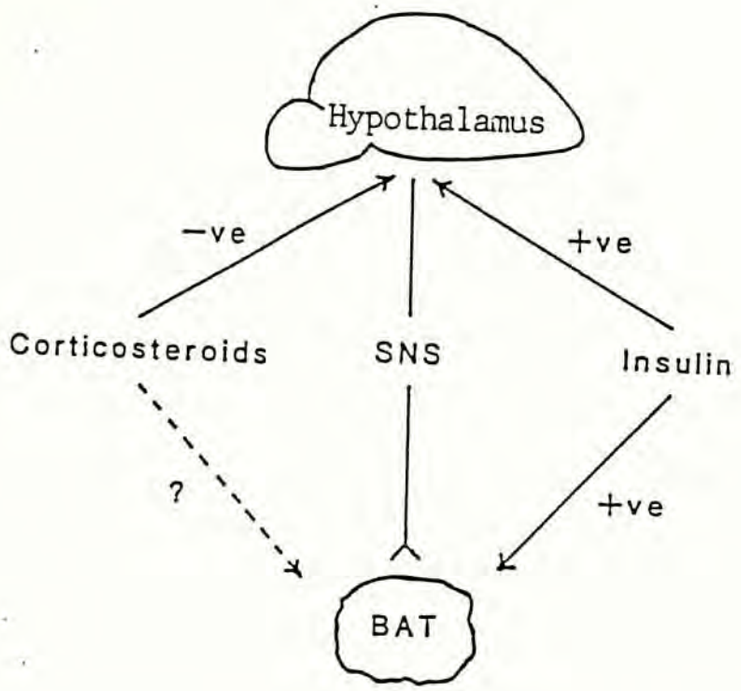



Fig. 1.5 A schematic representation of the routes by which insulin and corticosteroids may influence the activity of BAT (SNS = sympathetic nervous system). (Adapted from Trayhurn 1986.)

## 12. Pregnancy and Lactation and BAT

### 12.1. Energy Balance During Pregnancy and Lactation

Pregnancy and lactation represent a time of physiological stress to the mother due to increases in metabolism associated with the production of new tissue such as placenta, foetal membranes, foetus and mammary gland during pregnancy, and with production of milk during lactation. Certain adaptive mechanisms take place during pregnancy and lactation in order to maintain maternal energy balance. Recently detailed studies on energy expenditure during pregnancy have indicated that there is enhanced efficiency of metabolism in rodents (Schneider and Wades 1987; Kava, West, Lukasil, Prinz and Greenwood 1986; Vernon and Finley 1988; Villarroya and Mempel 1987) and humans (Whitehead, Black and Wiles 1981). In women, there is a decrease in basal metabolic rate in the first half of pregnancy (Durnin, McKillop, Grant and Fitzgerald 1985).

In animal studies, it has been suggested that the energy efficiency is increased during pregnancy and the energy conserved is initially stored as fat for later mobilization during lactation to meet the energy needs. However, some studies have shown no enhancement of energy efficiency during pregnancy. Pregnant mice pair-fed to that of unmated controls show no change in efficiency. On

the other hand pregnant mice fed ad libitum show increased efficiency associated with increased energy intake (Richard and Trayhurn 1985). In several other species no change in maternal energy efficiency during pregnancy has been reported (Close, Noblet and Heavens 1985; Eisen and Leatherwood 1976; Nagy and King 1984). In hamsters the strategy for balancing energy storage and expenditure during pregnancy is quite different (Schneider and Wade 1987; Wade, Jennings and Trayhurn 1986). Syrian hamsters do not increase their food intake or decrease metabolic efficiency but they increase hoarding and store large amounts of metabolic fuels outside the body. Their lipid stores also fall in the later stage of gestation (Wade et al. 1986). A great loss of carcase lipid even with a high food intake has been reported in Djungarian hamsters during pregnancy (Schneider and Wade 1987). In rats lactation promotes negative energy balance regardless of the level of energy intake or increase in energy efficiency (Roberts and Coward 1984).

## 12.2. Some Metabolic Changes During Pregnancy and Lactation

During pregnancy there is an increase in accumulation of fat in preparation for the energy requirement for lactation (Steingrinsdottir, Greenwood and Brasel 1980; Moore and Brasel 1984). This enhanced fat deposition can be the result of increased energy intake (Richard and

Trayhurn 1985), or hormonal changes such as elevated insulin level (Flint, Sinnet-Smith, Clegg and Vernon 1979; Flint 1985) or increased numbers of insulin receptors in adipose tissue (Flint et al. 1979). In white adipose tissue lipoprotein lipase activity is increased at mid pregnancy, thus increasing the uptake of triglycercides from the blood to the white adipose tissue (Steingrinsdottir et al. 1980; Flint, Clegg and Vernon 1983). Moreover, adrenergic stimulation of lipolysis in white adipose tissue decreases in pregnancy and increases in lactation (Guesnet, Massoud and Demarne 1987). In white adipose tissue fatty acid synthesis decreases during lactation due to changes in insulin concentration (Vernon and Finley 1988). These data suggest that in white fat an enhancement of fat storage and reduction of fat utilization occur during pregnancy but are reversed during lactation.

Lipogenesis in BAT increases in mid pregnancy (Aguis and Williamson 1980) decreases in late pregnancy and lactation (Villarroya and Mampel 1986; Aguis and Williamson 1980) and returns to normal during weaning in rats (Aguis and Williamson 1980). A significant reduction in the utilization of glucose for fatty acid synthesis in BAT is observed during lactation (Villarroya and Mempel 1987). There is hypertrophy of BAT during pregnancy because of progressive lipid accumulation, and atrophy during lactation (Villarroya, Felipe and Mampel 1986; Trayhyrn et al. 1982a; Andrews, Richard, Jennings and

Trayhurn 1986). In addition, blood flow is altered during pregnancy, thus shifting the energy away from consumption in BAT to storage in white adipose tissue (Kava et al. 1986).

## 12.3. Role of BAT in Pregnancy and Lactation

The importance of BAT in regulation of energy balance and thermogenesis is discussed in previous sections 9., 10. and 11. Changes in BAT during pregnancy were first reported by Teodoru and Grishman (1961). Later, thermogenic function of BAT during pregnancy and lactation was studied extensively by Trayhurn and his coworkers. The absence of any change in the thermogenic activity of BAT until the later stage of gestation appeared to make it very unlikely that BAT plays any particular role in early pregnancy (Andrews et al. 1986; Villarroya et al. 1986). In mice the thermogenic activity measured by GDP binding, cytochrome oxidase activity and respiration of BAT mitochondria are suppressed in late pregnancy and lactation (Trayhurn et al. 1982a; Trayhurn 1983 and 1985). Others have confirmed the suppression of thermogenesis (Villarroya et al. 1986; Tatelman, Steinberg and winick 1985; Andrews et al. 1986; Gerardo, Moore, Stern and Horwitz 1985) and cytochrome oxidase activity during lactation (Villarroya et al. 1986; Andrews et al. 1986). The fall in GDP binding is observed in late pregnancy

(Andrews et al. 1986; Tatelman et al. 1985). The decrease in GDP binding continues during the entire lactation period -lowest activity is found in mid and late lactation (Trayhurn et al. 1982a; Andrews et al. 1986; Villarroya et al. 1986 and Gerardo et al. 1985).

Although changes in GDP binding and cytochrome oxidase activity during pregnancy and lactation have been reported, there are very few reports on the changes in UCP content (Ashwell et al. 1983b). A significant depletion of UCP content in mid lactation (9-12 days post partum) and at weaning (21 days post partum) has been reported (Ashwell et al. 1983b). A detailed study of the changes in UCP content during the entire breeding cycle and post weaning period would be of interest in tracing sequential changes in thermogenic capacity.

## 12.4. Mechanism of Regulation of Thermogenesis during Pregnancy and Lactation

During late pregnancy and lactation, there is a significant increase in plasma PRL (Amenomori, Chen and Meites 1970), which coincides with the reduction of thermogenic activity of BAT. Thus it is suggested that PRL may cause the suppression of thermogenesis. In a study using pituitary transplants to induce hyperprolactinaemia GDP binding is shown to be decreased (Gerardo et al. 1985). This decrease is only partial compared to a

remarkable suppression during lactation. The PRL concentration in pituitary transplanted rats was not reported in this study. In Syrian and Siberian hamsters, which have seasonal changes in PRL level, Bartness, Wade and Goldman (1987) manipulated the PRL level in these animals. They showed that during a long photoperiod Syrian hamsters have high level of PRL and treatment with bromoergocryptine, a dopamine agonist, caused increases in BAT protein, cytochrome oxidase activity, and retroperitoneal lipoprotein lipase activity. There are no change in BAT lipoprotein lipase activity and noradrenaline stimulated $O_2$ consumption. However, bromoergocryptine treatment elevates noradrenaline stimulated $O_2$ consumption in Siberian hamsters. The results suggest that the changes in energy balance associated with photoperiod in these species cannot be attributed simply to changes in serum PRL concentration but also possibly involves with changes of BAT thermogenic function. Direct measurement of thermogenic function by GDP binding and UCP content were not reported and therefore the effect of PRL on thermogenic function of BAT is not fully established. Recently, pituitary implantation-induced hyper-prolactinaemia has been shown to prevent short photoperiod-induced changes in BAT mass and mitochondrial GDP binding (Kott, Moore, Fournier and Horwitz 1989). Further evidence suggesting that PRL may be involved in the regulation of thermogenic function of BAT comes from the observation that there is an inverse relationship between litter size and BAT function (Isler,

Trayhurn and Lunn 1984). These workers estimated the heat generated during milk synthesis and concluded that reduction in BAT activity is not merely in response to metabolic heat production in the mammary tissue but suggested that BAT function in lactating rats may be under endocrinological control by the lactation-promoting hormone PRL (Matheij, Gruisen and Swarts 1979).

Despite the considerable increase in the energy intake during pregnancy and lactation in small mammals the protein and RNA content of BAT are lower in pregnant rats, indicating down regulation of the tissue activity (Abelenda and Puerta 1987). During pregnancy, therefore, hyperphagia does not enhance BAT activity. The capacity for NST during lactation is also suppressed in mice (Trayhurn 1983). Lactating rats show a lower thermoregulatory ability evaluated by measurement of colonic temperature, evaporative water loss and survival time during terminal heating (Knecht, Toraason and Wright 1980). Roberts and Coward (1985) have suggested that obligatory heat production is increased substantially during lactation and this may explain why BAT thermogenesis is suppressed. The impairment of DIT and NST in lactating mammals may be related to changes in sympathetic activity in BAT. Significant decreases in noradrenaline turnover in lactating rat (Villarroya, Felipe and Mampel 1987) and mice (Trayhurn and Wusteman 1987b), and noradrenaline content in BAT of mice (Trayhurn and Wusteman 1987b) have been reported. However

noradrenaline turnover in BAT is not altered in lactating golden hamsters (Trayhurn and Wusteman 1987a) and it was suggested that sympathetic activity and BAT thermogenesis are dissociated during pregnancy and lactation in this species.

Other mechanisms may also be involved in the changes of thermogenic activity during pregnancy and lactation. The decrease in BAT thermogenic activity is associated with a parallel decrease not only in the iodothyronine 5'-deiodinase (or called 5'-deiodinase) activity but also in the actual $T_3$ content of the tissue suggesting that this hormone may play an important role in the thermogenic activity of the tissue (Vina, Giralt, Obregon, Iglesias, Villarroya and Mampel 1988), perhaps by modulating the level of UCP gene expression, as suggested by Bianco and Silva (1987a and b). The reduction of sympathetic activity occurring in rat BAT during mid-lactation (Villarroya et al. 1987) would be expected to reduce 5'-deiodinase activity in BAT. However, the activity of 5'-deiodinase is decreased during late pregnancy when sympathetic activity is unlikely to be substantially depressed.

## 13. Factors Controlling the Thermogenesis

The adaptive response of brown fat during development, cold adaptation and hyperphagia is influenced by two major factors: (1) neuronal control - sympathetic stimulation of the tissue is in itself sufficient as an initial signal for hypertrophy, hyperplasia, and selective synthesis of the UCP in BAT; (2) hormonal involvement - this may be required as an additional stimulus.

### 13.1. Sympathetic Nervous Control

BAT has a rich sympathetic innervation, with nerve endings on both cells and blood vessels (see review Nechad 1986), which suggests that SNS may have a role in the control of BAT. Several studies have shown that the central sympathetic outflow to brown fat is activated by cold exposure and by overfeeding (see reviews Barnard et al. 1980; Girardier and Seydoux 1986).

The role of SNS in the control of BAT is established from many studies.

#### 13.1.1. Studies of Administration of Noradrenaline

In the 1970s there were technical difficulties in maintaining a continuously elevated noradrenaline level in

order to study the chronic effect of noradrenaline on BAT. Repetitive daily injection of the catecholamine resembles more closely intermittent cold stress rather than continuous cold exposure, and this probably accounts for the discrepancy in results reported in the 1970s and 1980s. Repetitive administration of noradrenaline (Desautels and Himms-Hagen 1979; Le Blanc and Villemaire 1970) for 2 weeks or more to warm adapted rats typically caused a 50% increase in the wet weight, total tissue protein content and cytochrome oxidase activity of interscapular BAT and an insignificant increase in the amount of protein in the 32 kDa region (Desautels and Himms-Hagen 1979). Although the rapid increase in GDP binding observed within 1 h of cold exposure (Desautels and Himms-Hagen 1979) can also be seen within 1 h of the commencement of noradrenaline infusion, this appears to be transient, because no permanent increase is seen in the chronically treated animal (Desautels and Himms-Hagen 1979).

However in a recent study Mory, Bouillaud, Combes-George and Ricquier (1984) have demonstrated that constant infusion with noradrenaline by mini-osmotic pumps implanted in rats greatly increased both mitochondrial mass and the specific mitochondrial concentration of UCP. The DNA content of the interscapular brown fat of adult rats is doubled by chronic isoproterenol treatment (Mory, Ricquier and Hemon 1980). Implantation of phaeochromocytoma causes an increase in weight, DNA

content, total and mitochondrial protein content, GDP binding activity and UCP content in BAT (Ricquier et al. 1983). The morphological regression of brown adipocytes is prevented by noradrenaline in in vitro culture (Cinti, Cigolini, Sbarbati, Zancanaro and Bjorntorp 1987). Addition of noradrenaline stimulates cytochrome oxidase activity of differentiating brown adipocytes, indicating that noradrenaline is involved in the differentiation process (Nechad, Nedergaard and Cannon 1987).

### 13.1.2. Control of the Fuel Supply to BAT by SNS

Fatty acid is known to be the main fuel for thermogenesis in BAT (Joel 1966) and in addition it is a regulator of the respiratory rate in brown fat mitochondria (Locke and Nicholls 1981). Lipolysis in BAT is regulated primarily by the noradrenaline released from sympathetic nerve endings. Direct electrical stimulation of the sympathetic nerves also enhances the synthesis of fatty acid and glycerol in BAT of rat (Minokoshi, Saito and Shimazu 1988). The refeeding-induced increase in lipolysis of interscapular BAT in fasted rats is abolished by sympathetic denervation of interscapular BAT (Mercer and Williamson 1988). Surgical denervation of interscapular BAT also prevents the utilization of free fatty acid in cold stimulated BAT (Laury, Chapey and Portet 1987).

Moreover, noradrenaline may also play a role in BAT glucose metabolism. It has been shown in the mouse that noradrenaline increases the uptake of 2-deoxyglucose in BAT (Cooney, Caterson and Newsholme 1985). Although noradrenaline alone does not modify glucose transport in brown adipocytes *in vitro*, it potentiates insulin-induced glucose oxidation (Ebner, Burnol, Ferre, Saintaurin and Girard 1987).

### 13.1.3 Sympathetic denervation

Sympathetic denervation causes biochemical and morphological changes in BAT. It decreases the turnover of triglycerides in BAT in contrast to sympathetic stimulation which has the opposite effect (Shimazu and Takahashi 1980; Takahashi and Shimazu 1981). The denervated BAT contains larger lipid droplets, some of which are unilocular (Minokoshi, Saito and Shimazu 1986). Selective loss of UCP from mitochondria of surgically denervated BAT of cold acclimated mice in the absence of change in the tissue DNA content has been reported (Desautels, Dulos and Mozaffari 1986). Sympathetic denervation abolishes the cold induced increase in UCP concentration and BAT hyperplasia indicating an intact innervation is required for both synthesis and maintenance of a high mitochondrial UCP content in cold adapted animals (Desautels *et al*. 1986). Rothwell and Stock (1984d) also demonstrated that the denervation of BAT reduces GDP binding.

## 13.2. Hormonal Control

### 13.2.1. Thyroid Hormone

Thyroid hormones can affect BAT thermogenesis. Hypothyroidism leads to a decrease in the thermogenic response of BAT to electrical stimulation of nerve and catecholamine in adult rats. In BAT obtained from a thyroidectomized ovine foetus, the catecholamine-stimulated respiration is augmented by infusion of $T_3$ (Klein, Reviczky and Padbury 1984).

The increase in UCP content as a result of cold response is suppressed in hypothyroid rats (Bianco and Silva 1987a and b; Triandafillou, Gwilliam and Himms-Hagen 1982; Ricquier et al. 1984) and thyroxine replacement restores this cold effect. However excessive thyroid hormone (thyrotoxicosis) causes a paradoxical reduction of the mitochondrial concentration of UCP. It was concluded that thyroid hormone has a permissive role in the function of BAT and expression of UCP (Sundin 1981; Triandafillou et al. 1982). Fellenz, Triandafillou, Gwilliam and Himms-Hagen (1982) confirmed that a maintenance amount of thyroxine is needed for BAT growth and for increases in cytochrome oxidase activity, protein, GDP binding activity and 32 kDa polypeptide during cold response. Adequate $T_3$ level in BAT is also required for the increases in lipogenic enzymes and α-glycero-phosphate dehydrogenase (Bianco and Silva 1987b).

BAT contains a propylthiouracil-insensitive type II $T_4$ 5'-deiodinase (Leonard, Mellen and Larsen 1982), which converts $T_4$ to $T_3$. This enzyme provides over 50% of the intracellular $T_3$ in BAT and is activated by the adrenergic system via the α-receptors (Silva and Larsen 1983). During cold adaptation, 95% of $T_3$ nuclear receptors are occupied and there is an increase in the conversion of $T_4$ to $T_3$ (Bianco and Silva 1988). The cold-induced activation of 5'-deiodinase is prevented by prazosin (Bianco and Silva 1988). The increase in thyroxine deiodinase activity in BAT in response to cold exposure has been shown to be due to a noradrenaline-induced increase in expression of the gene (Jones, Henschen, Mohell and Nedergaard 1986). This suggests that adrenergic stimulation activates the deiodinase leading to saturation of the nuclear $T_3$ receptors which in turn is required for the realization of the full thermogenic potential.

In the Syrian hamster there is remarkable increase in serum $T_3$ and 5'-deiodinase activity in BAT in response to cold (Kopecky, Sigurdson, Park and Himms-Hagen 1986; Himms-Hagen 1986; Sigurdson and Himms-Hagen 1988) and chronic administration of $T_3$ causes an increase in $O_2$ consumption (Sigurdson and Himms-Hagen 1988). The decline in $T_3$ concentration upon return to a warm temperature also correlates well with the decline in BAT 5'-deiodinase activity. These indicate that $T_3$ exerts a thermogenic effect during cold-acclimation in hamster.

During 'cafeteria' feeding, there is a rise in circulating $T_3$ concentration (Rothwell and Stock 1979a). However the metabolic significance of $T_3$ in DIT remains unknown and it probably exerts only a permissive role (see Himms-Hagen 1983).

The mechanism whereby thyroid hormones influence BAT thermogenesis is not well understood. Thyroid hormones are known to regulate $\beta$-adrenergic receptor number in several tissues including BAT (Williams and Lefkowitz 1977; Seydous, Giacobino and Girardies 1982). Post-receptor intracellular metabolism also appears to be influenced by thyroid status (Seydous et al. 1982). $(Bu)_2$cAMP-stimulated cellular respiration is increased in the $T_3$ treated thyroidectomized ovine foetus (Klein et al. 1984). These suggest thyroid hormones may act via alteration of intracellular post-receptor events (Silva and Larsen 1983; Bianco and Silva 1988).

### 13.2.2. Insulin

In diabetic animals there is atrophy of BAT and there is a failure to exhibit DIT unless insulin is given (Rothwell and Stock 1981b; Seydoux et al. 1984). The re-activation of lipolysis in BAT during refeeding is prevented by inhibition of insulin secretion (Mercer and Williamson 1988). A link between the development of

insulin resistance in BAT and impairment in the thermogenic response to acute cold exposure in ob/ob mouse has been discussed (section 11.2.4.). Geloen and Trayhurn (1989) have demonstrated that insulin increases UCP content in a dose dependent manner in Streptozotocin-induced diabetic mice indicating a direct relationship between insulin and UCP content. These suggest an important role for insulin in the regulation of BAT.

Metabolism of glucose in BAT is extremely sensitive to insulin (Cooney et al. 1985; Ferre, Burnol, Leturque, Terretaz, Penicaud, Jeanrenaud and Girard 1986; Ebner et al. 1987). In the presence of physiological hyperinsulinaemia, BAT glucose utilization can represent 10% of the total glucose turnover rate (Ferre et al. 1986). Glucose utilization by BAT is also suppressed in conditions of decreased insulin sensitivity (Ferre et al. 1986). Moreover, insulin stimulates total glucose metabolism by 5-fold and glucose transport by 8-fold in BAT (Ebner et al. 1987). A meal rich in carbohydrate induces a thermogenic effect (Kanarek and Orthen-Gambill 1982 and Kanarek et al. 1987). Furthermore, insulin has also been implicated in the thermogenic effect of food and catecholamines (Rothwell et al. 1983).

It has been proposed that insulin has two separate roles in the control of BAT thermogenesis: a direct action on the tissue itself to stimulate glucose metabolism and an indirect action by increasing sympathetic drive to the

tissue via the hypothalamus (Rothwell et al. 1983; Rothwell, Stock and Warwick 1985; Mercer and Williamson 1988).

### 13.2.3. Pituitary Hormones

Pituitary hormones are not directly involved in the regulation of BAT. Hypophysectomized rats can respond to mild cold by increasing BAT weight (Goubern et al. 1985), however, they do not adapt in a very cold environment (Fellenz et al. 1982). This effect appears to be secondary to disturbances in the thyroid and adrenocortical hormones (Fellenz et al. 1982). The role of PRL in the suppression of thermogenic function of BAT during lactation is discussed in section 12.

### 13.2.4. Glucocorticoids

Corticosteroids have a profound effect on thermogenesis of BAT (see review York 1989). Chronic treatment with corticosterone stimulates appetite and increases metabolic efficiency (Galpin et al. 1983a), and there are decreases in cytochrome oxidase activity and mitochondrial GDP binding of BAT (Galpin et al. 1983b). Although corticosterone can suppress the stimulation of BAT thermogenesis induced by diet, it does not affect the acute response to cold (Galpin et al. 1983b). A poorer DIT

found in older animals is reversed by adrenalectomy. (Rothwell and Stock 1984a). Adrenalectomy normalizes the cytochrome oxidase and mitochondrial GDP binding in some genetically obese animals (Holt and York 1984; Trayhurn and Mercer 1984). The role of adrenal gland in obese animal has been discussed previously (section 11.2.2.).

The mechanism by which glucocorticoids regulate BAT thermogenesis remains unclear. Scarpace, Baresi and Morley (1988a) reported that glucocorticoids do not decrease $\beta$-adrenergic receptor in adrenalectomized rats but suppress the adrenalectomy-induced increased adenylate cyclase activity. These data suggest that glucocorticoids regulate BAT thermogenesis by modulating the $\beta$-adrenergic pathway at the level of adenylate cyclase activation. However, others have reported that adrenalectomy increases the activity of SNS (Fukushima, Lupien and Bray 1985). Removal of adrenal corticosteroids increases sympathetic activity in animals with ventromedial hypothalamic lesions (Bruce et al. 1982; Debons, Zurek, Tse, Abrahamsen 1986) and in animals with paraventricular hypothalamic lesions (Tokunaga, Fukushima, Lupien, Bray, Kemnitz and Schemmel 1988). Therefore ventromedial and paraventricular nuclei are unlikely to be the site of action of glucocorticoids in controlling energy balance.

On the contrary, lesions in the lateral hypothalamus significantly increase BAT sympathetic activity (Arase,

Sakaguchi and Bray 1987), noradrenaline turnover (Yoshida, Kemnitz and Bray 1983) and GDP-binding activity (Lupien and Bray 1986; Lupien, Tokunaga, Kemnitz, Groos and Bray 1986). Acute subcutaneous injection of the antiglucocorticoid, RU-486, stimulates oxygen consumption and increases GDP binding activity in rats (Hardwick, Linton and Rothwell 1989). However the effect of antiglucocorticoid is abolished by surgical denervation of the sympathetic supply to the tissue. Cerebroventricular injection of antiglucocorticoid also stimulates oxygen consumption (Hardwick et al. 1989). From these studies it is suggested that corticosteroids may act by modulating the central sympathetic outflow probably from the lateral hypothalamus.

### 13.2.5. Corticotropin-Releasing Factor

Corticotropin-releasing factor (CRF) is an hypothalamic neuropeptide and is the primary physiological stimulus for the release of ACTH from the pituitary. There is evidence to show that CRF is also involved in the regulation of thermogenesis in BAT (LeFeuvre et al. 1987; Rothwell 1989). The thermogenic effect of CRF is not dependent on the pituitary. CRF may be responsible for the enhanced thermogenesis that follows adrenalectomy (Holt, Rothwell, Stock and York 1988) and adrenalectomy is associated with an increased turnover of CRF in the paraventricular nucleus (Vale, Rivier, Brown, Spiess,

Koob, Swamson, Bilezikjian, Bloom and Rivier 1983).

Chronic CRF treatment reduces food intake and GDP binding activity in rats (Hardwick et al. 1989). Morley, Levine and Rowland (1983) have demonstrated that injection of CRF into cerebral ventricles causes reduction in food intake. Acute administration of CRF into the third ventricle reduces food intake in normal rats and increases GDP binding of BAT from rats deprived of 21 h for food (Arase, York, Shimizu, Shargill and Bray 1988). Therefore CRF may influence thermogenesis via the action within the central nervous system (Brown, Fisher, Spiess, Rivier, Rivier and Vale 1982).

## 14. Aims of the Study

In the present study, the first goal was to purify and isolate UCP from BAT mitochondria of rats and use this purified UCP to produce antiserum and develop the ELISA method for UCP.

The second part of the study was to examine the changes in thermogenic function of BAT during pregnancy and lactation using the ELISA method. The role of PRL on the changes on thermogenic function during lactation was also investigated in order to provide more understanding of the mechanism of alteration in BAT thermogenic function during lactation.

# CHAPTER II

# ISOLATION AND PURIFICATION OF UCP AND DEVELOPMENT OF AN ENZYME LINKED IMMUNOSORBENT ASSAY FOR UCP

## 1. INTRODUCTION

Measurement of UCP in BAT has been regarded as an important parameter of thermogenesis. One of the main aims of the present study is to develop a specific assay to quantitate the UCP content of BAT. The ELISA technique was selected as the method of choice because of the following advantages: it does not use any radioactivity, the shelf life of reagents is long, the technique can handle a large number of samples, and only a small amount of BAT sample is required for the assay.

In this chapter the development and validation of the following methods are described and discussed : GDP binding study, purification of UCP and development of ELISA.

## 2. MATERIALS AND METHODS

### 2.1. Animals

All the animals used in the experiments were bred in the animal house of the Chinese University of Hong Kong. Sprague-Dawley rats were used in all of the experiments except where specified in the text. Female rats were used at about 6-8 weeks of age (except as specified in the text). Animals were kept in plastic cages (5 rats/cage except where specified in the text) at room temperature of 22±1°C. Tap water and rat chow (Rodent Laboratory Chow 5001, Purina Mills Inc., composition by weight: protein 23%, fat 4.5%, fiber 6.0%, ash 8%, mineral 2.5%) were provided ad libitum.

### 2.2. Collection of BAT

Rats were sacrificed by cervical dislocation and an incision was made on the dorsal aspect to expose the interscapular as well as the cervical BAT. BAT was dissected out and trimmed free from the adherent connective tissue, skeletal muscle and white adipose tissue.

### 2.3. Isolation of Mitochondria

The method used for the isolation of mitochondria was

that described by Cannon and Lindberg (1979) with minor modifications. Ice cold sucrose solution was used throughout the procedure. The composition of the sucrose solution was: 0.25 mol/L sucrose,

5 mmol/L K-TES,

pH was adjusted to 7.2 with 1 mol/L KCl solution

The procedure was as follows.

(a) BAT was minced into fine pieces using a pair of scissors.

(b) About 1 g of BAT was homogenized in 2 ml of sucrose solution by a motor driven teflon-glass homogenizer (Jencons Limited, H104/2/0325).

(c) The homogenate was filtered through 2 layers of gauze.

(d) The homogenate was centrifuged at 8,500 xg (IEC High Speed Centrifuge M25) and 4°C for 10 min.

(e) The lipid layer and supernatant were aspirated.

(f) Any fat adhering to the wall of the test-tube was wiped off with tissue.

(g) The pellet was resuspended and centrifuged at 700 xg Centrifuge CR3000) and 4°C for 10 min.

(h) The supernatant was transferred into a clean tube and pellet was resuspended with sucrose solution.

(i) The supernatant and the suspension of the pellet were centrifuged at 700 xg and 4°C for 10 min.

(j) The supernatant was collected and the pellet containing nuclei and cell debris was discarded.

(k) The supernatant was centrifuged at 8,500 xg and 4°C for 10 min.

(l) The pellet containing the mitochondria was washed with ice cold sucrose solution and centrifuged at 8,500 xg and 4°C for 10 min.

(m) The pellet was stored at -20°C until use.

Liver and skeletal muscle mitochondria were prepared by the same procedure as that for the BAT mitochondria. For the preparation of white adipose tissue mitochondria it was necessary to use a higher centrifugal force, 12,000xg, in steps (d), (k) and (l).

## 2.4. Electron Microscopy (EM) of Isolated BAT Mitochondria

Purity of mitochondria isolated from BAT was studied by EM. The steps involved in the preparation of mitochondria for EM were similar to those used conventionally for other tissues, namely fixing, staining and sectioning except that during fixation centrifugation was used to concentrate the mitochondrial pellet.

(a) The isolated mitochondria were fixed and mixed well in 2% glutaraldehyde in 0.1 mol/L phosphate buffer, (pH 7.2) at 4°C for 2 h.

(b) The mitochondria were pelleted by centrifugation at 8,500 xg at 4°C for 10 min and glutaraldehyde was aspirated.

(c) The pellet was washed and resuspended with ice cold 0.1 mol/L phosphate buffer (pH 7.2).

(d) It was centrifuged at 8,500 xg at 4°C for 10 min.

(e) The pellet was washed again with ice cold 2% w/v sucrose in phosphate buffer pH 7.2.

(f) Steps (d) and (e) were repeated twice.

(g) The pellet was post-fixed in 1% Osmium tetraoxide in 0.1 mol/L phosphate buffer (pH 7.2) for 3 h.

(h) It was rinsed twice for 10 min each time in 2% sucrose solution in phosphate buffer.

(i) The pellet was dehydrated through a graded series of acetone (50%, 70%, 90%, and 100% v/v). At each concentration the pellet was left for 10 min.

(j) The pellet was washed in 1 part 100% acetone and 1 part Epon-Araldite for 1 h.

(k) It was infiltrated with Epon-Araldite for 3 h. For the last 0.5 h the pellet was kept in a vacuum oven at room temperature.

(l) The pellet was embedded in Epon-Araldite containing 2.5% DMP30.

(m) The tissue block was thin-sectioned and the thin section mounted on the grid and observed under an electron microscope.

## 2.5. Measurement of Protein and Cytochrome C Oxidase Activity

### 2.5.1. Measurement of Protein Concentration

The protein concentration was determined by the method of Lowry, Rosebrough, Farr and Randall (1951) as modified for the centrifugal analyser (Cobas Bio, Hoffman-La Roche & Co., Basle). The linear range of the assay was 100-700 mg/L and the samples were diluted with distilled water when necessary. The method is as follows:-

Reagent A : 2 ml of 2 g/100 ml $CuSO_4.5H_2O$

2 ml of 4 g/100 ml sodium potassium tartrate ($5H_2O$)

96 ml of 3 g/100 ml $Na_2CO_3$ in 0.1 mol/L NaOH

Reagent A was freshly made with the above stock solutions before each assay.

Reagent B : Folin and Ciocalteu's phenol reagent (Sigma) was diluted with an equal volume of distilled water before assay.

Protein standard : Protein standards (200, 300, 400 mg/L) were made from bovine serum albumin (Sigma).

The parameters used for setting the Cobas Bio centrifugal analyser are listed as follows.

| | |
|---|---|
| (1) Units | mg/L |
| (2) Calculation factor | 0 |
| (3) Standard 1 conc | 200 |
| (4) Standard 2 conc | 300 |
| (5) Standard 3 conc | 400 |
| (6) Limit | 700 |
| (7) Temperature [deg C] | 25.0 |
| (8) Type of analysis | 6 |
| (9) Wavelength [nm] | 750 |
| (10) Sample volume [$\mu$l] | 10 |
| (11) Diluent volume [$\mu$l] | 20 |
| (12) Reagent volume [$\mu$l] | 300 |
| (13) Incubation time [sec] | 900 |
| (14) Start reagent volume [$\mu$l] | 30 |
| (15) Time of first reading [sec] | 1.0 |
| (16) Time interval [sec] | 180 |
| (17) Number of readings | 16 |
| (18) Blanking mode | 1 |
| (19) Printout mode | 1 |

Ten $\mu$l of sample was incubated with 300 $\mu$l of Reagent A for 15 min at 25°C. It was then mixed with 30 $\mu$l of Reagent B and further incubated for 45 min. Then the absorbance was read at 750 nm (water was used as blank).

In some experiments TritonX-100 was used to solubilize the membrane bound protein. This detergent has been reported to interfere with the Lowry method by forming a yellow precipitate. This was examined by measuring the protein concentration of BSA solutions containing different amounts of TritonX-100 (Table 2.1). TritonX-100 at concentrations as low as 0.025% increased the measured protein values by 23%. In order to avoid this interference, a modified assay as described by Markwell, Haas, Tolbest and Bieber (1981) was used. In this method SDS was included in the alkaline reagent and the concentration of copper tartrate used was higher. Reagent A was prepared by mixing 100 parts of solution containing 2.0% $Na_2CO_3$, 0.4% NaOH, 0.16% sodium tartrate, 1.0% SDS and 1 part of 4% $CuSO_4.5H_2O$ solution. Reagent A was freshly made before use. The parameter setting of the centrifugal analyser was the same as described earlier.

When BSA solution containing different amounts of TritonX-100 was analysed by this method, it was found that when the concentration of TritonX-100 was lower than 5% (Table 2.2.) there was no interference; at higher concentrations of TritonX-100 the apparent protein concentration was higher than expected. Therefore in all experiments described below the modified method was used and the concentration of TritonX-100 in the samples was kept below 5%.

Table 2.1. : Effects of TritonX-100 on protein determination by the method of Lowry *et al*. (1951). BSA solution was mixed with different concentrations of TritonX-100 solution. Values are means of duplicates.

| % of TritonX-100 in sample | Observed value (mg/L) |
|---|---|
| 0.000 | 345 |
| 0.025 | 423 |
| 0.050 | 436 |
| 0.091 | 444 |
| 0.110 | 475 |
| 0.200 | 491 |

Table 2.2 : The effect of addition of 1% SDS in Reagent A (section 2.5.) on determination of protein in samples containing TritonX-100 by the modified Lowry method (Markwell *et al*. 1981). Values are means of duplicates.

| % of TritonX-100 in sample | Observed value (mg/L) |
|---|---|
| 8 | 626 |
| 7 | 576 |
| 6 | 529 |
| 5 | 452 |
| 4 | 445 |
| 3 | 437 |
| 2 | 440 |
| 1 | 442 |
| 0 | 452 |

### 2.5.2. Measurement of Cytochrome C Oxidase Activity

Cytochrome C oxidase (EC 1.9.3.1) activity of BAT mitochondria was determined by the method of Wharton and Tzagoloff (1967). The rate of oxidation of ferrocytochrome C is measured by the rate of decrease in the absorbance of its α-band at 550 nm.

Reagents:

(1) Potassium phosphate buffer, 0.1 mol/L, pH 7.0.

(2) Ferrocytochrome C, 1%. Cytochrome C (Sigma, No. C-2506, Type III: from horse heart) was dissolved in 0.01 mol/L phosphate buffer, pH 7.0. The solution was reduced with a few milligrams of potassium ascorbate. Excess ascorbate was removed by dialysis in 2 L of 0.01 mol/L phosphate buffer, pH 7.0 for 24 h with 3 changes of buffer. The reduced cytochrome C was stored at -20°C.

(3) Enzyme standard, lyophilized bovine heart cytochrome oxidase (EC 1.9.3.1) was purchased from Sigma (No. C-5771). A stock enzyme solution of 7.5 μmol/ml was prepared by dissolving the lyophilized enzyme in 0.1 mol/L potassium phosphate buffer (pH 7.0). The stock solution was stored at -20°C in aliquots and diluted

to 1.25 and 0.83 μmol/ml with phosphate buffer before assay.

Procedure

A centrifugal analyzer (Cobas-Bio) was used to measure the enzyme activity. The parameter settings used were as follows:

| | |
|---|---|
| (1) Units | μmol/ml |
| (2) Calculation factor | 0 |
| (3) Standard 1 conc | 0.0 |
| (4) Standard 2 conc | 1.00 |
| (5) Standard 3 conc | 1.25 |
| (6) Limit | 700 |
| (7) Temperature [deg °C] | 37.0 |
| (8) Type of analysis | 3 |
| (9) Wavelength [nm] | 550 |
| (10) Sample volume [μl] | 10 |
| (11) Diluent volume [μl] | 30 |
| (12) Reagent volume [μl] | 100 |
| (13) Incubation time [sec] | 120 |
| (14) Start reagent volume [μl] | 30 |
| (15) Time of first reading [sec] | 1.0 |
| (16) Time interval [sec] | 10 |
| (17) Number of readings | 30 |
| (18) Blanking mode | 1 |
| (19) Printout mode | 2 |

Ten $\mu$l of sample was incubated with 100 $\mu$l of potassium phosphate buffer at 37°C for 120 sec. 30 $\mu$l of reduced ferrocytochrome C was added and the absorbance at 550 nm was recorded every 10 sec for 30 readings. The rate of enzyme activity was obtained from the linear part of the reaction curve. The activity of cytochrome C oxidase in the sample was obtained by comparison with the reaction rate of the cytochrome C oxidase standard.

## 2.6. GDP Binding Assay of BAT Mitochondria

In 1976 Rafael and Heldt first found that the purine nucleotide binding capacity of brown fat mitochondria from neonatal and developing guinea-pigs varied according to the animals' requirement for thermogenesis control. Thereafter many studies have shown that the nucleotide binding capacity correlates with the nonshivering thermogenic activity. GDP binding capacity measured by Scatchard analysis is shown to vary (Nicholls 1976; Rial and Nicholls 1983) in many situations such as development, cold or warm adaptation, 'cafeteria' feeding, and in some genetically obese animals. GDP binding activity has been regarded as the primary method for measuring thermogenic activity in animals. In the present studies this assay was used to determine the thermogenic capacity in rats and to monitor the purity of UCP during isolation.

The theory of saturable binding was used to calculate the maximum number of GDP binding sites. Saturable binding exists when micromolecules (ligands) interact with macromolecules (receptors), and the relationship can be described by the law of mass action. When the concentration of free macromolecule (M) and of free ligand (F) reaches an equilibrium with the concentration of bound ligand (B), the interaction can be characterized by the dissociation constant (kd):

$$kd = (M \times F) / B \qquad (1)$$

Equation 1 can be rewritten as

$$kd = ((Bmax - B) \times F) / B \qquad (2)$$

where Bmax is the total concentration of macromolecules or the maximum number of binding sites.

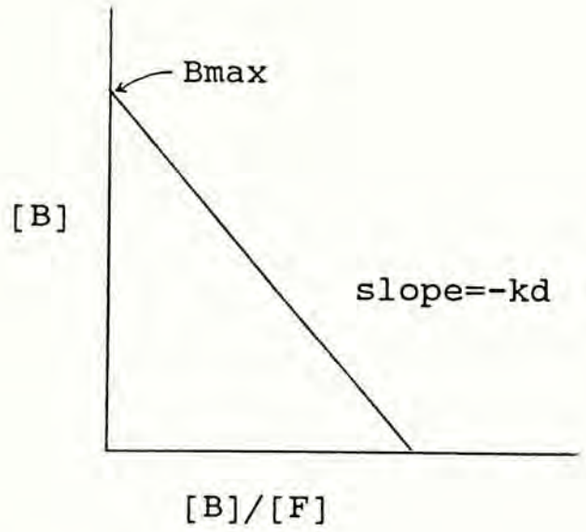
When equation 2 is rearranged:

$$B/F = (1/kd)\ (Bmax - B)$$

$$Bmax - B = B/F \times kd$$

$$B = Bmax - kd\ (B/F) \qquad (3)$$

When B is plotted against B/F as in Equation 3, the intercept on the Y axis gives Bmax, the maximum number of binding sites, and the slope represents -kd (See figure below) (Scatchard 1949). The line of best fit in the Scatchard plot was determined by a linear regression method using the Abstat (Anderson-Bell) program.




### 2.6.1. GDP Binding Assay of Mitochondria by Centrifugation Method

GDP binding assay was used to monitor the purity of UCP during the purification steps. The method used was similar to that described by Desautels, Zaror-Behrens and Himms-Hagen (1978) with minor modifications. Mitochondria were isolated as described in section 2.3.

Incubation medium :

| | mmol/L |
|---|---|
| Sucrose | 100 |
| N-Tris (hydroxymethyl-2-aminoethane sulfonate) | 20 |
| Choline chloride | 10 |
| EDTA disodium salt | 1 |
| Rotenone | 0.005 |
| Potassium atractyloside | 0.1 |

pH was adjusted to 7.1 with KOH

The procedure used was as follows:

(a) All the reagents were diluted from stocks with incubation medium before the GDP binding assay.

(b) Freshly isolated mitochondria (0.5 mg of protein in 100 $\mu$l) were incubated with 50 $\mu$l of 20 pmol of $^{3}$H-GDP (about 450,000 dpm per sample, specific activity 10.8 Curies/mmol, Amersham) and with different amounts of GDP (0 to 2000 pmole in 100 $\mu$l, Sigma).

(c) For non specific binding, 200 nmole of GDP was added in addition to the $^{3}$H-GDP.

(d) $^{14}$C-sucrose (about 50,000 dpm in 50 $\mu$l, Amersham) was added to correct for trapping of free $^{3}$H-GDP in the pellet.

(e) The total volume of the incubation mixture was 0.3 ml and the mixture was incubated at room temperature (22°C).

(f) After 15 min of incubation, 0.2 ml of cold incubation medium was added and the mixture was centrifuged at 8,500 xg in an Eppendorf centrifuge (Model 5413) for 5 min.

(g) The supernatant was aspirated.

(h) The tissue pellet was dissolved in 0.2 ml of tissue solubilizer (quarternary ammonium hydroxide, Beckman 450) by incubating at 55°C for 45 min.

(i) The tissue suspension was vortexed well and an aliquot (0.15 ml) was transferred into 2 ml of scintillation cocktail (0.7% PPO in toluene).

(j) The sample was counted in a Beckman scintillation counter (LS 9800) for 5 min using dual label channels. The channel settings for $^{3}H$ and $^{14}C$ were 0-400 and 400-670 respectively. The quenching effect from the background in each sample which causes a shift of the energy spectrum of radiation was also considered and measured by H number (by counting the energy shift of known external standard caused by the quenching effect of the sample background). The automatic quench compensation program would use the H number to reset the counting windows so as to adjust the shift of the energy end point and obtain an optimum value for each sample.

(k) The amount of specific GDP binding was calculated as the amount of $^{3}H$-GDP bound to the tissue after correcting for trapped $^{3}H$-GDP (included in the buffer) and non-specific binding. The amount of unbound $^{3}H$-GDP trapped in the pellet was calculated by counting the $^{14}C$-sucrose which was an extra-

mitochondrial marker in the pellet.

### 2.6.2. GDP Binding Activity by Equilibrium Dialysis

The conventional method of GDP binding described in section 2.6.1. can be used only for large particles, such as mitochondria, which can be pelleted by high speed centrifugation, thus separating the free and bound $^{3}$H-GDP. During purification, UCP was separated from the mitochondrial membrane by dissolving the latter in a detergent. Therefore equilibrium dialysis was used for the GDP binding study. In this method UCP was retained on one side of a dialysis membrane and GDP solution was placed on the other side. The unbound GDP could pass freely through the membrane to another side because of its low MW. Once GDP bound to UCP it would remain on one side. At equilibrium, the concentration of $^{3}$H-GDP inside the tubing denotes that the concentration of bound plus free $^{3}$H-GDP and that in the bath represents the concentration of free $^{3}$H-GDP. Hence the bound $^{3}$H-GDP could be calculated.

This method was used to assay GDP binding activity of UCP during different stages of purification. The dialysis tubing employed was Spectrapor No.4, tubing size 10 mm, MW cutoff 12-14 kDa. One ml of sample from different stages of UCP purification, that is, the mitochondria (protein concentration 5 g/L), the supernatant after extraction with TritonX-100 (5.5 g/L) and purified UCP (1 g/L) were

transferred into the pre-wet tubing which was then sealed with a plastic clip. Two dialysis tubings were prepared for each sample, one for specific binding and the other for non-specific binding. The dialysis bath contained 0.256 nmol/L of $^{3}$H-GDP (5,800,000 dpm) in 20 ml of standard buffer. The dialysis bath for non-specific binding consisted of cold GDP (21.5 $\mu$mol/L) in addition to the same concentration of $^{3}$H-GDP. All the tubings for specific binding were placed in the same dialysis bath and all the tubings for non-specific binding were placed in another bath. The equilibrium dialysis was carried out at 4°C and the bath was constantly mixed with a magnetic stirrer. During dialysis aliquots (50 $\mu$l) were taken from the bath and from the mixture inside the tubing. The radioactivity in these aliquots was measured after dissolving them in 200 $\mu$l of Beckman tissue solubilizer, digesting the mixture for 1 h at 55°C, and then adding 6 ml of scintillation cocktail. Equilibrium was achieved when the radioactivity of the mixture inside the tubing increased and reached a plateau: this occurred after 5 days of incubation. The specific binding was obtained by subtraction of the non-specific binding from the total binding.

### 2.6.3. GDP Binding by Microfiltration Method

The method described in section 2.6.2. for GDP binding of BAT mitochondria requires a large amount of UCP and is too time consuming. Therefore a microfiltration method for GDP binding was used to determine the Bmax and the kd of GDP binding to purified UCP.

The basic principle of microfiltration is the use of a filter membrane to retain the protein on one side whilst the free ligand, which is smaller than the pore size, is freely filtered. A centrifugal force is applied to enhance the filtration rate. After about 30 min of centrifugation the free ligand can be separated from the bound ligand.

Amicon Centricon Disposable Microconcentrator (Amicon Cat. No. 4201) with filter membrane Amicon Centricon-10 (cutoff MW 10 kDa) was used to retain GDP bound to UCP. The total volume of incubation medium was 300 $\mu$l containing $^{3}$H-GDP (1,180,000 dpm), $^{14}$C-sucrose (43,000 dpm), 110 $\mu$g of UCP and different concentrations of cold GDP (from 0 to 20 nmol, 0.01 mmol for non-specific binding). $^{14}$C-sucrose is also filtered freely through the filter membrane therefore its concentration in unfiltered fraction and in filtrate should be similar. After incubation at room temperature for 15 min the incubation medium was transferred to a microconcentrator and

centrifugated at 3,000 rpm at room temperature for 30 min. The volume of the unfiltered fraction and the filtrate were measured by weight. A 30 μl aliquot of the filtrate or unfiltered fraction was mixed with 200 μl of Beckman tissue solubilizer and with 6 ml of scintillation cocktail and counted in a dual channel scintillation counter. The specific $^{3}$H-GDP binding was obtained by subtracting the value of $^{3}$H-GDP in unfiltered fraction from the value of free $^{3}$H-GDP trapped.

## 2.7. Experiments Designed for Validation of GDP Binding Assay

In order to validate the GDP binding assay in the present study several experiments were designed to study the changes in GDP binding activity in BAT under different conditions.

### 2.7.1. GDP Binding Activity in BAT Mitochondria after Noradrenaline Treatment

Seven week old rats were kept at room temperature and randomly divided into 2 groups of 4 rats each. One group was injected intraperitoneally with 25 μg/100 g BW of noradrenaline (Levophed 1 mg/ml, Winthrop, UK). Noradrenaline was diluted to 0.1 mg/ml with 0.9% NaCl solution before injection. The control group was injected with the same volume of vehicle. One h after injection the

animals were killed by cervical dislocation, BAT was removed and weighed. BAT from each group was pooled and mitochondria isolated as described in section 2.3. The maximum number of GDP binding sites and the kd were determined by the method described in section 2.6.1.

### 2.7.2. GDP Binding Activity in BAT Mitochondria after Cold Acclimation and Noradrenaline Treatment

Six week old rats weighing from 200 to 250 g were kept at 4°C in a cold room for 10 days. Rat chow and water were provided ad libitum. After 10 days, the rats were divided into 2 groups of 4 rats each. One group of rats was injected i.p. with 25 μg/100 g BW of noradrenaline (Levophed 1 mg/ml, Winthrop, UK). The control group was injected with vehicle. A third group of rats (n=4) was kept at room temperature (22±1°C) and received i.p. injection of vehicle. One h after injection, all the rats were killed by cervical dislocation. Then BAT was dissected out, mitochondria isolated and GDP binding determined as described respectively in sections 2.3. and 2.6.1.

### 2.7.3. Effect of Food Restriction on Cold Acclimated Rats

Four week old rats were divided into 3 groups of 4 rats each. All rats were kept individually in cages. Two groups of rats were cold acclimated and kept at 4°C. One

group of cold acclimated rats (Group 2) was provided with rat chow and water ad libitum. Another group (Group 1) was kept at room temperature (22±2°C) with free access to chow and water. The food consumption of this group was recorded daily and the mean daily chow consumption was calculated. This amount was given to a second group (Group 3) of cold acclimated rats on the following day. At the end of the 5 day experimental period the rats were killed by cervical dislocation. The body weight was recorded and the BAT removed. Mitochondria were isolated and GDP binding determined as described in sections 2.3. and 2.6.1.

### 2.7.4. GDP Binding Activity of BAT Mitochondria of Rats of Different Ages

GDP binding activity of BAT mitochondria in 4 and 11 week old rats was studied. The animals were sacrificed and the BAT was analysed as described in sections 2.3. and 2.6.1.

## 2.8. Isolation and Purification of UCP

UCP was isolated and purified by the method of Lin and Klingenberg (1980 and 1982) with some modifications. The isolation and purification of UCP involved differential detergent extraction of the mitochondria, followed by

affinity column chromatography on hydroxylapatite, and finally ultrafiltration to concentrate the protein. The extremely hydrophobic nature of this inner membrane protein renders it soluble in the neutral detergent TritonX-100, but insoluble in Lubrol PX, a detergent used to remove the matrix proteins. The temperature stability of UCP makes it possible to produce a high yield of high-purity UCP by these relatively simple procedures and allows hydroxylapatite chromatography at room temperature to denature and remove essentially all the other TritonX-100 soluble proteins. Lin and Klingenberg (1980) employed sucrose density gradient centrifugation in the final stages of the preparation, principally to reduce the content of free TritonX-100. The method of sucrose density gradient was tried several times, and it was found that the free TritonX-100 was not completely removed by this method. Moreover, this method is time consuming and the success depends greatly upon very careful manipulation and ultracentrifugation. Therefore a simpler method, using Bio-Beads (Bio-Rad SM-2), was included to remove free TritonX-100.

The procedure, which is basically similar to that of Lin and Klingenberg (1980 and 1982), is described below. The standard buffer was used throughout the experiment.

Standard buffer : EDTA 1 mmol/L
$Na_2SO_4$ 20 mmol/L
MOPS 20 mmol/L
pH 6.7

(1) About 20 mg of mitochondrial protein isolated (as described in section 2.3.) from 8 g of BAT taken from 20 cold acclimated rats was dissolved in 3.2% w/v Lubrol PX (Sigma) in standard buffer. It was stirred continuously at 4°C for 1 h with a magnetic stirrer.

(2) The mixture was centrifuged at 110,000 xg for 15 min at 4°C and the supernatant was discarded.

(3) The pellet was washed with sucrose solution (sucrose 0.3 mol/L, Tris 10 mmol/L, EDTA 2 mmol/L and pH 7.2) to remove the remaining Lubrol PX. Then it was centrifuged again as in step (2).

(4) The pellet was dissolved in TritonX-100 (Sigma). The ratio of protein to TritonX-100 was 5:7 by weight. The mixture was further stirred for 1 h at 4°C.

(5) It was centrifuged at 110,000 xg at 4°C for 15 min. The supernatant was transferred into a hydroxylapatite column (Bio-Rad Cat. No. 130-0420). The column, 18 cm in length and 2 cm in diameter, was prewashed with standard buffer before application of the supernatant.

(6) The UCP was eluted by standard buffer using a peristaltic pump (Pharmacia P-1).

(7) The fractions were collected (50 x 3 ml) using a

fraction collector (Pharmacia Frac-100) and the protein peak was monitored by measuring absorbance at 280 nm using an optical unit (Pharmacia Single Path Monitor UV-1).

(8) The fractions which contained the protein peak were pooled (about 25 ml).

(9) The pooled protein solution was treated with Bio-Beads at 4°C for 1 h with constant and gentle stirring to remove unbound TritonX-100, and was centrifuged at 3,000 rpm for 5 min to separate the Bio-Beads and protein solution.

(10) The protein solution was then concentrated by pressure ultrafiltration in an Amicon Ultrafiltration Cell (Amicon, Mass. Model 8200) at 50 lb/sq.in. using a 62 mm Diaflo ultrafiltration membrane (Amicon YM 10, cutoff MW 10 kDa), which had been previously washed in double distilled water.

(11) The final volume of protein solution was about 1.5 ml and the quantity of protein was assayed by the modified method of Markwell et al. (1981).

## 2.9. Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The purity of the isolated UCP was monitored by SDS-PAGE. A vertical slab gel method was used with a neutral pH discontinuous buffer SDS-PAGE consisting of stacking gel and resolving gel with 2.5% and 7.5% of acrylamide gel concentrations respectively. The method employed is described below.

Preparation of reagents:

(1) Acrylamide-bisacrylamide (30:0.8); 30 g of acrylamide and 0.8 g of bisacrylamide were dissolved in a total volume of 100 ml. The solution was filtered through Whatman No. 1 filter paper and stored at 4°C in a dark bottle.

(2) N,N,N',N'-tetramethylethylenediamine (TEMED, Sigma); this was used as supplied.

(3) Ammonium persulphate (1.5%); was freshly prepared before use by dissolving 0.15 g of ammonium persulphate in 10 ml of water.

(4) Stacking gel buffer stock (0.5 M Tris); 6.0 g Tris-base was dissolved in 40 ml water and the pH was adjusted to

6.8 with 1 mol/L HCl, and the final volume was made up to 100 ml. It was filtered through Whatman No. 1 filter paper.

(5) Resolving gel buffer stock (3.0 M Tris, pH 8.8); 36.3 g Tris was dissolved in 50 ml water, pH was adjusted to 8.8 with 1 mol/L HCl, and the final volume brought to 100 ml with water.

(6) Reservoir buffer stock (0.25 mol/L Tris, 1.92 mol/L glycine, 1% w/v SDS, pH 8.3); 30.3 g Tris, 144 g glycine and 10 g of SDS were dissolved in 900 ml water, pH was adjusted to 8.3, and the final volume brought to 1000 ml.

The recipe for preparation of one gel (16X14 $cm^2$ with 1.5 mm thickness) is as follows.

| | Stacking gel (ml) | Resolving gel (ml) |
|---|---|---|
| Acrylamide-Bisacrylamide | 2.5 | 7.5 |
| Resolving gel buffer | - | 3.75 |
| Stacking gel buffer | 5.0 | - |
| 10% SDS | 0.2 | 0.3 |
| 1.5% Ammonium persulphate | 1.0 | 1.5 |
| Water | 11.3 | 16.95 |
| TEMED | 0.015 | 0.015 |

Gel mixture preparation:

The resolving gel solution was prepared by mixing the acrylamide, gel buffer, SDS solution and water in an Erlenmeyer flask. It was mixed well and degassed for an hour. Ammonium persulphate and TEMED were added. It was mixed well and immediately poured into a preset casting stand (LKB 2001, vertical electrophoresis unit) to a level of 3 cm below the top. Butanol saturated water was overlaid to seal the acrylamide from contact with the air and was allowed to form a smooth surface on the gel top. The gel was allowed to polymerize for 1 h without disturbance. The unpolymerized acrylamide was washed off with distilled water. The water was then drained out by placing the gel upside down.

The stacking gel was prepared as in the recipe using a method similar to that for the preparation of resolving gel. Just after transferring the stacking gel into the casting stand, a 10 wells comb was inserted into the gel. The gel was stored at 4°C until use, which was usually less than a week.

Preparation and application of sample:

The BAT mitochondrial sample, constituting 50 $\mu$l of protein solution at concentration of 2g/L, was mixed with 20 $\mu$l 2-mercaptoethanol (Sigma), and 20 $\mu$l of 10% SDS

solution. The sample was not heat treated as in the conventional method because white precipitate was formed during heat treatment. Next 20 μl of bromophenol blue (Merck, 0.05% in 10% glycerol solution) was added as a tracer and mixed well. The wells were filled up with diluted reservoir buffer solution, and then the samples were applied. Using a microliter syringe, the required volume of each sample was added as closely as possible to the bottom of the well.

Molecular weight protein standard:

Protein standard was used as a molecular weight reference in the electrophoresis. This was obtained from a commercial supplier (Serva, Protein Test Mixture 4, containing carbonic anhydrase MW 29 kDa, egg albumin MW 45 kDa, bovine albumin MW 68 kDa and phosphorylase B MW 92.5 kDa). The standards were reconstituted with 1 ml of water and stored at -70°C in aliquots until use. The standard containing 1 μg of protein was mixed with 10 μl of 10% SDS solution, 10 μl of mercaptoethanol, and 10 μl of bromophenol blue. The mixture was then applied to the gel and used as reference in every run.

Running conditions of SDS-PAGE:

The LKB Vertical Electrophoresis Unit (LKB 2201) was used for electrophoresis. The reservoir buffer solution

was used in both the upper and lower buffer chambers. The power was provided by a LKB 2002 power supply unit. A constant current of 30 mA was applied before the buffer front arrived at the resolving gel when it was set at 40 mA. When the buffer front reached 1 cm from the gel end electrophoresis was terminated. During the electrophoresis the buffer in the lower buffer tank was kept cold by a cooling system set at 4°C (MultiTemp Thermostatic Circulator, LKB 2209).

Fixing, staining and destaining of gel:

Fixing and staining of the gel was performed in 0.1% (w/v) Coomassie blue (PAGE blue G-90 'Electran', BDH) in 30% methanol and 10% acetic acid (by volume) in distilled water for 1 h. The gel was destained with several changes of 30% methanol and 10% acetic acid in distilled water, until a clear background was obtained.

Drying gel:

The gel was dried for a permanent record in a sandwich of cellophane dialysis membranes (LKB 2003-902) by a gel dryer (LKB 2003 Slab Gel Dryer).

## 2.10. Methods for Raising Anti-Rat-UCP Antibody and the Characterization of Antiserum

### 2.10.1. Raising Rabbit Anti-Rat-UCP Antibody

Four rabbits were used for raising anti-rat-UCP antibody. The fur on the back of the rabbits was shaved off. Blood was taken from the ear veins before immunization to be used as normal rabbit serum. Purified UCP (200 μg) was mixed thoroughly with 2 ml Freund's complete adjuvant (Sigma), and injected intradermally into each rabbit at 10 different sites. The immunization was enhanced by repeated booster injections with 100 μg of UCP in 2 ml of Freund's incomplete adjuvant (Sigma) every two weeks for 3 months. Blood samples were obtained via ear veins and allowed to clot overnight at 4°C. Serum was collected and stored at -20°C until use.

### 2.10.2. Western Blot Analysis For Cross-Reactivity Study

A western blot allows the transfer of proteins from gel to a blotting filter on which they can be detected. Transfer can be done quickly and efficiently by electroblotting. In the present study the LKB 2005 Transphor Electroblotting Unit was used. SDS-PAGE was done as described in section 2.9. The transfer buffer and running conditions for the electroblotting were similar to

those described in the manual for the electroblotting unit, and are outlined below.

Transfer buffer: 25 mmol/L sodium phosphate

7.01 g $Na_2HPO_4.2H_2O$

13.4 g $NaH_2PO_4.2H_2O$

made up to 5 L with distilled water

adjusted pH to 6.5

Running conditions: cooling temperature 4°C

current up to 1.2 A

voltage 100%

experimental time 1.5 h

Nitrocellulose paper (Schleicher and Schuell membranfilter, BA 85) was used as the blotting filter. It was mounted on the gel and they were sandwiched between two layers of Whatman No.1 filter paper. A diagram of the arrangement of the nitrocellulose paper and the gel is shown in Fig 2.1. Electrophoresis was carried out as described above under 'running conditions'.

### 2.10.3. Immuno-Autoradiographic Method for Detection of Specificity of Rabbit Anti-Rat UCP Antiserum

After the proteins are transferred onto the nitrocellulose paper, the desired protein can be detected immunologically. The specific binding of antibody and

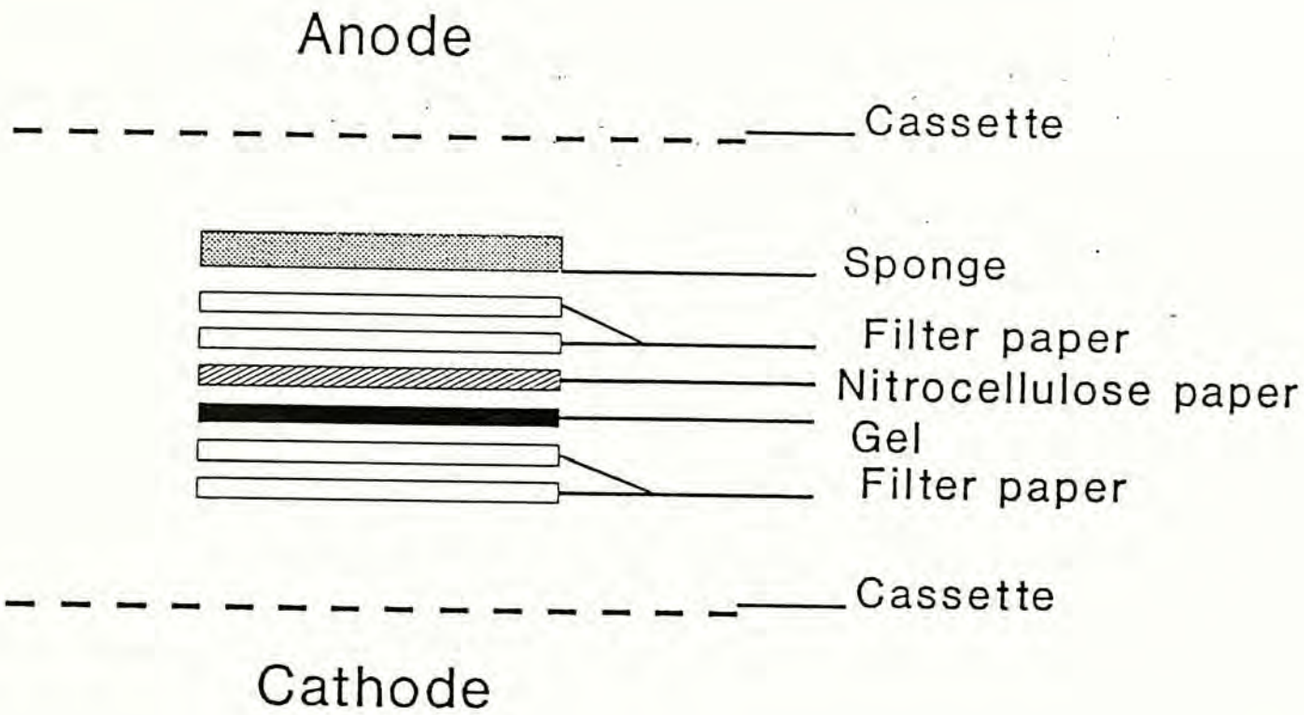



Fig. 2.1 Schematic diagram of the electroblotting technique. The gel and filter papers were sandwiched between two cassettes. Running conditions were as descibed in the text, section 2.10.2.

antigen can be qualitatively or quantitatively assayed by the binding of a secondary antibody, or by a specific binding protein, which is commonly labelled with $^{125}I$ or linked to an enzyme. In the present study Protein A labelled with $^{125}I$, which can specifically bind to immunoglobulin, was used to detect the binding of antibody to antigen. The binding was visualized by autoradiography.

To study the cross-reactivity of the antiserum, mitochondrial proteins of BAT, liver, skeletal muscle, white adipose tissue, and the mitochondrial proteins of BAT obtained from different rodent species were tested. After electrophoresis proteins were electroblotted onto nitrocellulose paper and the paper was washed with 5% w/v non-fat dried milk (Carnation Company) in PBS with 0.1% v/v Tween 20 (Sigma) for 2.5 h at 37°C, with constant shaking, to block the non-specific binding. The nitrocellulose paper was incubated with 10 ml of diluted antiserum (1:200 dilution in 1% w/v BSA in PBS). Another nitrocellulose paper, which contained the same mitochondrial proteins, was incubated with normal rabbit serum (1:200 dilution in 1% w/v BSA) as a control. The paper was washed 7 times with 0.1% v/v Tween-20 in PBS (PBS-Tween). It was then incubated with 1 $\mu$Ci $^{125}I$-labelled Protein A (Amersham) in 15 ml of solution (10 mmol/L $MgCl_2$, 1 mmol/L 2-mercaptoethanol in PBS) for 3 h at 37°C with constant shaking. Then the paper was washed

with 7 changes of PBS-Tween to remove the unbound $^{125}I$-labelled Protein A. The paper was mounted together with a film (Kodak Diagnostic Film X-OMAT, Cat. 165 1454) in a light-tight exposure cassette with two intensifying screens. The film was exposed at -70°C for several h (24-72 h) until a clear visible image formed on the film. The film was developed by the usual developing method.

The species specificity of the antiserum was also examined. BAT was collected from several species including guinea pigs, hamsters and mice. The cross-reactivity of the antiserum was carried out as described in previous paragraph.

**2.11. Enzyme Linked Immunosorbent Assay For UCP**

There are many methods to assay a specific protein by immuno-reaction. The most classic and common methods are radioimmunoassay or radioimmunometric assay in which a radioisotope is used to label the antigen or antibody. However there are several disadvantages in using radioisotopes. These include: limited shelf life, radiation hazard and the problem of disposal of radioactive waste. Recently, use of nonisotopic labels has become increasingly common in both research and clinical laboratories. The sensitivity of these assays is similar to that of classical radioimmunoassay and the immunochemical mechanisms and principles are also similar:

the only difference is the label. The labels most commonly used are enzymes and fluorescent substances which can be covalently bound to antibodies or ligand. The binding can be detected by enzymatic or chemical reaction via the formation of chromogen or a fluorescent product.

In the present study a heterogeneous enzyme immunoassay technique - ELISA was used. One of the reaction components is nonspecifically adsorbed to a solid phase, such as a microtiter plate. The binding of one of the reactants to a solid phase facilitates separation of bound and free fractions. In the present case a competitive ELISA was used. UCP was pre-coated on the microtiter plate and allowed to react with antibody, and at the same time either UCP standards or the BAT samples with unknown UCP concentration were added. The added UCP can displace the binding of antibody to the UCP coated on the plate so that with increasing UCP in the sample, binding of antibody to the UCP coated on the solid phase decreases: the binding between antibody and the UCP coated on the plate is inversely proportional to the concentration of the UCP in the sample. The unbound antibody is washed away. Protein A which is conjugated to $\beta$-galactosidase, binds specifically to immmoglobulin, was used to detect the antibody. The enzyme catalyzes the substrate, ortho-nitrophenyl-$\beta$-D-galacto-pyranoside to form a coloured compound which can be measured by spectrophotometry. The details of the assay are described

below.

Solutions for the ELISA :

(a) Phosphate buffered saline (PBS) : 0.8% NaCl, 0.02% KCl, 0.102% $Na_2HPO_4$ and 0.02% $KH_2PO_4$ were dissolved in distilled water and pH was adjusted to 7.2. PBS mixed with 0.1% v/v Tween 20 (Sigma) was used as washing solution.

(b) The conjugate of Protein A linked with $\beta$-galactosidase obtained from Amersham (UK) was diluted 500 times with PBS containing 0.1% v/v Tween 20, 10 mmol/L $MgCl_2$, 0.1 mmol/L 2-mercaptoethanol and 1% w/v bovine serum albumin.

(c) Substrate solution : a stock substrate solution (30 mmol/L ortho-nitrophenyl-$\beta$-D-galactopyranoside (ONPG), purchased from Amersham) was prepared by dissolving 9 mg/ml in PBS and heating the mixture to 40-50°C. The stock solution was stored at -20°C. When required it was diluted 10-fold to 3 mmol/L with PBS containing 10 mmol/L $MgCl_2$ and 1 mmol/L 2-mercaptoethanol.

(d) Standard : purified UCP was diluted in PBS to give a range of standards from 1 to 500 ng in a volume of 25 $\mu$l.

Treatment of Samples for the ELISA Assay :

200 μl of BAT homogenate was mixed with 400 μl of sucrose solution (used in the isolation of mitochondria, section 2.3.), and the mixture was centrifuged for 5 minutes at 8,500 xg. The supernatant was discarded and 50 μl of 10% TritonX-100 was added to solubilize the pellet. The sample was vortexed for 2 minutes and allowed to stand at room temperature for 1 h. Then 900 μl of potassium phosphate buffer (0.1 mmol/L, pH 7.2) was added to the sample and was further diluted 5 or 10 times with PBS for ELISA.

Procedure :

(a) Coating of the microtiter plate: purified UCP solution was diluted with PBS to give a concentration of 1 μg in 50 μl. One μg of UCP was added to the wells of the microtiter plate (Dynatech Laboratories Inc., Immunlon-2) and incubated overnight at 4°C.

(b) The plate was washed 5 times with washing solution using an automated microtiter plate washer (Dynatech Ultrawash II) to remove the unbound UCP.

(c) Non-specific binding sites on the plate were blocked by adding 100 μl of 1% w/v bovine serum albumin solution in PBS and incubated at room temperature for

1 h. Then the BSA was removed from the wells.

(d) BAT samples containing UCP (25 µl) were preincubated with 25 µl of a 1 in 500 dilution of rabbit anti-UCP antiserum at room temperature for 1 h. The mixture was then transferred into the wells of the microtiter plate and incubated at 37°C for 2 h with constant rotatary shaking.

(e) After incubation, the plate was washed 5 times with the washing solution.

(f) 50 µl of diluted enzyme labelled protein A was added to each well and incubated at 37°C for 3 h with constant shaking.

(g) The plate was washed with washing solution.

(h) 100 µl of substrate (3 mmol/L of ONPG) was added and incubated at 37°C with constant shaking for 1 h. The length of incubation was sufficient to give a maximum absorbance of about 0.8 in the lowest standard.

(i) The reaction was stopped by the addition of 50 µl of 1 mol/L $Na_2CO_3$.

(j) The absorbance was read at 410 nm by a microtiter plate reader (Dynatech Laboratories, Inc. MR 600).

## 2.12. Experiment Designed to Validate the ELISA

To evaluate the newly developed ELISA, an experiment was designed to measure UCP content during cold adaptation.

Four groups of female rats (7-8 weeks old) were kept in plastic cages (5 per cage) at 4°C. Food and water were provided ad libitum. They were sacrificed by cervical dislocation after 0, 1, 4 or 6 days of cold acclimation, and the interscapular and cervical BAT were removed. BAT was homogenized and stored for ELISA of UCP as described in section 2.11.

## 2.13. Statistical Analysis

The results were expressed as means ± SEM. The statistical significance of differences between groups was assessed by analysis of variance (ANOVA) and Duncan's multiple-range test. When only two groups were compared, the Student's t test was used to calculate the statistical significance.

## 3. RESULTS

### 3.1. EM of Isolated BAT Mitochondria

An electron micrograph of isolated mitochondria is shown in Fig. 2.2. The micrograph shows that the preparation consisted mainly of mitochondria and that most of them were intact, thus indicating the reliability of the present method of isolation.

### 3.2. GDP Binding Assay of BAT Mitochondria

A study was performed to find out the optimum time for the GDP binding assay. Mitochondrial protein (0.5 mg) was incubated with $^{3}$H-GDP (450,000 dpm) and the binding was stopped at time intervals of 2, 4, 15 and 30 min by the addition of cold incubation medium. The results are shown in Fig. 2.3. There was a gradual increase in GDP binding with increasing time of incubation which reached a plateau after 15 min. This was selected as the time of incubation in subsequent assays.

The GDP binding assay of mitochondria from rats kept at room temperature was performed by the centrifugation method and Bmax and kd were obtained by a Scatchard plot.

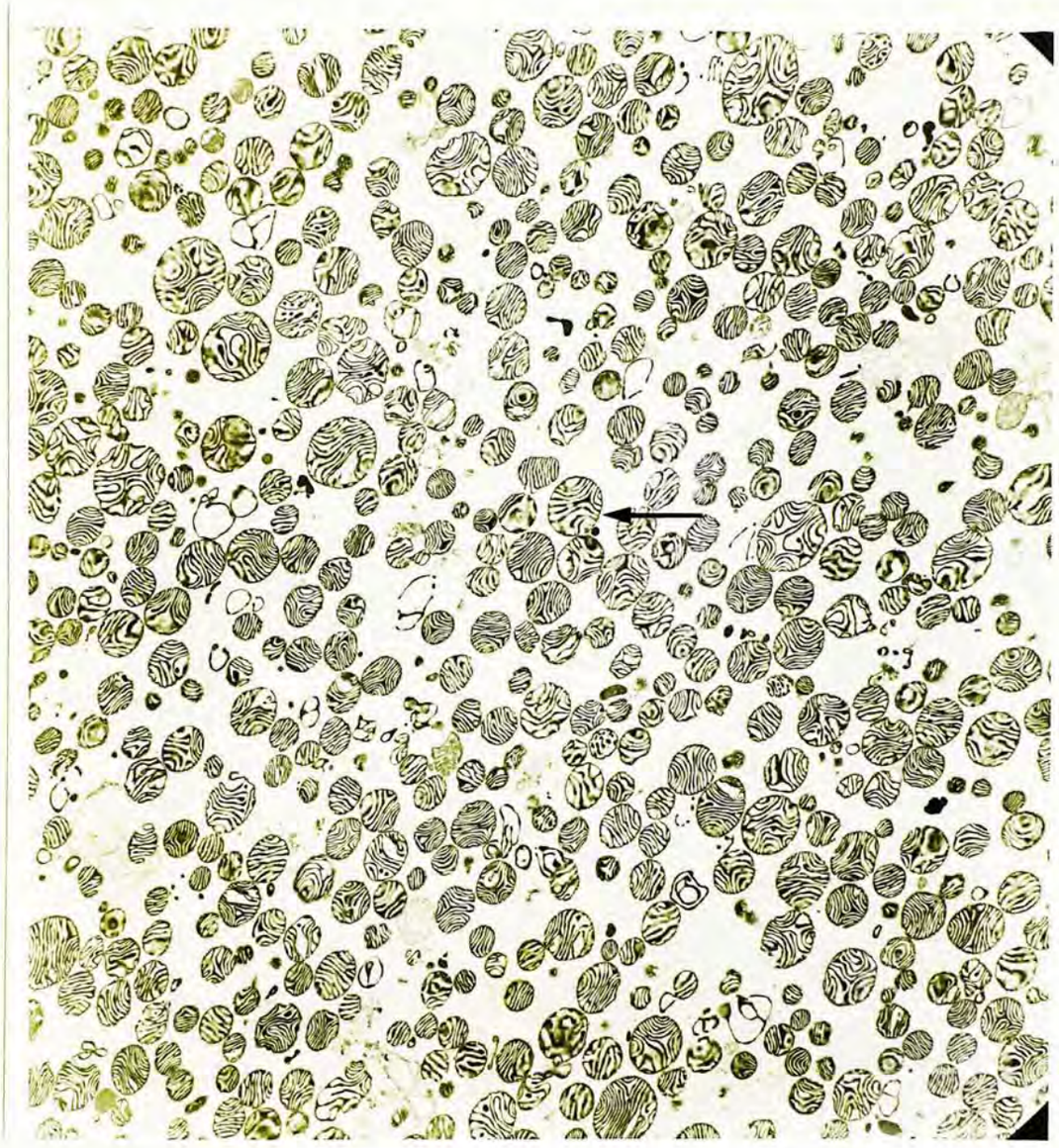


Fig. 2.2 Electron micrograph of mitochondria isolated from BAT (magnification X3650). Arrow indicates a mitochondrion. The method is described in section 2.4.

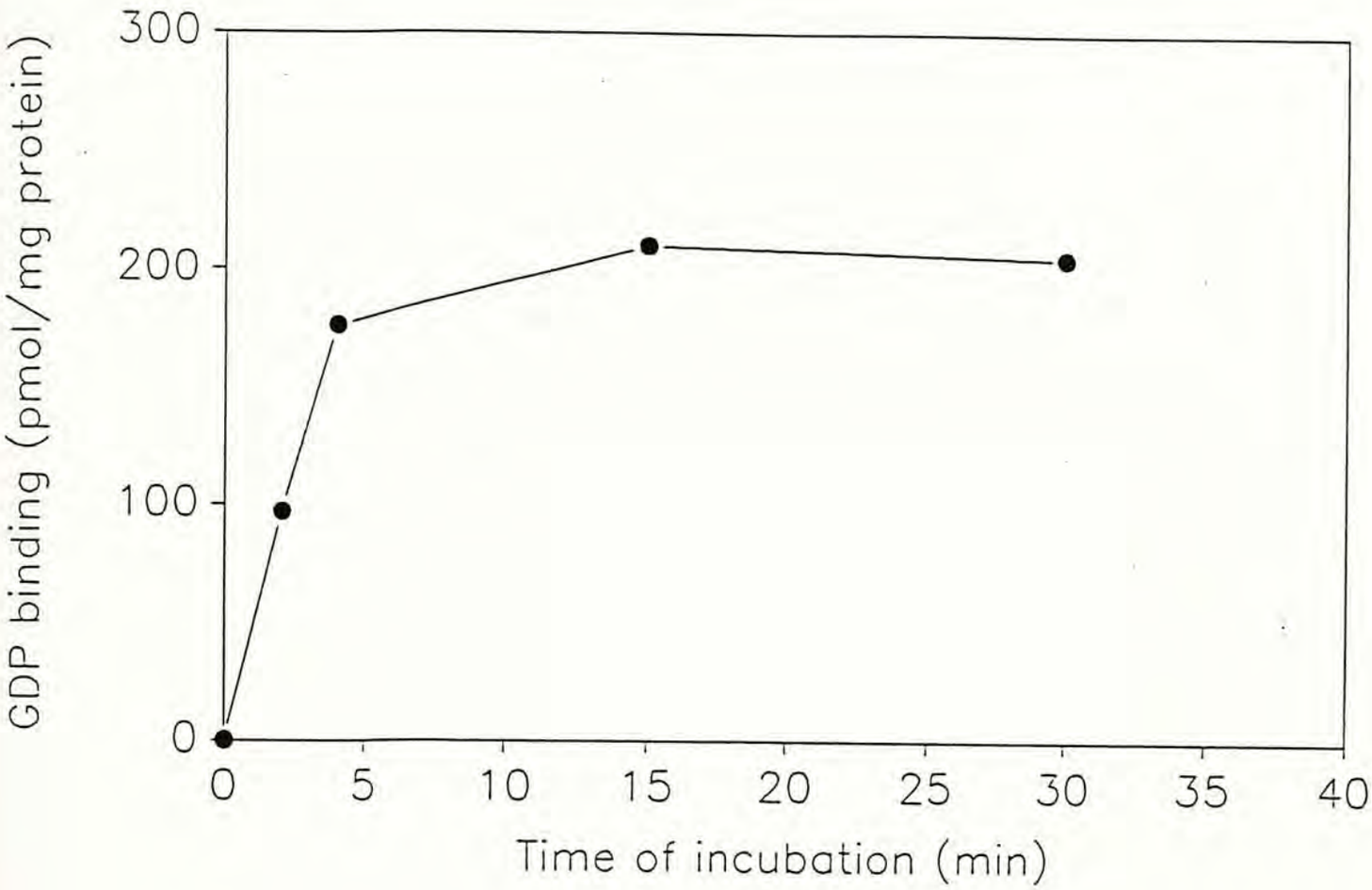



Fig. 2.3 The effect of incubation time on the binding of GDP to mitochondria. Mitochondria were incubated with a mixture of cold and labelled GDP for different times. The reaction was stopped by addition of cold incubation buffer and the mixture was centrifuged for 5 min at 8,500 xg.

When the concentration of GDP bound was plotted against the ratio of concentration of bound and free in a Scatchard plot (Fig. 2.4), there was a linear relationship between bound and bound/free. The maximum number of GDP binding sites, and the dissociation constant (kd) were 330 pmol/mg protein and 2.20 μmol/L respectively.

## 3.3. Experiments Designed for Validation of GDP Binding Assay

### 3.3.1. GDP Binding Activity of BAT Mitochondria after Noradrenaline Injection

The effect of noradrenaline treatment in GDP binding activity was studied. One h after noradrenaline injection the animals were killed and GDP binding was determined. The Scatchard analysis is shown in Fig. 2.5. There was a significant increase of about 51% in Bmax of GDP binding from 286.8 (control group) to 432.6 pmol/mg protein after noradrenaline treatment. The slopes of the lines from these two groups in the Scatchard plot were almost parallel to each other indicating that there was no change in kd (2.06 and 1.95 μmol/L in control and noradrenaline groups respectively).

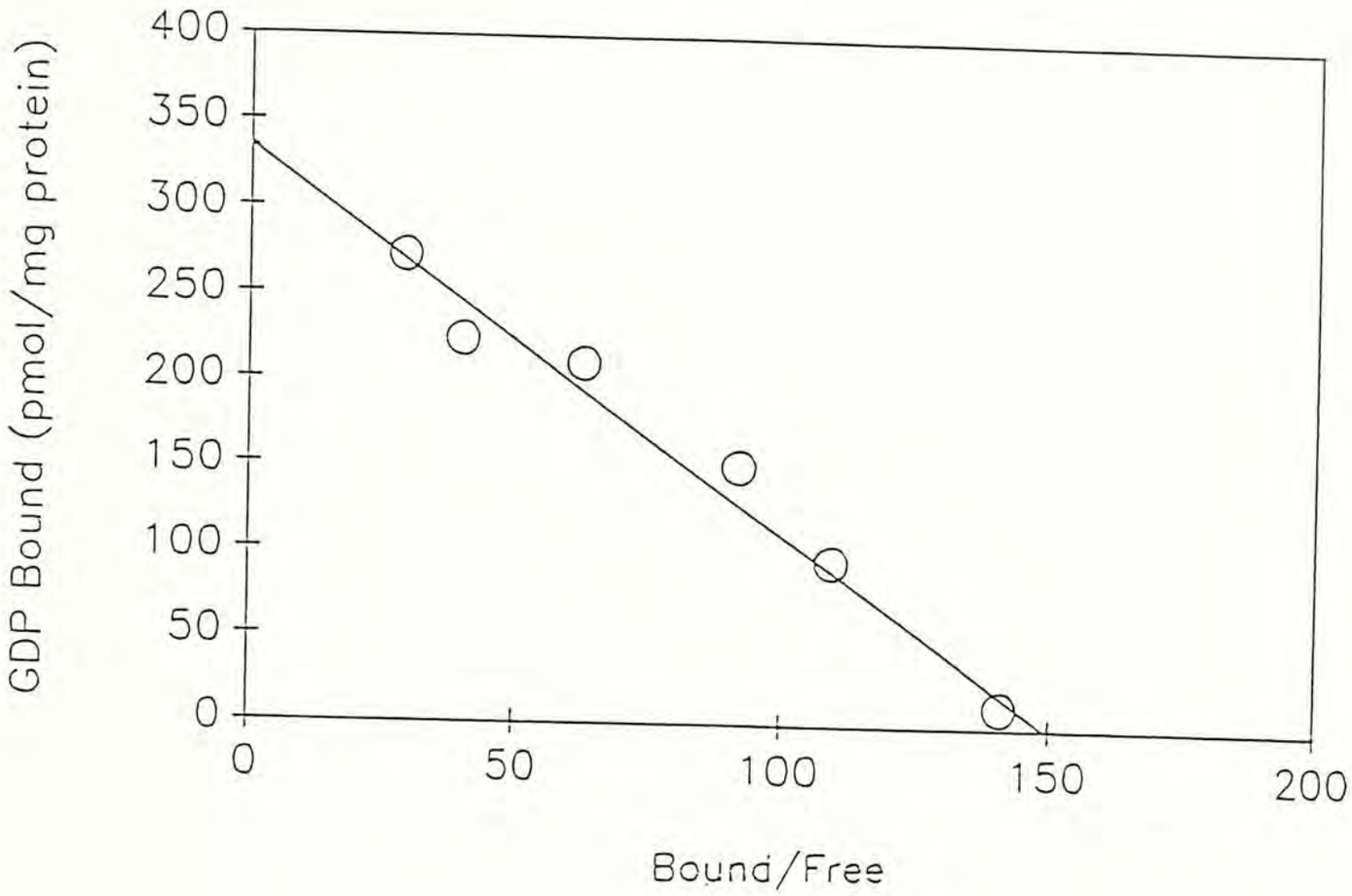



Fig. 2.4 Scatchard plot of GDP Bound vs GDP Bound/Free. The intercept on the Y axis gives the maximum number of GDP binding sites (Bmax) and the slope gives the -kd.

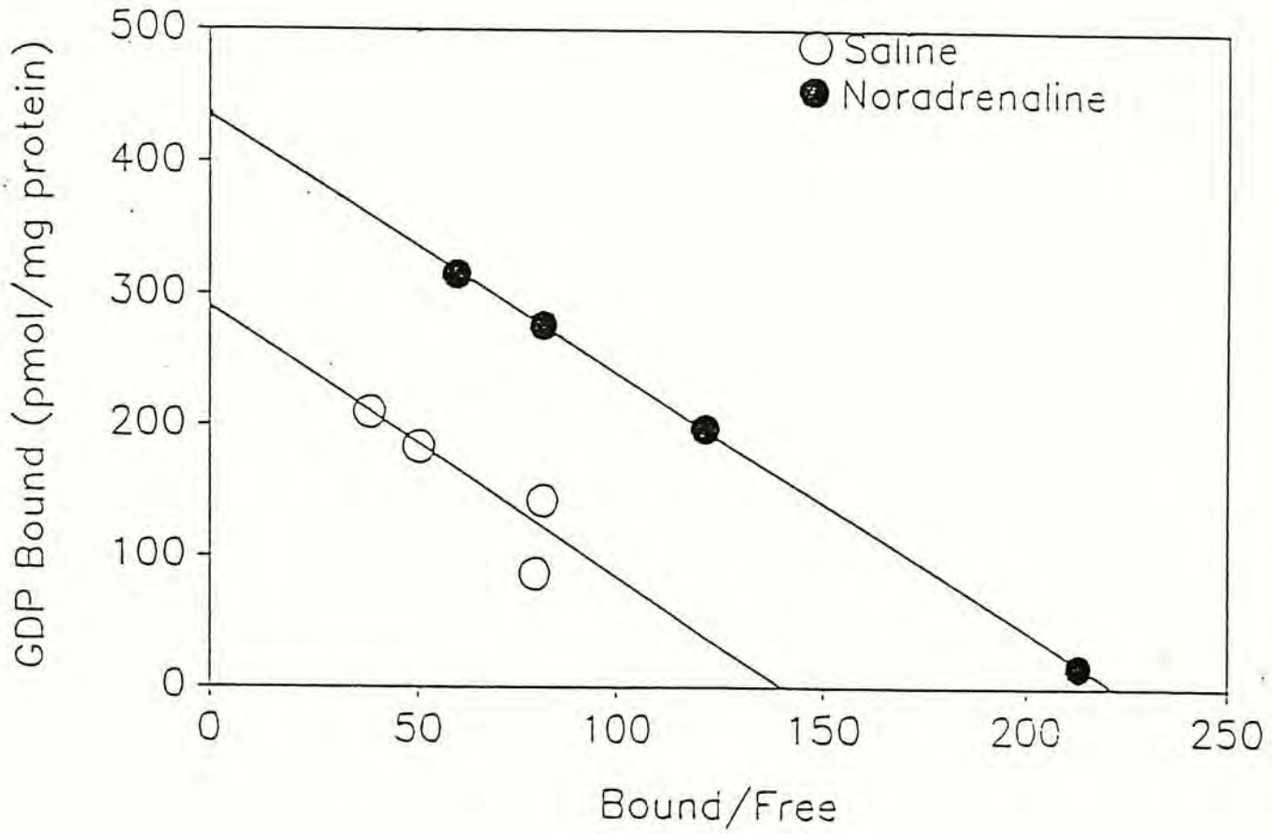



Fig. 2.5 Scatchard plot of GDP binding of BAT mitochondria one h after noradrenaline or saline treatment.

### 3.3.2. GDP Binding Activity of BAT Mitochondria after Cold Acclimation and Noradrenaline Treatment

Cold acclimation and noradrenaline have been demonstrated to be effective factors in enhancing thermogenic capacity of BAT as shown by an increase in GDP binding. The effect of combining these factors on the GDP binding was studied here. The results of GDP binding after cold acclimation and noradrenaline treatment are shown in Fig. 2.6. After 10 days there was a significant increase, more than 4-fold, in Bmax of GDP binding from 338 pmol/mg protein in the rats kept at 22°C (control) to 1385 pmol/mg protein in the cold acclimated group. The kd value increased from 2.20 to 3.41 μmol/L after cold adaptation. However, noradrenaline treatment did not increase Bmax or alter kd value in cold adapted rats.

### 3.3.3. Effects of Food Restriction on Cold Acclimated Rats

During cold acclimation, two factors that may cause BAT hypertrophy are an increase of food intake or an increase of sympathetic activity and these two proceed pari passu. The present experiment was designed to study the effects of food intake on BAT thermogenesis during cold acclimation. Groups of rats were kept at 4°C and fed ad libitum or pair fed with rats kept at 22°C. The results are summarized in Table 2.3. The food consumption during the 5 days of the experimental period

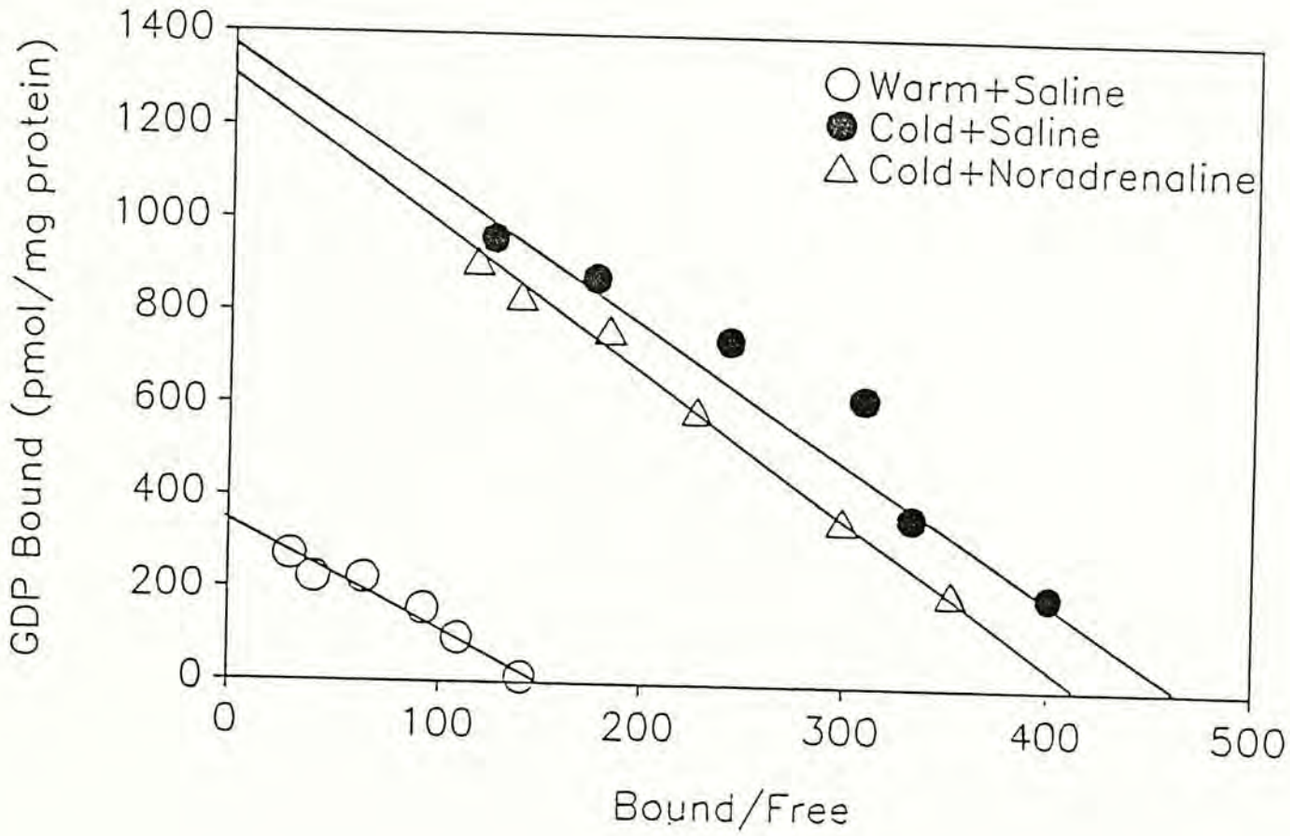



Fig. 2.6 Scatchard plot of GDP binding to BAT mitochondria from (1) rats kept at 22°C after saline injection, (2) cold adapted rats after saline injection, and (3) cold adapted rats after noradrenaline injection. The binding assay is as described in section 2.6.1.

Table 2.3 Effects of food restriction on body weight, food intake and BAT parameters of cold acclimated rats.

| | Group 1 (Warm ad libitum) | Group 2 (Cold ad libitum) | Group 3 (Cold pair fed) |
|---|---|---|---|
| BW (g) | | | |
| initial | 74.0±0.6 | 74.9±1.0 | 76.9±1.6 |
| final | 106.1±1.6 | 86.7±5.8[a] | 72.5±0.9[be] |
| net gain | 32.1±1.5 | 11.8±4.9[c] | -4.4±0.5[be] |
| Food intake (g/day.rat) | 14.3±0.7 | 18.8±0.9[c] | 14.0±0.5[d] |
| BAT | | | |
| weight (mg) | 145.2±17.0 | 188.3±7.8 | 121.8±12.2[d] |
| # mitochondria protein (mg/BAT) | 8.25 | 11.6 | 9.09 |
| Bmax (pmol/Lmg protein) | 384±15 | 945±61 | 755±46 |
| Kd (μmol/L) | 2.02±0.2 | 2.87±0.34 | 2.89±0.23 |

Data are presented as means±SE and each group had 4 rats; statistical significance by Student's t test: [a] Groups 1 vs 2, $p<0.05$
[b] Groups 1 vs 3, $p<0.001$
[c] Groups 1 vs 2, $p<0.01$
[d] Groups 2 vs 3, $p<0.01$
[e] Groups 2 vs 3, $p<0.05$
# Protein content of the pooled mitochondria from 4 rats.

was significantly higher in the cold adapted group with free access to chow (Group 2) than the warm adapted rats fed ad libitum (Group 1), or pair fed cold adapted group (Group 3). During the 5 days of the experiment Group 1 gained 32.1±1.5 g in weight whereas Group 2 gained 11.8±4.85 g, which was significantly less than that of Group 1 ($p<0.05$). Group 3 lost an average of 4.4±0.5 g. The weight of interscapular BAT was significantly lower in Group 3 than that of Group 2. The mean BAT weight in Group 1 was lower than that of Group 2 but it was not statistically significant. The Bmax of GDP binding of BAT mitochondria from Group 2 and 3 were higher compared with Group 1. The Bmax of GDP binding of BAT mitochondria from Group 2 was also slightly higher than Group 3. This showed that food restriction could only slightly suppress the cold induced increase in Bmax of GDP binding. The kd values of Group 2 and 3 were similar and they were higher than Group 1. The results of this experiment confirmed the previous finding in Section 3.3.2. that cold exposure could increase Bmax, and induced hypertrophy of BAT. Although restricted food intake limited the absolute BAT weight and the mitochondrial protein, the thermogenic function shown by GDP binding was only slightly suppressed.

### 3.3.4. GDP Binding Activity of BAT Mitochondria from Rats of Different Ages

To study the effect of age on the thermogenic function of rats, GDP binding of mitochondria in 4 and 11 week old rats was studied. The results are presented in Fig.2.7. There was a linear relationship between the concentration of bound GDP and the ratio of bound to free GDP. The maximum GDP binding sites of mitochondria (Bmax) of 4 and 11 week old rats were 146.6 pmol/mg protein and 148.5 pmol/mg protein respectively. The dissociation constants (kd) were 1.56 μmol/L and 1.39 μmol/L respectively. Bmax and kd values were similar in the two age groups studied in this experiment.

## 3.4. Isolation and Purification of UCP

The method of isolation and purification of UCP was similar to that of Lin and Klingenberg (1980 and 1982) with some minor modifications. Column chromatography using hydroxylapatite was the major purification step. When TritonX-100 treated mitochondrial extract was applied to the column the elution profile (Fig. 2.8), monitored at 280 nm, and by protein assay showed that there was only one peak. The peaks of absorbance and protein assay coincided and appeared from fraction 8 to fraction 13. These fractions were pooled, TritonX-100 was removed by

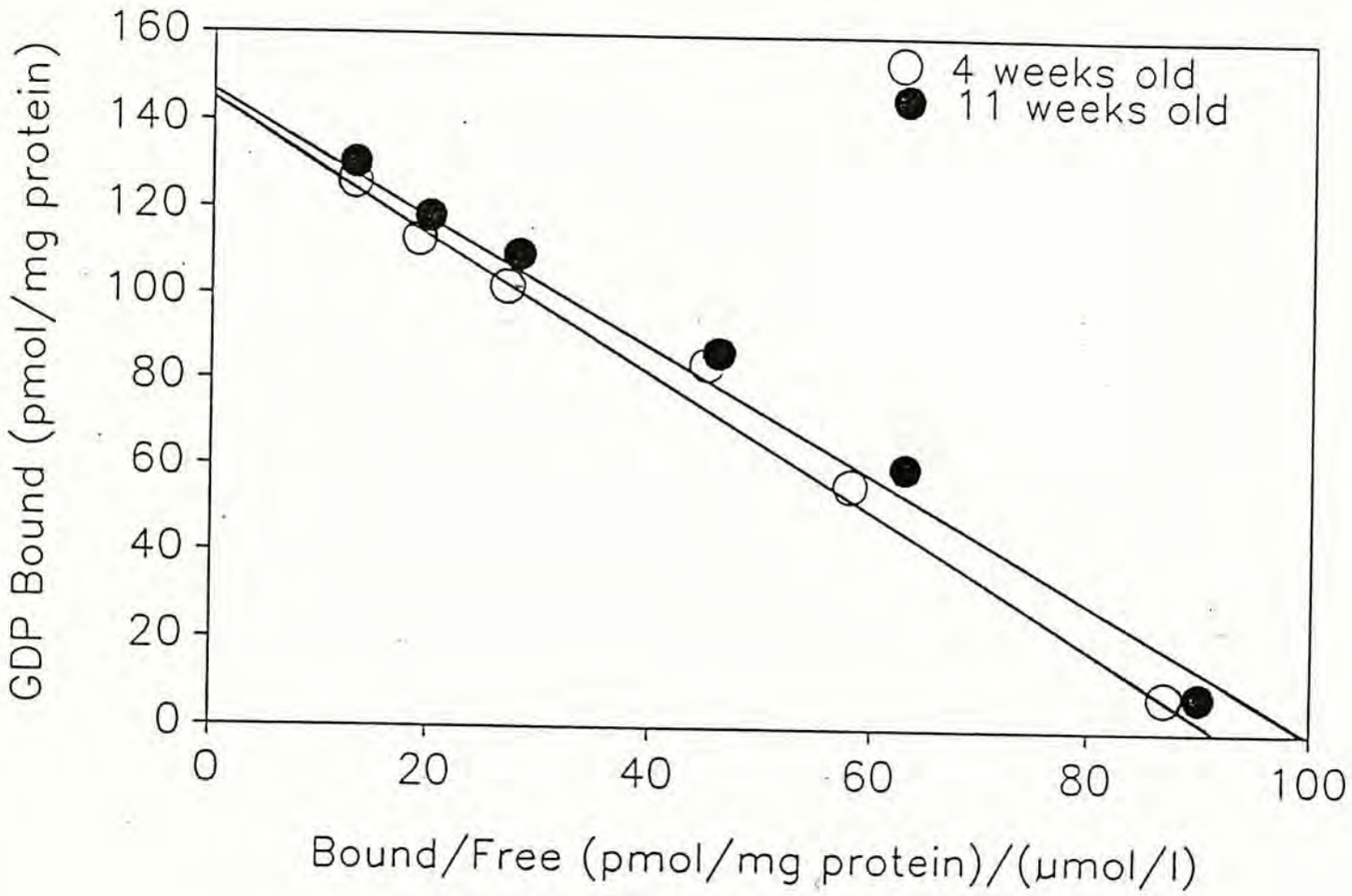



Fig. 2.7 Scatchard plot of GDP binding to BAT mitochondria form 4 week old and 11 week old rats. The assay is as described in section 2.6.1.

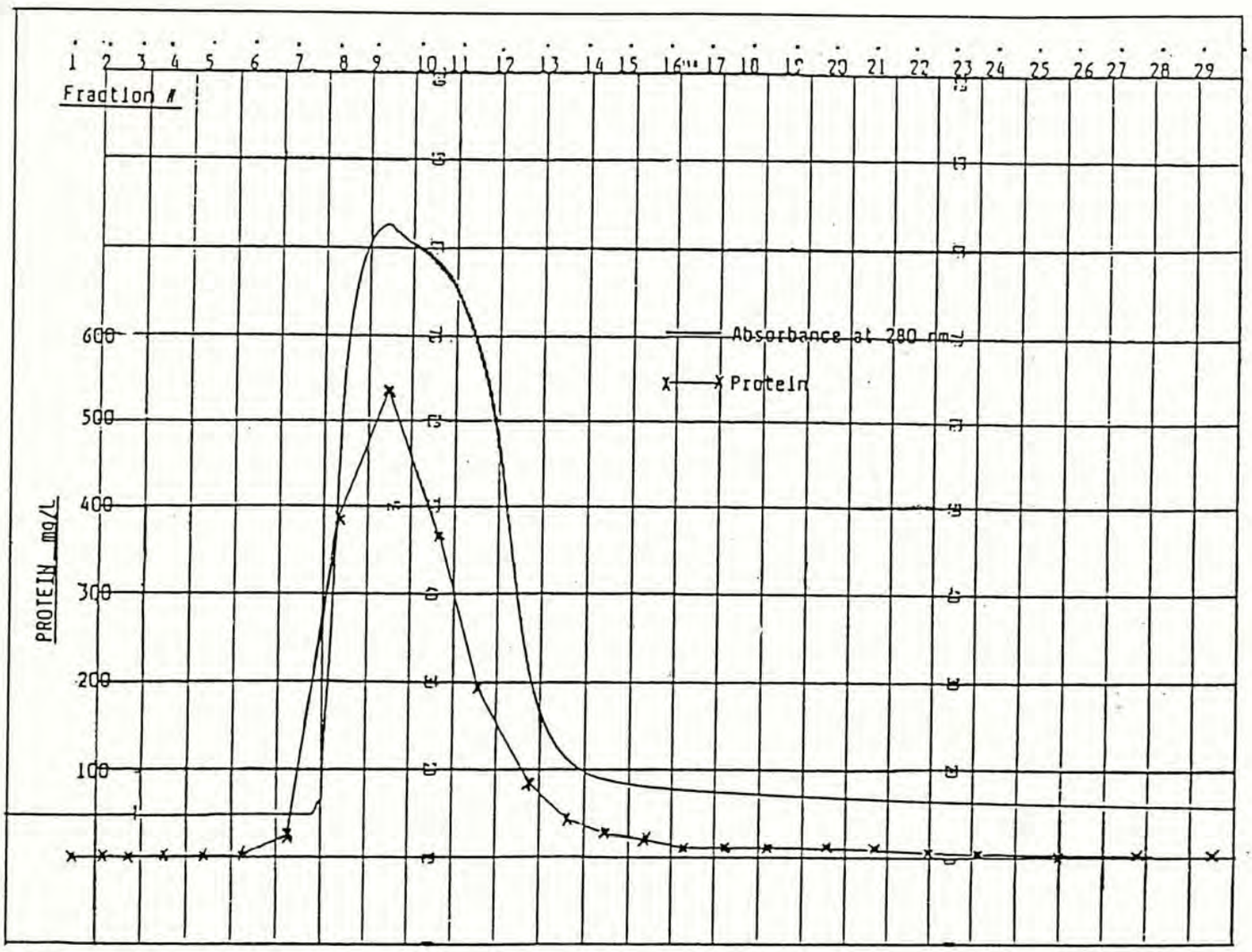



Fig. 2.8 Elution profile of hydroxylapatite column chromatography monitored at 280 nm. The protein concentration was assayed by a modified method (Markwell et al. 1981).

Bio-Beads and the protein was then concentrated by using the Amicon Concentrator.

The presence of UCP at different stages of the purification was detected by SDS-PAGE, and determined by GDP binding activity. The results from 4 separate preparations of rat UCP are shown in Table 2.4 (in page 148). During the Lubrol PX step, about 50% of the mitochondrial proteins were removed. After extraction with TritonX-100 about 20% of the total mitochondrial protein remained. The final yield as a percentage of total mitochondrial protein varied from 0.96 to 1.95% in these 4 preparations.

### 3.4.1. Results of SDS-PAGE

The purity of the mitochondrial proteins during each step of isolation was determined by SDS-PAGE. The 4 molecular weight standards were run in parallel to identify the 32 kDa protein. A plot of the distance travelled by the standard proteins in the SDS-PAGE vs the log of molecular weight (Fig. 2.9) shows a linear relationship between the two. The results of SDS-PAGE of untreated mitochondria (Fig. 2.10, lane 3), Lubrol PX solubilized proteins (Fig. 2.11, lane 2, 3 & 4), TritonX-100 extracted (Fig. 2.10, lane 1 & 2) and purified UCP (Fig. 2.12, lane 1 & 3) are shown. By comparison of the protein bands of untreated mitochondria with those of the Lubrol PX extract, it can be seen that Lubrol PX extracted

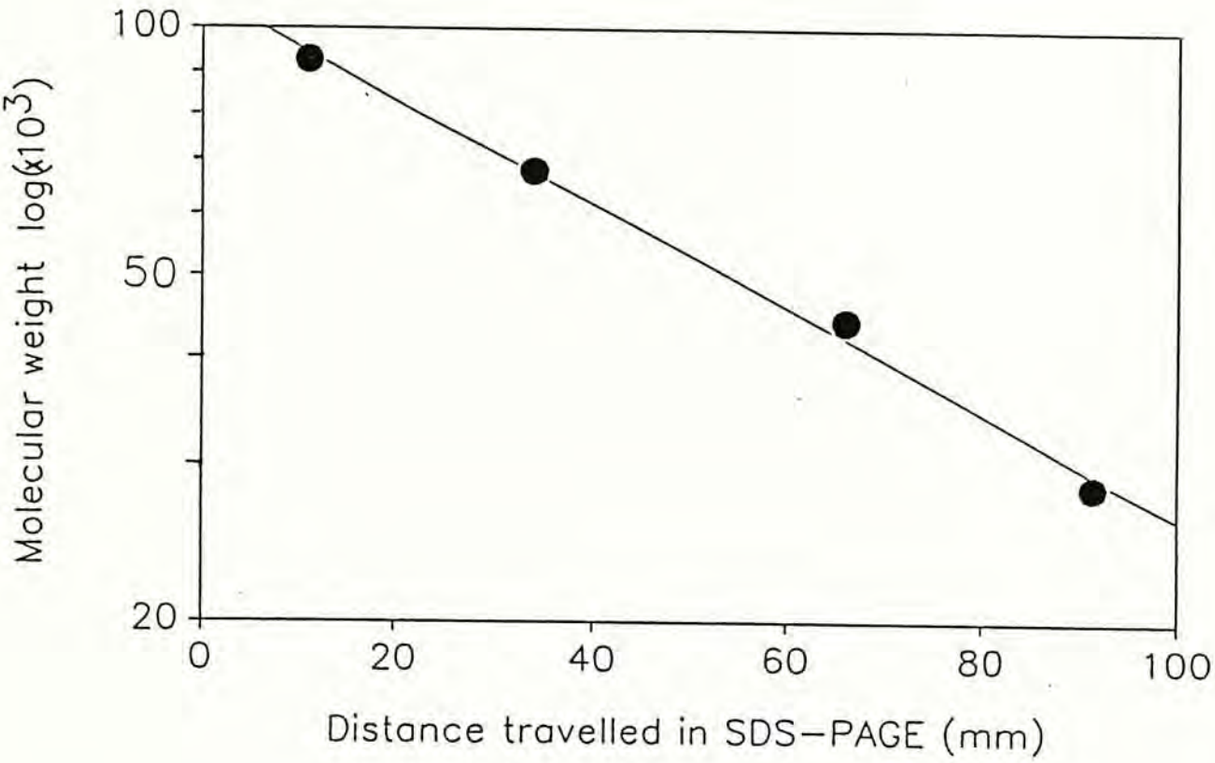



Fig. 2.9 A plot of molecular weight vs distance travelled in SDS-PAGE of protein molecular weight standards. The method is as described in section 2.9.


Fig. 2.10 SDS-PAGE of UCP during different stages of purification. Lanes 1 and 2 show TritonX-100 extracts of mitochondria (15 and 7.5 $\mu$g of protein respectively) . and lane 3 shows untreated mitochondria (40 $\mu$g). The method is described in section 2.9. Arrow indicates the 32 kDa range.

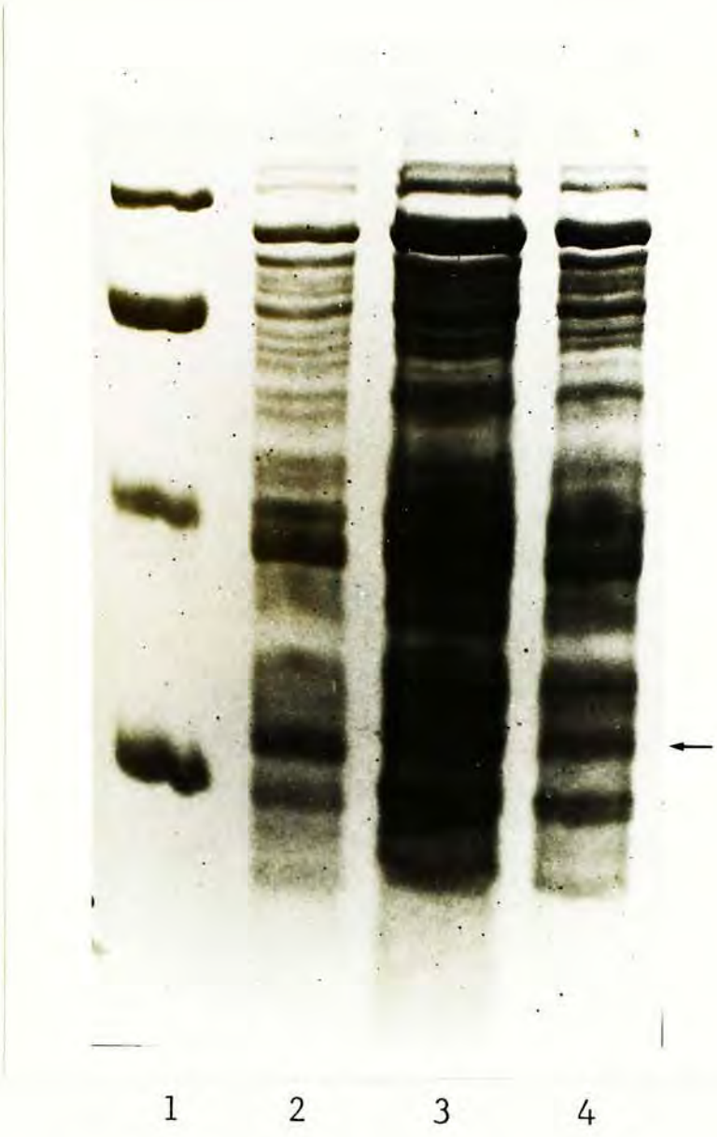



Fig. 2.11 Photograph of SDS-PAGE of mitochondrial protein after Lubrol PX extract. Lane 1; protein molecular weight standards; lane 2, 3 and 4; Lubrol PX extracts - 20, 50 and 30 $\mu$g of protein respectively. The SDS-PAGE is described before (section 2.9.).


Fig. 2.12 SDS-PAGE of UCP: lane 1, 1.75 μg of purified UCP; lane 2, molecular weight marker;lane 3, 8.1 μg of UCP. The arrow indicates the position of protein MW range of 32 kDa.

Table 2.4 : Rat BAT mitochondrial preparation and isolation of UCP.

| Experiment | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| Number of rats | 5 | 15 | 20 | 20 |
| Tissue weight (g) | - | - | 16.3 | 14.9 |
| Total mitochondria (mg protein) | 50.8 | 205 | 382.4 | 301 |
| Lubrol PX Supernatant (mg protein) | 25.6 | 112 | 248.6 | 182.5 |
| TritonX-100 Supernatant (mg protein) | 10.7 | 24 | 48 | 50 |
| After hydroxylapatite (mg protein) | - | 5.82 | - | - |
| Purified UCP after concentrating (mg protein) | 0.75 | 1.97 | 7.46 | 3.82 |
| Final yield of total mitochondrial protein (%) | 1.45 | 0.96 | 1.95 | 1.3 |

most of the mitochondrial proteins and possibly extracted some of the UCP as shown by the presence of a 32kDa protein (Fig. 2.11). Lin and Klingenberg (1982) found that no UCP was extracted by Lubrol WX. Lubrol PX was used in the present experiments as only this detergent was available commercially. Lubrol PX is known to sequester TritonX-100 by forming mixed micelles and therefore it was removed from the residual sedimented membranes by washing with sucrose solution in order to minimize the amount of TritonX-100 necessary for the subsequent solubilization. The TritonX-100 extract contained fewer protein bands than the Lubrol PX extract (see Fig. 2.10 and 2.11).

The final protein concentrate showed only one major band of protein at appropriate 32 kDa (see Fig. 2.12). Lane 3 was deliberately overloaded in order to demonstrate the absence of contaminants in the purified protein. There was a very faint band at 64 kDa which would correspond to a dimer of UCP.

### 3.4.2. Results of GDP Binding Activity

During the isolation and purification of UCP, aliquots were taken at the different stages to monitor GDP binding capacity. This was measured by equilibrium dialysis (section 2.6.2.) and results are shown in Table 2.5. The protein content decreased from 228.8 mg at the start to 9.82 mg in the final step whereas GDP binding

Table 2.5 : Purification of UCP during different stages of preparation.

| Purification steps | Protein content (mg) | GDP Binding (pmol/mg protein) | Purification factor | Recovery % |
|---|---|---|---|---|
| Mitochondria | 228.8 | 2.76 | 1 | 100 |
| Lubrol PX extract | 183.4 | - | - | - |
| TritonX-100 extract | 51.9 | 10.68 | 3.9 | 87.8 |
| Purified UCP | 9.82 | 42.39 | 15.4 | 65.9 |

activity increased several-fold from 2.76 to 42.39 pmol/mg protein. The purification factor increased 3.9-fold after TritonX-100 extraction and 15.4-fold after the final step. The final recovery was 65.9%.

Scatchard analysis of GDP binding of UCP would appear to be the suitable way to assess the purity of UCP. Furthermore, the binding capacity and the affinity should reflect the intactness of the solubilized protein. Using the microfiltration method (Section 2.6.3.), Bmax and kd were obtained. Bmax of purified UCP was 30,050 pmol GDP/mg protein or 0.939 mol GDP/mol protein (using 32 kDa as MW of UCP), and kd was 13.64 μmol/L (Fig. 2.13). When Bmax of purified UCP was compared with the BAT mitochondria from the cold adapted rat (Bmax=1385 pmol/mg protein; kd=3.41 μmol/L), it was about 21 times higher. Therefore the binding capacity was greatly increased in the purified UCP, but the dissociation constant of the purified UCP was higher, indicating that the binding affinity decreased during the process of purification.

### 3.5. Rabbit Anti-rat-UCP Antibody and the Characterization of Antiserum

Four rabbits were injected subcutaneously with purified UCP several times. Before each booster injection blood was collected from the ear vein to monitor antibody titre. Antibody titre was determined by

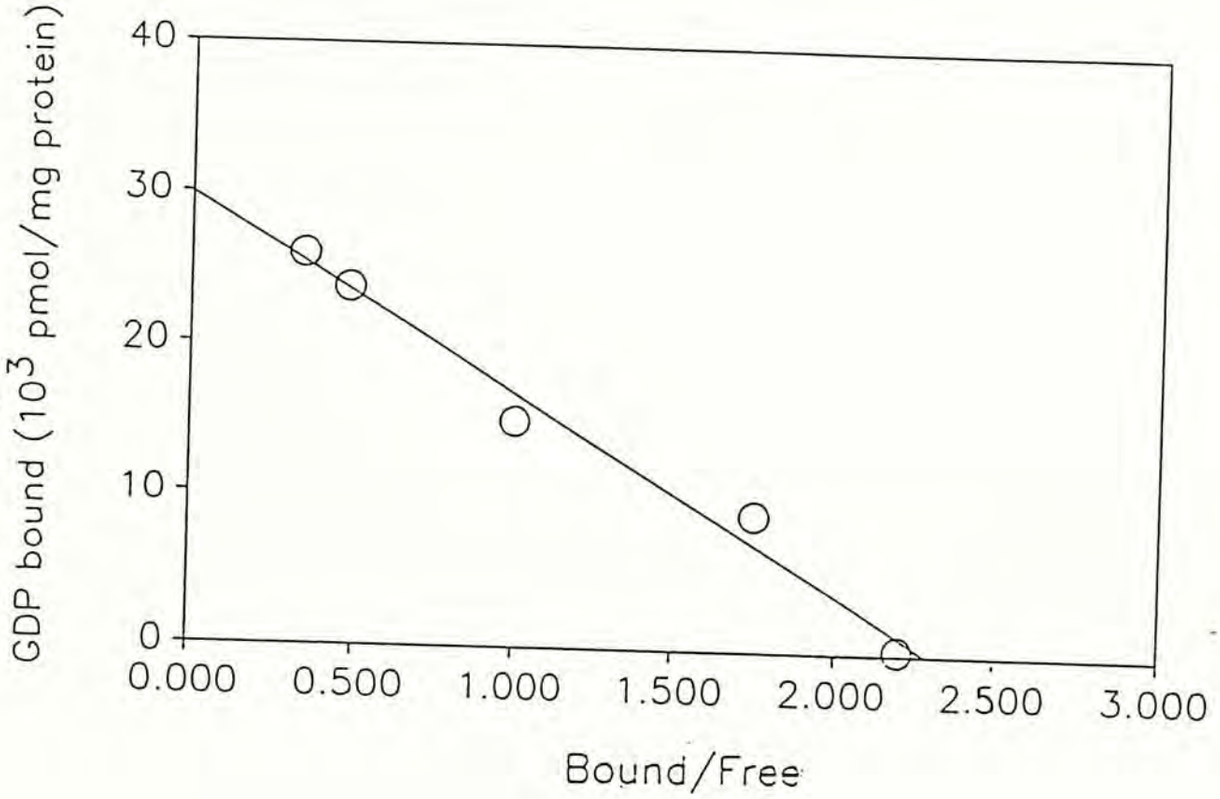



Fig. 2.13 Scatchard plot of GDP binding to purified UCP by the microfiltration method (see section 2.6.3.). Bmax was 30,050 pmol/mg protein and kd was 13.64 μmol/L.

incubating the diluted antiserum with UCP coated to the microtitre plate and the binding was detected by Protein A linked to $\beta$-galactosidase. After 5 injections of UCP, the serum of all four rabbits showed binding to UCP (Fig. 2.14). The measured absorbance decreased with increasing dilution of antiserum. Rabbit 2 antiserum had the highest absorbance and was selected for further evaluation and characterization for use in the ELISA assay.

### 3.5.1. Immuno-autoradiography for Specificity of Rabbit Anti-rat-UCP Antiserum

#### 3.5.1.1. Cross-reactivity of the Rabbit Anti-rat-UCP Antiserum to Mitochondrial Proteins of BAT and from other Tissues

To check the completeness of transfer of protein by electroblotting the following experiment was done. After electrophoresis, the SDS-PAGE gel was cut into 2 halves (I and II in Fig. 2.15 A and B) and each half contained identical samples including mitochondria from liver, white fat and BAT, and UCP. One half of the gel was electroblotted (AI and BI) whilst the other was stained with Coomassie blue (AII and BII). After electroblotting AI and BI were also stained with Coomassie blue. This revealed some high MW proteins (above 100 kDa) remaining in the gel (Fig. 2.15 B) as shown by the presence of faint bands. However, proteins with lower MW were successfully

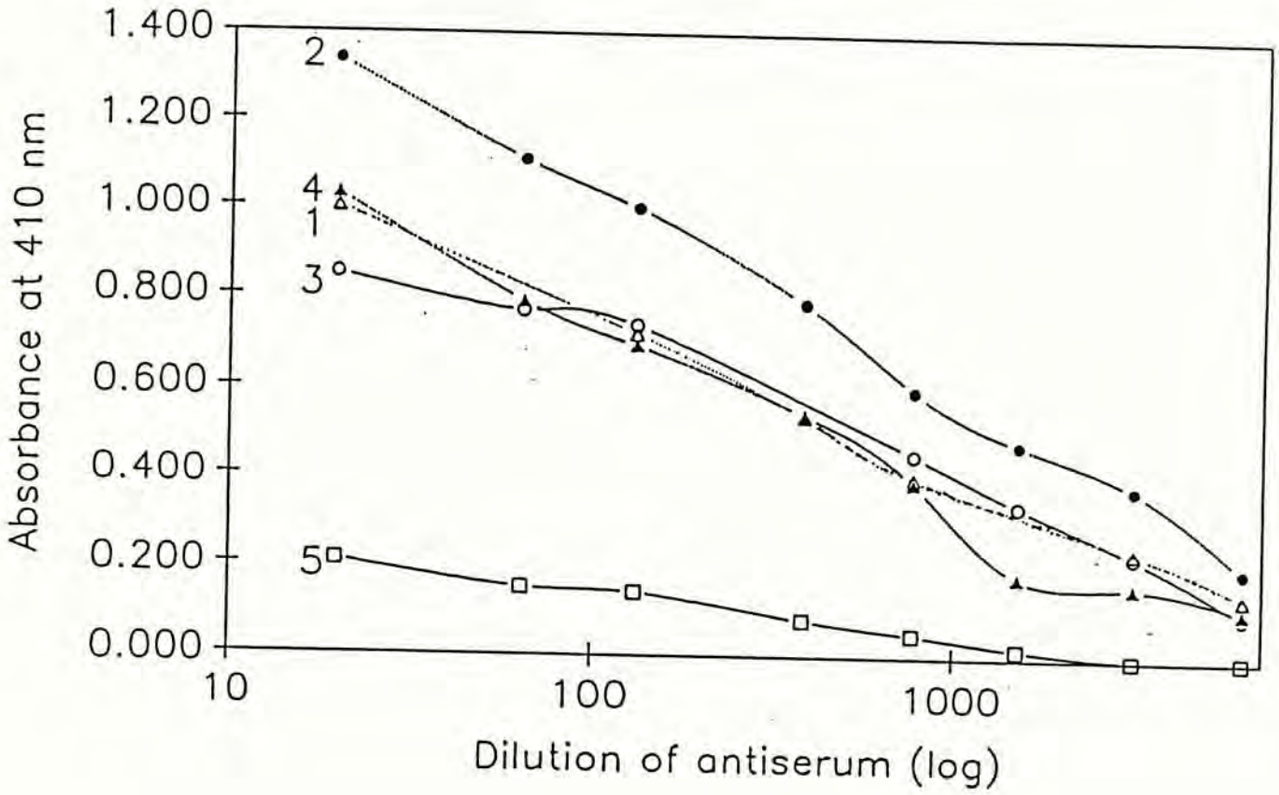



Fig. 2.14 The dilution curves of 4 different antisera. Diluted antisera were incubated in the UCP coated microtitre plate and binding was detected by Protein A linked to β-galactosidase. The numbers 1, 2, 3 and 4 denote sera from four different rabbits after several immunizations with UCP. Number 5 was the pooled sera from normal rabbits before immunization.

Fig. 2.15 These photographs show the results of experiment for checking the completeness of electroblotting by coomassie blue staining. After electrophoresis the gel was cut into two halves (I and II), each half containing the identical samples. One half (AI and BI) was electroblotted and then stained with coomassie blue, and the other half (AII and BII) was stained with coomassie blue without electroblotting. Lane A1 and A2 (32 and 64 $\mu$g of mitochondrial protein from white fat respectively), A3 and A4 (5 and 10 $\mu$g of purified UCP respectively), A5 and B5 (1$\mu$g of protein molecular weight standard); lane B1 and B2 (32 and 64 $\mu$g of BAT mitochondrial protein respectively), B3 and B4 (32 and 64 $\mu$g of liver mitochondrial protein from respectively).

A I

A II

A

1 2 3 4 5 1 2 3 4 5

B I

B II

B

1 2 3 4 5 1 2 3 4 5

transferred to the nitrocellulose paper.

After transfer to the nitrocellulose paper by electroblotting, the proteins were reacted with antiserum. The results of immuno-autoradiography of mitochondrial proteins from BAT, liver, skeletal muscle and white fat are shown in Fig. 2.16. In lane 4, only one prominent band formed, at approximately 32 kDa, whereas mitochondrial proteins from the other tissues: skeletal muscle, white fat and liver did not produce any band (lane 1, 2 and 3 respectively).

Since the method used for UCP purification was similar to that for ADP-ATP translocase (Lin and Klingenberg, 1980), there was the possibility of contamination by ADP-ATP translocase in the UCP preparation. As mitochondrial proteins from skeletal muscle (lane 1), white adipose tissue (lane 2) and liver (lane 3), even in large amounts, did not react with the antiserum contamination with ADP-ATP translocase was unlikely.

#### 3.5.1.2. Cross-reactivity of the Rabbit Anti-rat-UCP Antiserum to BAT Mitochondrial Protein from Different Rodent Species

The BAT mitochondrial proteins of different rodent species including hamster, mouse, guinea pig and rat were tested against the antiserum (Fig. 2.17). Mitochondrial proteins were separated by SDS-PAGE and then transferred

1 2 3 4

Fig. 2.16 Immunoblotting of mitochondrial proteins from skeletal muscle (172 μg; lane 1), white adipose tissue (823 μg; lane 2), liver (832 μg; lane 3) and UCP (21.7 μg; lane 4). Arrow denotes the 32 kDa range.

1A 1B 2A 2B 3A 3B 4A 4B 5

Fig. 2.17 Cross — reactivity of rabbit anti-rat-UCP antiserum to BAT mitochondrial proteins from different species of rodents by immunoblotting technique. Two different amounts of mitochondrial protein were loaded into the gel for each species: lane A=22.8 μg and lane B=45.6 μg of protein. lane 1, hamster; lane 2, mouse; lane 3, rat; lane 4, guinea pig; lane 5, 3.19 μg of rat UCP. Arrow indicates the 32 kDa range.

to the nitrocellulose paper by electroblotting. The nitrocellulose paper was treated with antiserum and the binding of antigen and antibody was determined by $^{125}I$-Protein A. The gel was loaded with protein, 22.8 μg (lane A) and 45.6 μg (lane B), from each species. All the species studied showed a positive result and, moreover, only the mitochondrial protein located at the position about 32 kDa reacted with the rabbit anti-rat-UCP antiserum. Thus the rabbit anti-rat-UCP cross-reacted with other rodent species and was specific to the UCP of these species. Hamster and mouse had a strong signal similar to rat whereas guinea pig showed a very weak reaction.

### 3.5.1.3. Dose Response of Rabbit Anti-rat-UCP Antiserum to UCP

The dose response of rabbit anti-rat-UCP antiserum to UCP is shown in Fig. 2.18. When increasing amounts of UCP (0.64 to 10.2 μg of protein) were applied to the gel, there was a dose related response in the signal (Fig. 2.18, lane 1 to 6). Normal rabbit serum showed a negative response to UCP (lane 7 and 8) irrespective of the amount of UCP applied (1.28 or 10.2 μg).


Fig. 2.18 An immunoblot of increasing amounts of UCP with anti-UCP or normal rabbit sera. The nitrocellulose paper containing lanes 1 to 6 (0.64, 1.28, 2.55, 5.10, 7.70 and 10.20 μg of UCP respectively) was treated with antiserum (Rabbit 2). Lanes 7 and 8 (1.28 and 10.2 μg of UCP respectively) were treated with pooled normal rabbit sera. Arrow denotes 32 kDa range (method see section 2.10.3., exposure time 47 h).

## 3.6. ELISA of UCP

### 3.6.1. Determination of Maximum Amount of UCP Binding on Microtitre Plate

In order to determine the amount of UCP which can be coated on the microtitre plate in the ELISA method, different amounts of UCP were coated on the plate overnight at 4°C. The unbound UCP was washed away and antiserum at a dilution of 500-fold was used to detect the binding of UCP on the plate. Results are shown in Fig. 2.19. Absorbance increased as the amount of UCP in the well increased (Fig. 2.19) and reached a plateau at about 0.5 $\mu$g. In order to ensure a sufficient amount of UCP coated on the plate, one $\mu$g of UCP was used thereafter to coat the microtitre plate.

### 3.6.2. Antibody Dilution Curve

To find a suitable dilution of antibody for the assay, antiserum was serially diluted and tested. The UCP coated plate was incubated with 50 $\mu$l of different dilutions of antiserum. The binding of antibody to UCP was measured by incubating with Protein A linked with $\beta$-galactosidase.

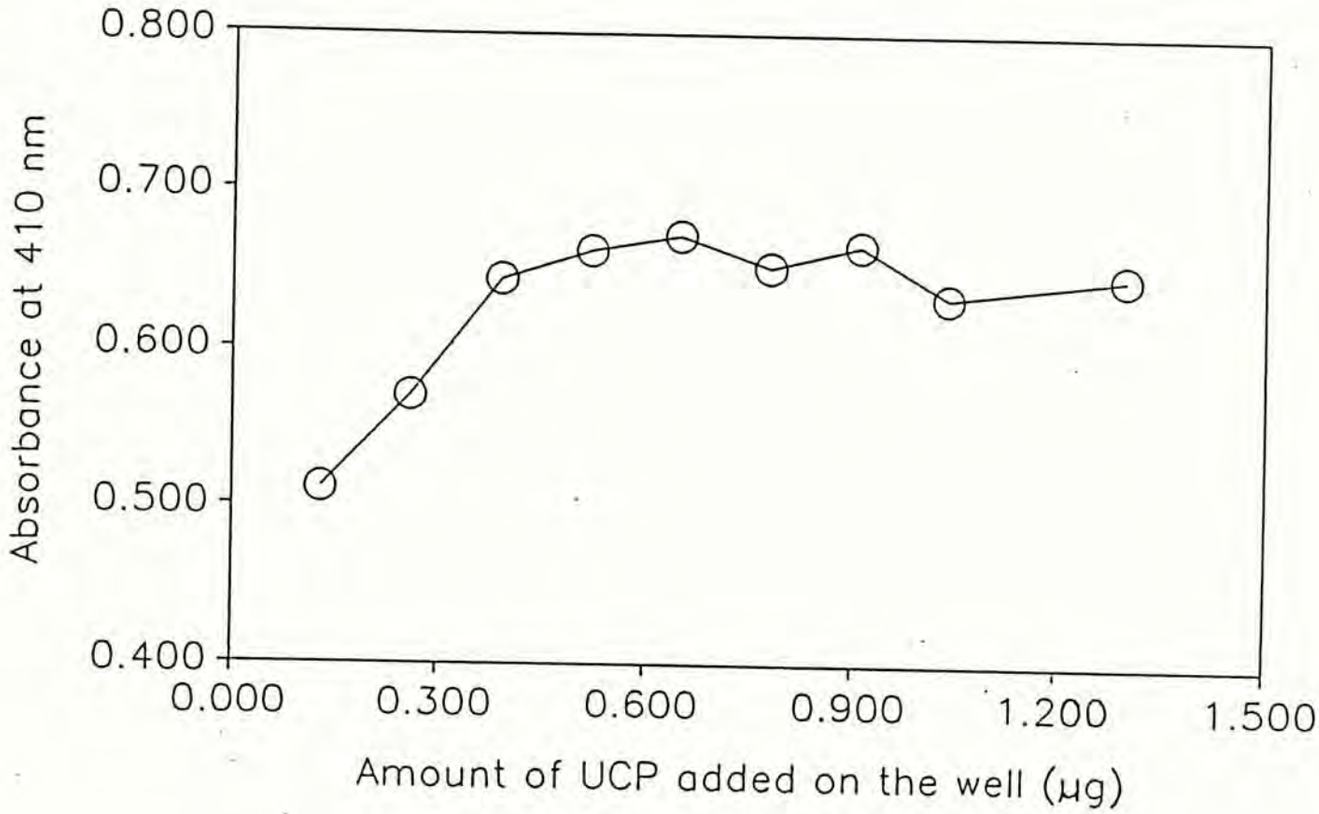



Fig. 2.19 Amount of UCP coated on the well vs absorbance measured after 1 hr. For methods see section 2.11., antiserum dilution used was 500-fold.

The antibody dilution curve is shown in Fig. 2.20. Increasing the dilution of antiserum decreased the absorbance at 410 nm whereas there was very little change in absorbance when normal rabbit serum was used. Since a 500-fold dilution of antiserum gave sufficient absorbance, of about 1.0, it was used in subsequent assays.

### 3.6.3. Incubation Time for Enzyme - Substrate Reaction

The optimal incubation time for the enzyme - substrate reaction to give sufficient absorbance in the final step of ELISA was studied. The 500-fold diluted antiserum was incubated with UCP coated microtitre for 2 h. Protein A linked to $\beta$-galactosidase was added and followed by the addition of ONPG. The absorbance of the same sample was read at different incubation times and the results are shown in Fig. 2.21. There was a gradual increase in absorbance with increasing time of incubation. After 1 h of incubation the absorbance was about 1.0 and this was chosen for the enzyme reaction in the ELISA.

### 3.6.4. Competitive ELISA

A competitive ELISA method was used to measure the UCP content of BAT homogenate. The cross-reaction to UCP, liver and BAT homogenate was studied. Wells pre-coated with UCP were incubated with mixture of the antiserum (500 dilution) and different amounts of UCP, BAT mitochondria or liver mitochondria. The results are shown in Fig. 2.22.

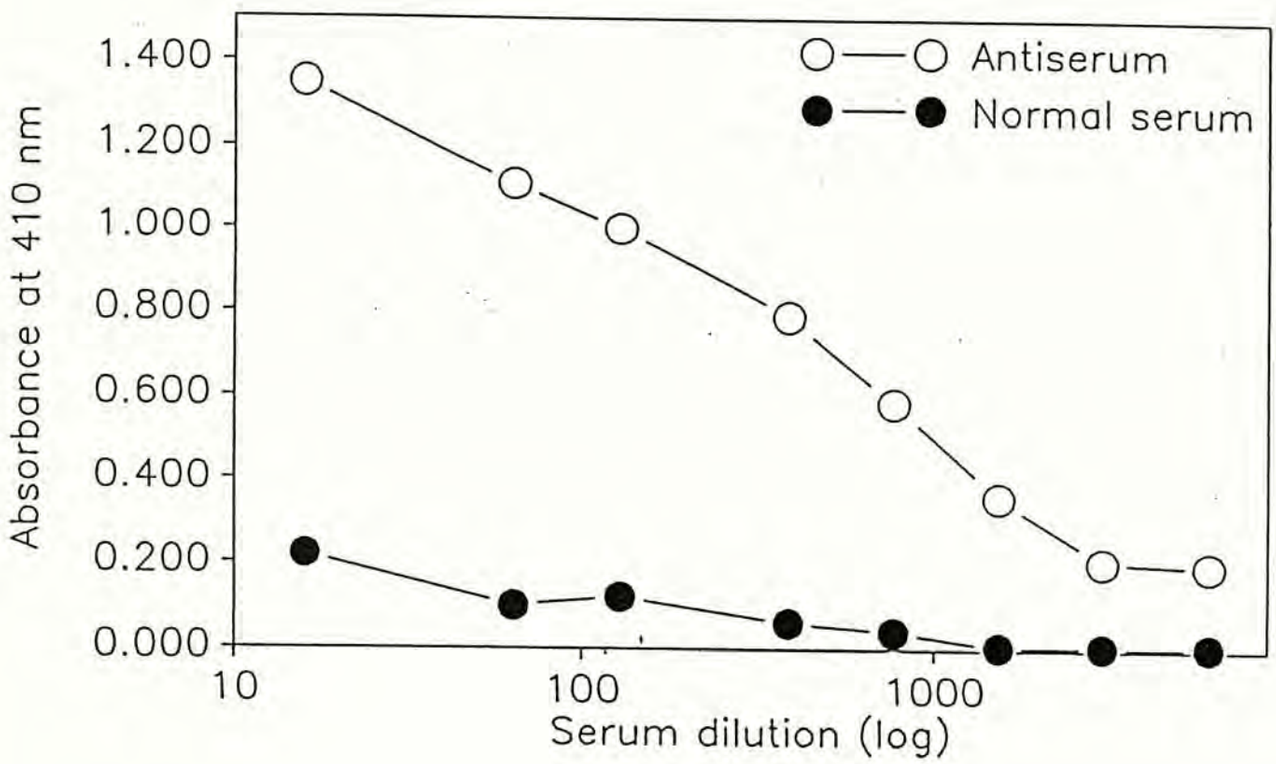



Fig. 2.20 Antibody dilution curve. Microtiter plate was coated with 1 $\mu$g of UCP per well and non-specific binding sites were blocked by bovine serum albumin. After washing, serial dilutions of antiserum were added and incubated for 2 h. Protein A linked to $\beta$-galactosidase was then added to identify the bound antibody.

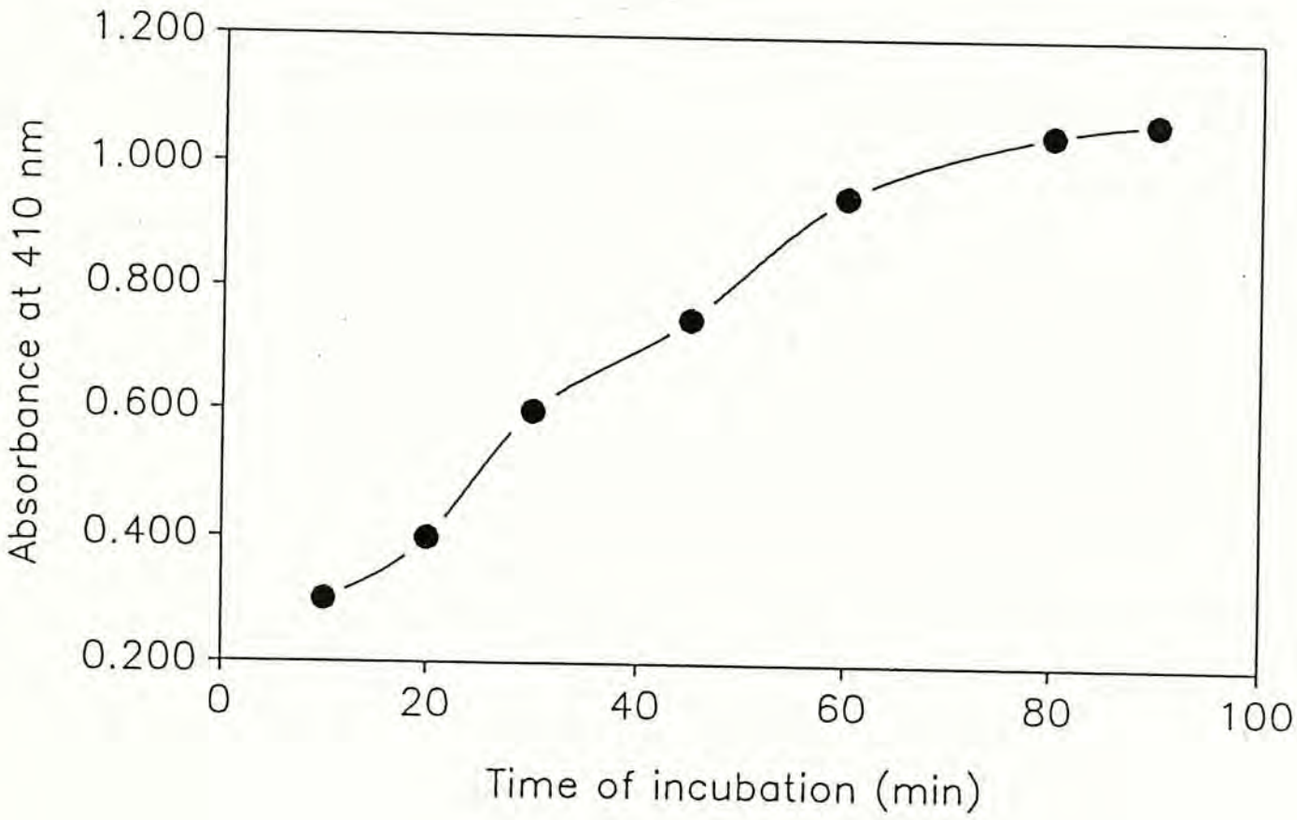



Fig. 2.21 The time course of absorbance change. (For methods see section 2.11, antiserum dilution was 500-fold).

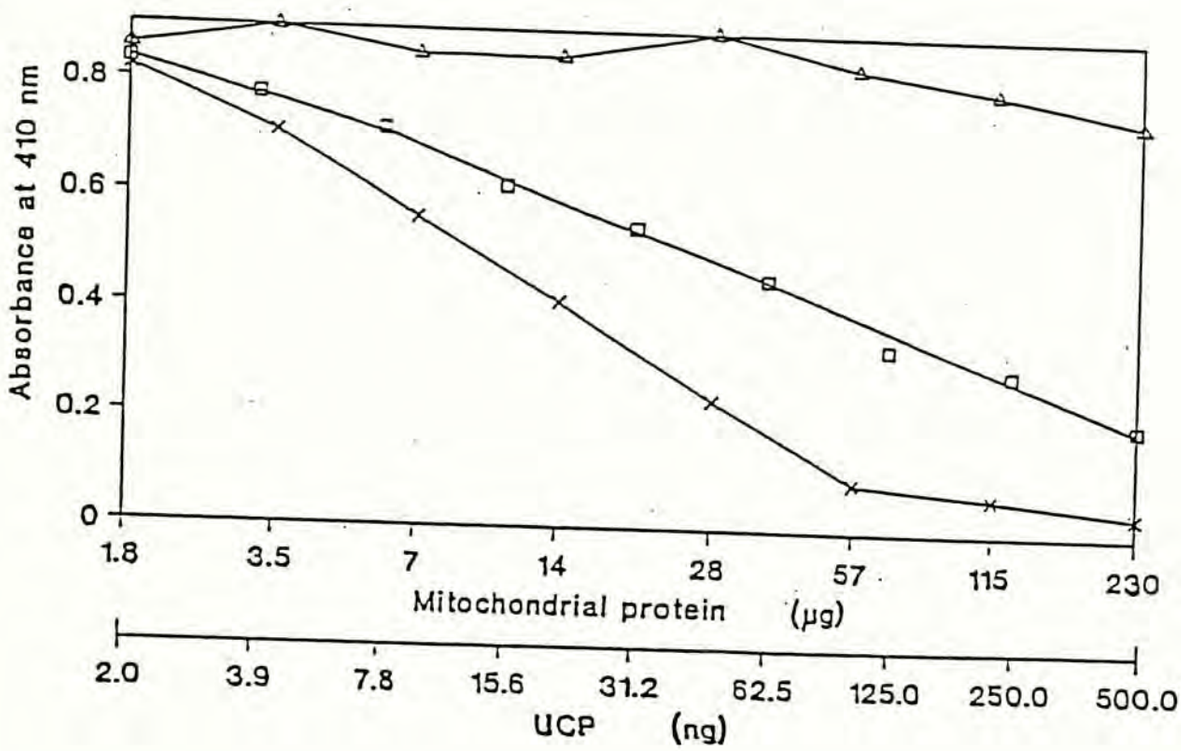



Fig. 2.22 ELISA of purified UCP (⊟), BAT homogenate (✳) and liver mitochondria (△). Samples were incubated with the antiserum (1:500 dilution) for 1 h and then transferred to a microtitre plate pre-coated with 1 µg of UCP. After incubation for 2 h Protein A linked to β-galactosidase and ONPG were used for quantitation.

With increasing amounts of UCP or the BAT homogenate there was a linear decrease in the absorbance. However, liver mitochondria could not displace the binding of the antibody to the UCP which was coated on the well and there was no significant change in absorbance with increasing amounts of liver mitochondria.

### 3.6.5. Precision of ELISA

The intra-assay and inter-assay precision of ELISA were studied. The intra-assay precision was done in the same microtitre plate. Four different amounts of UCP were used and at each level 6 replicates were measured. To determine the inter-assay precision of ELISA, samples obtained from warm (kept at 22°C) or cold adapted (10 days at 4°C) rat were assayed in 4 different assays.

Table 2.6 shows the results of the intra-assay precision of ELISA for UCP. The CV varied between 6.0% and 9.5% depending on the amounts of UCP assayed. The inter-assay precision was 14.1% at 503 μg/BAT and was 12.7% at 1105 μg/BAT (Table 2.7).

Table 2.6 : Intra-assay precision of ELISA for UCP. Sample size at each concentration of UCP was 6.

| Amount of UCP (ng) | Mean±SD (ng) | CV (%) |
|---|---|---|
| 20 | 21±2.0 | 9.5 |
| 156 | 151±12.3 | 8.2 |
| 312 | 320±19.2 | 6.0 |
| 500 | 510±45 | 8.8 |

Table 2.7 : Inter-assay precision of ELISA for UCP. Two samples of BAT homogenate (one from warm adapted rat and the other from 10 days cold adapted rat) were assayed 4 times. In each assay the samples were assayed in duplicate and the mean was taken as the result for that assay.

| Sample | Mean (μg/BAT) | SD | CV (%) |
|---|---|---|---|
| Warm rat | 503 | 71 | 14.1 |
| Cold rat | 1105 | 140 | 12.7 |

## 3.7. Experiment Designed for Validation of ELISA by Measuring UCP in Cold Acclimated Rats

After cold exposure for 4 or 6 days there was a significant fall in body weight (Table 2.8). A significant increase in the interscapular and cervical BAT weight was observed after 4 or 6 days of cold acclimation. The total protein of BAT and the pelleted mitochondrial protein of BAT were significantly elevated after 4 or 6 days of cold exposure. The total UCP content of BAT also rose dramatically after cold acclimation (4 days or 6 days). Total cytochrome C oxidase activity decreased after 1 day of cold exposure. However there was an increase in this activity after a longer period (4 days or 6 days) of cold acclimation. The ratio of UCP to cytochrome C oxidase activity and the ratio of UCP to mg mitochondrial protein were all statistically significantly higher after cold exposure.

Table 2.8 : Effects in rats of cold acclimation on body weight and BAT parameters.

| Period at 4°C (day) | 0 | 1 | 4 | 6 |
|---|---|---|---|---|
| Body weight (g) | 179.5±2.9 | 167.6±6.1 | 165.6±4.6* | 153.2±3.3*** |
| Weight of interscapular and cervical BAT (mg) | 322±21 | 307±10 | 451±46* | 446±27** |
| Total protein mg/BAT | 21.0±1.5 | 26.8±2.0 | 38.8±2.3*** | 39.5±3.2*** |
| Pelleted Mitochondrial protein (mg/BAT) | 6.02±0.31 | 5.81±0.42 | 7.22±0.40* | 7.52±0.27** |
| Total UCP content (μg/BAT) | 513±20 | 546±29 | 979±54*** | 892±71*** |
| Total cytochrome C oxidase activity (μmol cytochrome C oxidized/min) | 799±73 | 439±46** | 1047±69 | 1087±79* |
| UCP (μg/Cytochrome C oxidase activity) | 0.659±0.047 | 1.245±0.092*** | 0.958±0.107* | 0.826±0.051* |
| UCP (μg/mg mitochondrial protein) | 85.5±6.0 | 94.1±5.8 | 136.0±8.0*** | 118.9±7.8*** |

Data are expressed in means±SEM and there were 5 rats in each group. Significantly different compared with Day 0 value by ANOVA and Duncan's multiple range test: *$p<0.05$, **$p<0.01$, ***$p<0.001$.

# 4. DISCUSSION

## 4.1. GDP Binding Assay of BAT Mitochondria

The method of mitochondrial isolation from BAT (Cannon and Lindberg 1979) was found to be reliable as judged by electron microscopy. The preparation appeared to be homogeneous and contained mainly mitochondria.

The Bmax value of GDP binding of BAT mitochondria from control rats found in the present study (Fig. 2.4) is similar to that reported by others (McDonald, Horwitz, Hamilton and Stern 1988b; Allars, Holt and York 1987; Peachey et al. 1988; Nedergaard and Cannon 1985). Values reported for kd vary widely. Brooks et al. (1980) and Sundin and Cannon (1980) reported kd values ranging from 0.12 to 0.8 μmol/L, different from the kd value of 13 μmol/L observed in cold adapted rats by Ricquier, Mory, Nechad and Hemon (1979). The kd values have been shown to vary from 4 to 34 μmol/L depending on the pH of the incubation medium in cold adapted hamsters (Nicholls 1976). The kd of 2.20 μmol/L found here in control rats kept at room temperature lies within the range reported.

After noradrenaline injection, there was a significant increase in Bmax. This increase was nearly 50% and close to the results reported by Brooks, Rothwell and Stock (1982). However, Desautel and Himms-Hagen (1979) reported a doubling of Bmax after noradrenaline treatment.

There was a 4-fold increase in Bmax of GDP binding in cold acclimated rats, in agreement with findings reported by others (Desautels et al. 1978; Ashwell et al. 1983a; Nedergaard and Cannon 1985; Peachey et al. 1988). The kd value increased from 2.20 to 3.41 μmol/L after cold adaptation. This suggests that there was a decrease in the affinity of GDP after cold treatment. Peachey et al. (1988) also reported an increase in kd to 0.73 μmol/L from 0.56 μmol/L in 5 days cold acclimated rats. Noradrenaline administration (25 μg /100 g BW), to cold acclimated rats did not induce a further rise in Bmax of GDP binding (Fig. 2.6).

Many studies have demonstrated that cold acclimation for a day causes an increase in noradrenaline turnover in BAT. Acute cold exposure for an hour was also shown to cause a comparable elevation of noradrenaline turnover in BAT (Young et al. 1982; Murazumi, Yahata & Kucoshima 1987; Crist & Romsos 1987). Cottle, Nash, Veress & Ferguson (1967) showed that BAT from cold acclimated rats has a greater noradrenaline concentration than controls, while in other studies (Kennedy, Gannibd & Hamolsky 1977; Laury, Beauvallet & Portet 1982; Young et al. 1982) noradrenaline content was not altered by cold treatment. Although there are discrepancies in the literature regarding noradrenaline content of BAT in cold adapted rats, there is unanimous agreement that there is an increase in the turnover of noradrenaline in BAT during cold acclimation. The lack of further enhancement in GDP binding by

noradrenaline may be due to the already high turnover of noradrenaline in cold adapted rats. The other possible explanation is that thermogenic capacity may have already reached its full capacity in chronic cold acclimated rats.

Food restriction decreased BAT mass, protein content, mitochondrial protein, and GDP binding activity in rats kept at room temperature (Rothwell and Stock 1982). The suppression of hypertrophy of BAT seen in cold adapted rats by food restriction in the present study was also observed by Johnson, Murry, Young and Landsberg (1982) who reported that there was no alteration in noradrenaline content in BAT with or without food limitation in cold adapted rats. This led them to conclude that increased food intake contributed to BAT hypertrophy in cold exposure independent of sympathetic activity. However, in their experiment only BAT weight and protein content were reported and the thermogenic function of BAT was not measured. The present finding clearly shows that the cold induced increase in thermogenesis in BAT was not affected by food restriction. On the other hand, the hypertrophy of BAT seen during cold acclimation was suppressed when food was restricted. Therefore increased food intake during cold adaptation plays only a minor role in the increased thermogenic function of BAT.

No difference in the Bmax of GDP binding in BAT mitochondria of rats aged 4 to 11 weeks (Fig. 2.7) could

be demonstrated. The Bmax in these animals is similar to those reported by Brooks et al. (1982) and McDonald et al. (1988a). McDonald et al. (1987) have reported that there is no difference in GDP binding between 5 and 26 months old in Sprague Dawley rats. However others have shown that in rodents there is a decrease in BAT thermogenesis with age (Horan, Little, Rothwell and Stock 1988, in BN/BiRij rats; McDonald et al. 1988, in Fischer rats; Ashwell et al. 1985, in mice and rats) as shown by a marked decline in GDP binding, protein content, and mitochondrial cytochrome oxidase activity. Furthermore, there is an age-associated decrease in $\beta$-adrenergic receptors and adenylate cyclase activity in rats (Scarpace et al. 1988b). Since only two age groups of rats were studied here, and their age difference was only 7 weeks, a definite conclusion cannot be drawn about the effect of age on GDP binding capacity of BAT but it would appear likely that thermogenic function declines with age.

From the results of the above studies, it is clear that the GDP binding assay can detect changes in thermogenic function under various conditions : cold acclimation, noradrenaline stimulation, and food restriction. Therefore the GDP binding assay described here can be considered to be a reliable method for monitoring the GDP binding capacity of UCP and was used subsequently to monitor activity of UCP during various stages of its isolation and purification from BAT mitochondria.

## 4.2. Isolation and Purification of UCP

The results with four separate preparations of rat UCP are shown in Table 2.4. These are similar to the finding of Lean, M. (personal communication) who obtained a final yield of 1.3 to 4.6%. The washing step after Lubrol PX extraction was employed here to reduce interference from mixed-miscelles of Lubrol PX and TritonX-100.

In SDS-PAGE, the purified UCP had only one major band at around 32 kDa (see Fig. 2.12). However, there was also a very faint band at 64 kDa corresponding to an aggregate of two molecules of the UCP. Lin and Klingenberg (1980 and 1982) found an internal cross-linking in a preexisting dimer. Other workers also have experienced difficulty in avoiding aggregation of membrane proteins in the presence of SDS (Lean, personal communication).

GDP binding capacity is a reliable index for monitoring possible degradation in the isolated UCP during purification of UCP. GDP binding activity was 2.76 pmol/mg protein in mitochondria and became 42.39 pmol/mg protein after the last purification step, an approximately 15-fold increase. This accords with the degree of purification achieved by Lin and Klingenberg (1980). The percentage recovery was 65.9%, a value slightly higher than that (58%) reported by Lin and Klingenberg (1980).

The Bmax and kd of purified UCP were determined by Scatchard analysis. Bmax of purified UCP was 30,050 pmol/mg protein and much greater than 1385 pmol/mg protein found for the intact mitochondria of cold adapted rats. The higher Bmax of purified UCP indicates that there was an increase in purity, and that the native conformation of UCP was well maintained during isolation. French, Gore and York (1988) have suggested that there is probably one binding site per molecule of UCP and this is supported by the results of equilibrium-dialysis in the present study. From the Scatchard plot, the maximum binding value of pure UCP was calculated to be 0.939 mol GDP/mol UCP, close to the value for one binding site per molecule of UCP.

It has been reported that the binding of GDP to UCP is pH dependent and kd increases with increasing pH (Lin and Klingenberg 1982; French et al. 1988). In the present study there was an increase in kd value of purified UCP (13.64 μmol/L) compared to intact mitochondria (3.41 μmol/L) and indicates that there was a decrease in binding affinity of purified UCP to GDP. Lin and Klingenberg (1982) have reported that kd for UCP was 2.5 μmol/L using equilibrium dialysis method. The difference between the present result and that of Lin and Klingenberg (1982) may be due to the following reasons: different assay methods used, structural change of UCP during purification or pH of the medium. Further investigations are required to find out the exact cause.

The high content of UCP in mitochondria (from 0.96% to 1.95% of mitochondrial protein shown in Table 2.4) is similar to the figure of 1.3% reported by Lin and Klingenberg (1980). In another study Lin and Klingenberg (1982) reported that UCP comprises 6-8% of total mitochondrial protein, 14% of membrane protein, and amounts to about 2.6 molecules of UCP per molecule of cytochrome $aa_3$. These emphasize the key role of UCP in the BAT.

Using the modified method of Lin and Klingenberg (1980), it has been possible to obtain reliably pure UCP with high GDP binding capacity. The washing step to remove Lubrol PX and the use of Bio-Beads to remove unbound TritonX-100 were simple steps which increased purification.

**4.3. Development and Evaluation of ELISA**

Four rabbits were injected with purified UCP and only the antiserum of the one with the highest titre (rabbit 2) was chosen for characterization and use in ELISA. The absence of cross-reactivity of this antiserum to mitochondria of various tissues such as white adipose tissue, liver, and skeletal muscle clearly indicated that the antiserum reacted specifically with UCP from BAT mitochondria, that is, the antiserum seemed to be tissue specific. The exclusive occurrence of UCP antigen in BAT has been reported by Cannon et al. (1982), who used anti-

UCP antiserum to test cross-reactivity with mitochondria from white fat, liver or heart muscle. Similar findings have also been shown by Lean et al. (1983); Henningfield, Gribskov and Swick (1985); Henningfield and Swick (1987). Study of the species specificity of the antiserum showed that it had a strong cross-reaction with UCP from other rodents. Henningfield and Swick (1987) have also found that anti-rat-UCP antiserum was not species specific, but cross-reacted with BAT from mammals such as monkey, and guinea pig. Controversial results have been reported for white adipose tissue mitochondria. Cannon et al. (1982) reported a slight reaction with hamster white adipose tissue but the reaction was regarded as nonspecific. Young, Arch and Ashwell (1984) have described UCP in the parametrial fat pads of mice. These inconsistencies may be due to differences in the polyclonal antibodies and the methods of assay used. In those cases where results suggested that UCP may occur in white adipose depots it is uncertain whether this is due to its normal presence or persistence of brown adipocytes (Henningfield and Swick 1987). UCP was not found in white fat from cold adapted rats (Fig. 2.16).

In the present study the SDS-PAGE was deliberately overloaded with liver mitochondrial protein to demonstrate the possible contamination with ADP-ATP translocase in the UCP preparation. The antiserum did not react with the liver mitochondria indicating lack of cross-reactivity with the ADP-ATP carrier and attesting to the purity of

the antigen.

The reaction of rabbit antiserum to UCP and BAT mitochondria was dose-dependent as shown in immuno-autoradiography and the ELISA, indicating that the antiserum could be used to measure the content of UCP in BAT mitochondria. The intra- and inter-assay precision in ELISA were acceptable.

Before the development of the specific immunoassay for UCP, SDS-PAGE and densitometric tracing were used to detect changes in the amount of the 32 kDa MW protein in BAT mitochondria (Ricquier and Kader 1976; Desautels et al. 1978; Gribskov et al. 1986). Recently measurement of UCP by immunoassay has been developed. Cold induced increase in UCP content measured by immunoassay has been reported (Nedergaard and Cannon 1985; Trayhurn et al. 1987; Peachey et al. 1988). The duration of cold exposure necessary to induce a detectable increase in UCP content varied from 1 to 24 h.

The reported values of UCP concentration vary from 8 to 43 μg/mg mitochondrial protein in rodents kept at room temperature (21 to 22 C°) (Nedergarrd and Cannon 1985; Henningfield and Swick 1987; Lean et al. 1983; Ashwell et al. 1985; Henningfield and Swick 1987; Sundin et al. 1987; Trayhurn et al. 1987; Peachey et al. 1988). In the present study it was 85.5±6.0 μg/mg mitochondrial protein in warm rats, a value higher than that reported in the literature.

The discrepancy may be due to different species and different immunoassay methods use.

In the present study, after 4 and 6 days of cold acclimation, there was a significant elevation in total UCP content and in UCP concentration but there was no detectable increase in UCP content or concentration after one day of cold exposure. In a study of lean Zucker rats by Peachey et al. (1988) there was a two-fold increase in UCP concentration after 1 day and a three-fold increase after 6 days. However other reported results (Lean et al. 1983; Nedergaard and Cannon 1985; Sundin et al. 1987) were closer to those found here. There was slightly more than a two-fold increase in UCP concentration after 4 days exposure to 4°C. The change in GDP binding seemed to be more prominent than UCP concentration during chronic cold adaptation. GDP binding increased 4-fold after 10 days cold acclimation in rats whereas UCP content increased about 2-fold after 4 days cold acclimation. Since the duration of cold exposure was different in these two studies, it is difficult to draw a conclusion as to whether there is a parallel change in UCP content and GDP binding in long term cold adaptation. Acute cold exposure from 2 to 4 h significantly increases GDP binding without a change in UCP concentration in Zucker rats (Peachey et al. 1988) and therefore causes a higher ratio of GDP binding to UCP. After 24 h of cold exposure, both GDP binding and UCP doubled, restoring the ratio to that in control animals (Peachey et al. 1988). In chronic cold

acclimation (5 days) there are parallel increases in both GDP binding and UCP content (Peachey et al. 1988). This supports the hypothesis that UCP exists in both non-functional (masked) and functional (unmasked) forms. The increase in binding in acute response to cold is due to the unmasking of existing UCP rather than to an absolute increase.

In the present studies the UCP data are presented as total UCP content per interscapular BAT pad (UCP μg/BAT) instead of the more commonly used expression i.e. in relation to mitochondrial protein (i.e. μg/mg mitochondrial protein). Since mitochondrial proteins may change in various physiological conditions such as cold and age, a change in the value of UCP μg/mitochondrial protein may arise from such changes. As stressed by Trayhurn in the 452th Scientific Meeting held in Dublin by the Nutrition Society (1988), the concentration of UCP in mitochondria determines the capacity of the proton conductance pathway, while the total amount in a BAT depot sets the thermogenic capacity of that particular depot (Trayhurn 1989). Therefore, in order to show the total thermogenic capacity of interscapular BAT, the total amount of UCP per interscapular BAT pad has been presented throughout.

Increases in total protein and BAT weight suggested that there was hypertrophy of BAT during cold acclimation. the higher cytochrome C oxidase activity also indicated there was an increase in the mass of mitochondria during

cold adaptation. The increase in the amount of UCP during cold adaptation may come from de novo synthesis which was suggested by Desautels and Himms-Hagen (1979) who used inhibitors of protein synthesis to prevent adaptation to cold. It was further confirmed by Ricquier et al. (1986) who reported an increase synthesis of mRNA encoding for the UCP in brown adipocytes during cold adaptation. The ratio of UCP to cytochrome C oxidase was higher in all cold exposed rats than that in control rats indicating a high thermogenic capacity in mitochondria of BAT after cold acclimation.

In summary the ELISA developed in our laboratory was a reliable tool to assay UCP content.

# CHAPTER III

# CHANGES IN BAT DURING PREGNANCY AND LACTATION AND ROLE OF PROLACTIN

## 1. INTRODUCTION

For nearly two decades, studies have been focused mainly on DIT and the role of BAT in the aetiology of obesity. However the importance of BAT in whole-body energy metabolism extends to other physiological situations. Recently several studies have highlighted the importance of BAT not only in DIT and NST, but also in the energy balance during pregnancy and lactation.

In late pregnancy in rats and mice, there is a reduction of GDP binding to BAT mitochondria (Tatelman et al. 1985; Andrews et al. 1986). During lactation in mice there is functional atrophy of BAT such as decreases in mitochondrial GDP binding and UCP content (Ashwell, Jennings and Trayhurn 1983b; Trayhurn and Jennings 1987). However there are no reports on the UCP content during various stages of pregnancy and lactation.

During late pregnancy and lactation, there is a dramatic rise in PRL concentration (Amenomori et al. 1970). The physiological actions of PRL include

proliferation of the mammary gland, initiation of milk secretion and maintenance of corpus luteum in the ovary. The rise in PRL level and fall in BAT thermogenic function during lactation coincide and this led to the suggestion that PRL may be involved in the suppression of BAT thermogenesis.

PRL secretion from the pituitary gland is tonically inhibited under normal circumstances by the influence of the hypothalamus. The hypothalamus secretes a PRL-inhibiting factor which travels to the anterior pituitary via the hypophysial portal system and there is increasing evidence to suggest that dopamine is the major component. Several methods can be used to alter serum PRL level either by introducing exogenous PRL or by suppression or stimulation of endogenous PRL secretion using drugs.

Metoclopramide is a non-selective dopamine receptor antagonist and acting by accelerating the turnover of dopamine (Hassan, Reches, Kuhn, Higgins and Fahn 1986) and it is a potent agent for enhancing PRL secretion. Metoclopramide enhances PRL secretion in rats (Scott, Lakin and Oliver 1987; van der Niepen, Dupon, Buydens, Smitz and Vanhaelst 1987; Lipham, Portor, Norred, Booth and Robbins 1987) and it was used in this study to investigate the effect of PRL on UCP content of BAT.

Bromocriptine, an ergot derivative comprising a

lysergic acid residue with a cyclic polypeptide moiety, has extensive clinical use as a highly successful agent for inhibiting PRL secretion (see review by Berde 1978; Johnson, Loew and Vigouret 1976; Lipham et al. 1987). This drug was used to suppress PRL secretion in normal rats, or rats with high PRL level induced by metoclopramide.

In this section UCP content of BAT throughout pregnancy, lactation and weaning is described and the role of PRL examined.

## 2. MATERIALS AND METHODS

### 2.1. Animal

Rats weighing from 250-300 g (age 3-4 months) were used in the present study. They were housed in a room at a temperature of 21 ± 1°C and fed ad libitum with laboratory chow and tap water.

### 2.2. Experimental Designs

#### 2.2.1. Effects of Pregnancy, Lactation and Post Weaning on BAT

Virgin female rats were mated with male rats of proven fertility. Pregnancy was confirmed by the presence of a vaginal plug in the bedding and its appearance was taken as the first day of pregnancy. Pregnancy was confirmed, where possible, by postmortem examination. The length of gestation in this colony was between 20-22 days. For each lactating rat, the size of the litter was made up to 10 by adding or removing litter. In one group (post weaning group) the litter was removed after 20 days of lactation and the rats were sacrificed 7 days later. Rats were kept individually throughout the experiment except during the time of mating. The daily food consumption was monitored. The amount of chow consumed was obtained by

subtracting the amount left after 24 h from the amount provided (50g chow). No correction was made for spillage.

The rats were studied at various times during pregnancy and lactation: at 10 days and 20 days of pregnancy, also at 3, 10, 16 and 20 days after the start of lactation and 7 days post weaning. Unmated female rats served as controls. At times indicated in the text the rats were bled by cardiac puncture under light ether anaesthesia and the serum was stored at -70°C for PRL assay. The animals were sacrificed by cervical dislocation and interscapular BAT was removed for analysis.

### 2.2.2. Effect of Metoclopramide on BAT

Metoclopramide, (Primperan, Laboratories Delagrange, Chilly-Mazarin) 5 mg/ml was used in this study. Rats were divided into three groups of 7 and received one of the following treatments: (1) 0.15 mol/L NaCl - control group, (2) metoclopramide 0.2 mg/kg BW or (3) metoclopramide 1.67 mg/kg BW. These injections were given subcutaneously twice daily for 5 days. The animals were killed on day 6 and BAT removed for analysis.

### 2.2.3. Effect of Metoclopramide and Bromocriptine on BAT

Bromocriptine mesilate (Parlodel, Sandoz Pharma-

ceuticals, Basle) was suspended (1 mg/ml) in 0.15 mol/L NaCl solution. Rats were divided into four groups of 7 rats each and they received one of the following treatments: (1) 0.15 mol/L NaCl - control group, (2) metoclopramide 1.67 mg/kg BW, (3) bromocriptine 2 mg/kg BW and metoclopramide 1.67 mg/kg BW or (4) bromocriptine 2 mg/kg BW. All treatments were given subcutaneously twice daily for 5 consecutive days. In this experiment the animals were bled by cardiac puncture 1 h after the injection of drugs on day 5 for the measurement of serum PRL concentration. The rats were killed on day 6 and BAT was taken for UCP assay.

### 2.2.4. Effect of PRL Injection on BAT

Rat-PRL (NIDDK-Rat-PRL-B-6, batch No. AFP-7545E) 1.335 mg/ml was dissolved in 0.03 mol/L of $NaHCO_3$ in 0.15 mol/L NaCl. Rats were divided into two groups of 8 each and they were injected with PRL (0.104 mg/rat) or saline subcutaneously twice daily for 5 consecutive days. On the morning of day 6, the animals were killed and interscapular BAT was collected from each rat for UCP analysis.

### 2.2.5. Continuous infusion of PRL

Continuous infusion of PRL was carried out by implantation of a mini-osmotic pump (Alzet, Model 2ML2) in

rats under the skin on the dorsal surface of rats. The mini-osmotic pump was filled with 2.1 ml of PRL solution (1.335 mg/ml of rat-PRL (NIDDK PRL-B-6) in 0.03 mol/L $NaHCO_3$ in 0.15 mol/L NaCl, pH 8.5) or vehicle. Before the implantation, the mini-osmotic pump was immersed in 0.15 mol/L NaCl for 2 h to let the pump imbibe the fluid in order to make the pump more effective in delivering the fluid. The mean pumping rate according to the instruction manual was 4.49 ± 0.21 $\mu$l/h (Mean ± SD) and the rate of PRL infusion was calculated to be 100 ng/min. Eight rats were infused with PRL and 8 rats received the vehicle.

The animals were anaesthetized by intraperitoneal injection with pentabarbitone 35 mg/kg BW and the surgery was performed under aseptic conditions. A 1.5 cm incision was made on the dorsal surface of the rat and a tunnel was made under the skin by loosening the connective tissue with a pair of blunt forceps. The mini-osmotic pump was then implanted and the incision was sutured with surgical silk. The animals were allowed to recover without further disturbance.

The implants were left in the rats for 5 days and on the morning of day 6 blood samples were taken by cardiac puncture and BAT was removed as previously described.

### 2.6.6. Measurements of BAT Parameters

BAT from interscapular region was obtained from rats in experiments and homogenized. Protein and UCP were assayed as described in Chapter II, Sections 2.5.1. and 2.11. respectively.

### 2.2.7. RIA of Serum PRL

Serum PRL was measured by RIA using reagents kindly supplied by the National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health (NIDDK, NIH). The methods of PRL RIA and iodination of PRL were similar to methods recommended by NIDDK. PRL (NIDDK-rat-PRL-I-5) was iodinated and purified by gel filtration chromatography on a sephadex G100 column.

(A) Iodination of rat-PRL

The basic principle in the iodination is the introduction of radioiodine into a molecule by oxidation of sodium $^{125}$Iodide to reactive $^{125}I_2$. Iodine will react with the aromatic ring structure of a molecule, such as tyrosine resulting in the incorporation of $^{125}$I. The oxidizing agent can be a strong oxidant or an oxidant produced by enzymatic reaction. There are three common

methods of iodination: chloramine-T, lactoperoxidase and Iodogen. Chloramine T was used for the iodination of PRL in this study.

(1) Reagents

(a) Sodium $^{125}$Iodide (Amersham), 0.5 mCi in 5$\mu$l was used for iodination.

(b) 0.5 mol/L sodium phosphate was adjusted to pH 7.6.

(c) Phosphate buffered saline: 0.01 mol/L $PO_4$, 0.15 mol/L NaCl, was adjusted to pH 7.6.

(d) Chloramine-T, 10 $\mu$l of 10 mg dissolved in 20 ml of phosphate buffered saline, prepared immediately before use.

(e) Sodium metabisulfite ($Na_2S_2O_5$), 100 $\mu$l of 5 mg dissolved in 20 ml of phosphate buffered saline, immediately prior to use.

(f) PRL (NIDDK-Rat-PRL-I-5) 5 $\mu$g was dissolved in 10 $\mu$l of 0.01 mol/L $NH_4HCO_3$, pH 8.5.

(2) Labelling procedure

(a) 0.5 mCi $^{125}$I was added to a small disposable plastic

vial which was used as the reaction vessel.

(b) 25 $\mu$l of 0.5 mol/L $PO_4$, pH 7.6, was added.

(c) Next 2 $\mu$g of PRL was added.

(d) 10 $\mu$l of Chloramine-T was then added. The vial was agitated for exactly 10-12 sec.

(e) 100 $\mu$l of sodium metabisulfite was added to stop the reaction. The entire reaction mixture was applied to a Sephadex G-100 column (about 10 cm long and packed in a 10 ml glass pipette and pre-equilibrated in phosphate buffered saline with 0.1% sodium azide). The column was eluted with phosphate buffered saline. Fractions of 0.5 ml were collected in small test tubes, containing 2 drops of 2% bovine serum albumin in phosphate buffered saline.

(f) From each fraction, 5 $\mu$l was aliquoted, diluted 100 -fold and 10 $\mu$l of the diluted fraction was transferred to a counting tube and counted in a gamma counter (Kontron) to locate the iodine peak.

(g) Two peaks were found in the elution profile (pattern is similar to Fig. 3.1). The first peak was $^{125}I$ labelled PRL and the second peak was excess $^{125}$iodide.

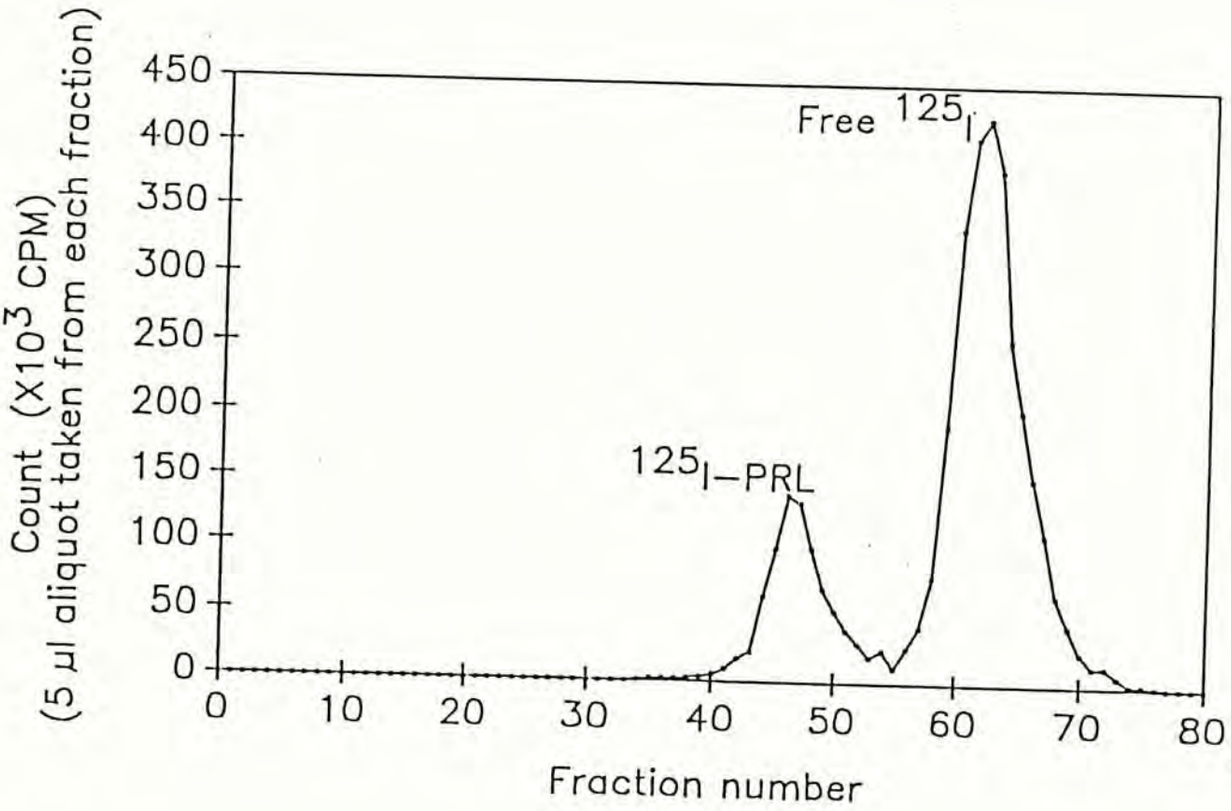



Fig. 3.1 Elution profile of free $^{125}$Iodide and $^{125}$I-PRL by G-100 gel filtration column chromatography.

(h) Fractions containing the first peak were pooled together and then stored in aliquots at -20°C for later use in PRL RIA.

(B) Reagents and Procedure for PRL RIA

(1) Reagents:

(a) Antiserum to rat-PRL (NIDDK-Anti-Rat-PRL-S-7) was diluted thousand fold in phosphate buffered saline (0.01 mol/L phosphate, 0.15 mol/L NaCl, 0.05 mol/L EDTA, 0.1% sodium azide and pH 7.6).

(b) Stock PRL standard (NIDDK-Rat-PRL-RP-3) was prepared by dissolving 10 $\mu$g in 1 ml of distilled water. Aliquots were stored at -70°C. Serial dilutions of PRL were made to give working standards ranging from 0.25 to 200 ng/ml.

(c) 2% of normal rabbit serum solution.

(d) Goat anti-rabbit immumoglobin/PEG precipitating solution (Diagnostic Products Corp. CA) was used to precipitate the antigen-antibody complex.

(e) $^{125}$I-PRL was diluted to contain about 20,000 dpm in 100 $\mu$l of phosphate buffered saline.

(2) Procedure:

(a) All samples were assayed in duplicate. 50 μl of serum sample or PRL standard was mixed with 150 μl of 1% BSA in phosphate buffered saline containing 0.1% $NaN_3$ in a disposable plastic vial. For samples with a low concentration of PRL, 100 μl serum was mixed with 100 μl of 1% BSA solution. The rest of the procedure was the same.

(b) 100 μl of 2% normal rabbit serum solution, 100 μl of $^{125}I$-labelled rat-PRL solution, and 100 μl of anti-rat-PRL antiserum were added and mixed well.

(c) The mixture was incubated overnight for 16 h at room temperature.

(d) After incubation, 200 μl goat anti-rabbit gamma globulin/PEG precipitating solution was added and mixed, and then centrifuged at 11,000 rpm for 5 min at 4°C.

(e) The supernatant was aspirated and the pellet was washed with 1 ml cold phosphate buffered saline and centrifuged at 11,000 rpm at 4°C for 5 min.

(f) The supernatant was aspirated and the pellet was counted for 2 min in a gamma counter. To determine

non-specific binding, normal rabbit serum was used instead of the antiserum. A typical standard curve is shown in Fig. 3.2. There is a gradual decrease in the ratio of percentage bound to total bound with increasing concentration of PRL.

### 2.2.8. PRL Receptors in BAT

PRL receptors in BAT were determined by radioreceptor assay. A large amount of BAT for membrane preparation was collected from rats cold adapted at 4°C for 5 days. The interscapular BAT was removed and frozen at -70°C prior to the preparation of microsomal membrane.

(A) Microsomal Membrane Preparation:

BAT was weighed and minced with scissors. It was homogenized by a motor driven homogenizer (Polytron) in ice-cold 0.3 mol/L sucrose (4:1, V/W) using a setting 7, for 6 X 30 sec. The homogenate was centrifuged at 10,000 xg at 4°C for 10 min. The supernatant was centrifuged at 105,000 xg (Beckman Ultracentrifuge L5-20, Rotor 42.1) for 1 h at 4°C. The pellet was resuspended in 25 mmol/L Tris buffer, pH 7.2 and stored at -70°C until use.

Liver and prostate gland were obtained from female and male rats respectively. Preparation of these tissues was similar to that for BAT membrane.

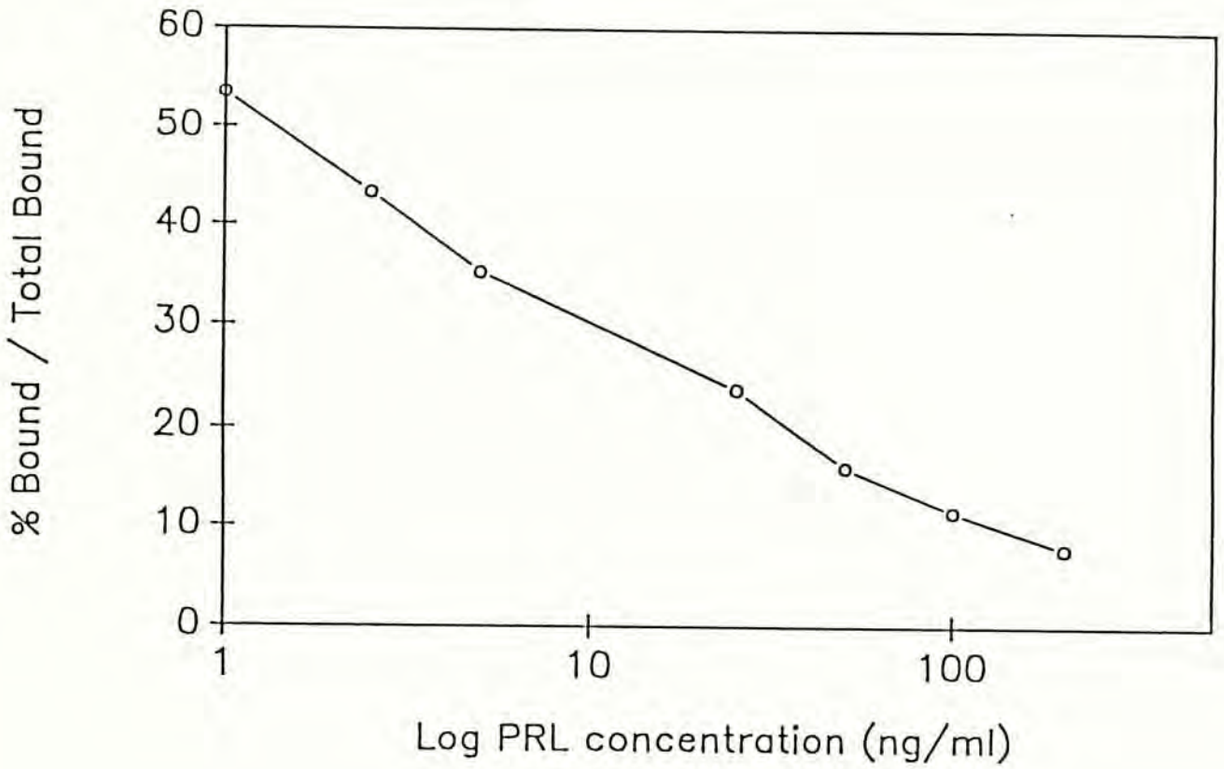



Fig. 3.2 A typical PRL standard curve. PRL concentration is plotted against % bound/total bound. Method is as decribed in section 2.2.7.

(B) Radioreceptor Assay:

(1) Solutions :

(a) Membrane buffer - Tris 25 mmol/L and pH was adjusted to 7.2 with 1 mol/L of HCl.

(b) Hormone buffer - Tris 25 mmol/L, BSA 0.5 g/100 ml and pH was adjusted to 7.2.

(c) Diluent - Tris 25 mmol/L, BSA 0.5 g/100ml, $CaCl_2$ 50 mmol/L and pH was adjusted to 7.2 with HCl.

(d) Stopping buffer - Tris 25mmol/L, BSA 0.4 g/100ml, $CaCl_2$ 56 mmol/L and pH was adjusted to 7.2 with HCl.

(2) Membrane : The frozen membrane was thawed at room temperature and was resuspended in membrane buffer.

(3) Ligand : PRL from two species (rat (NIDDK-Rat-PRL) and ovine (NIDDK-Ovine-PRL)) was used in the radioreceptor binding assay and iodination was carried as described below. Aliquots of $^{125}I$-rat-PRL or $^{125}I$-ovine-PRL were stored at -20°C. Before use, they were thawed and diluted to about 100,000 cpm/0.1 ml with hormone buffer.

(4) Iodination of PRL : A milder oxidation reaction using the lactoperoxidase enzyme was chosen for

iodination of PRL used in the receptor assay. Five μg rat-PRL or ovine-PRL (5 μl in 0.1 mol/L $NH_4HCO_3$, pH 8.5), 10 μl of $^{125}I$ (1 mCi) and 20 μl of 0.4 mol/L $CH_3COONa$ (pH 5.6) were mixed well. 5 μg/10 μl lactoperoxidase and 10 μl $H_2O_2$ (0.005%) were added, mixed immediately and incubated for 30 sec. The enzymatic reaction was stopped by addition of 100 μl stopping buffer and 50 μl BSA (5 g/100 ml). The reaction mixture was transferred to a G-100 gel filtration column (about 80 cm in length and 1 cm in diameter) and eluted with buffer; 1 ml fractions were collected in BSA coated glass tubes. Five μl from each fraction was transferred and counted in a gamma counter to locate the iodination peaks. There were 2 peaks of radioactivity (Fig. 3.1) (in page 194). Two fractions containing the highest count from the first peak were pooled and stored in small aliquots. The specific activity of labelled PRL was determined. Ten μl of the reaction mixture at the end of iodination was diluted 1,000 times with hormone buffer. 1 ml of this diluted solution was mixed with 2 ml of 1% trichloroacetic acid solution, and allowed to stand at 4°C for 2 h. The mixture was centrifuged at 3,500 rpm and 4°C for 20 min. The supernatant containing free $^{125}I$ was removed from the pellet which contained the labelled PRL. The radioactivity of the pellet was counted in a gamma counter. The specific activity of the labelled PRL

was calculated by dividing the radioactivity (μCi) by the protein content (μg). The specific activities for rat- and ovine-PRL were 33.4 and 260.0 μCi/μg respectively.

(5) PRL Receptor assay

All assays were performed in triplicate. Incubation was carried out in disposable polystyrene tubes. The incubation contained 200 μl diluent, 100 μl diluted membrane from rat liver, prostate gland or BAT, 100 μl hormone buffer (for specific binding) or 100 μl cold hormone (PRL 10 μg/ml in hormone buffer, for non-specific binding at every concentration of membrane protein) and 100 μl diluted ligand. Then it was mixed and incubated at room temperature for 16 h. The incubation was stopped by addition of 3 ml cold stopping buffer and centrifuged at 3,500 rpm for 45 min. The supernatant was decanted and the pellet was counted in a gamma counter. The non-specific binding to the tube was determined by adding 100 μl of membrane buffer instead of membranes.

**2.4. Statistical Analysis**

The results were expressed as mean ± SEM. The statistical significance of differences between groups was assessed by ANOVA and Duncan's multiple-range test.

# 3. RESULTS

## 3.1. Effects of Pregnancy and Lactation

### 3.1.1 Food Consumption and Body Weight

The daily food consumption in control, pregnant and lactation groups is illustrated in Fig. 3.3. There was no change in daily food intake in unmated controls during the 14 days. In the pregnant group there was a slight increase in daily food consumption from day 1 to 20 of pregnancy. When the mean daily food consumption was calculated as the average over 7 consecutive days, it was found that during mid and late pregnancy food consumption was significantly higher than that of the control group (Table 3.1), but not as high as in the lactating group. The mean daily food consumption during early (day 1-7), mid (day 8-14) and late (day 15-21) pregnancy were 22.0±0.2, 28.3±1.2, and 27.7±1.3 g/day respectively. The corresponding figure for the control group was 21.7±1.0 g/day.

The daily record of food consumption (Fig 3.3) shows that there was a gradual increase during lactation at the end of which the intake was almost 4 times higher in the unmated control group. When averaged over 7 days (Table 3.1), daily food intakes were 34.2±0.1 g/day in early lactation (day 1-7), 56.9±1.6 g/day in mid lactation (day 8-14) and 73.0±2.8 g/day in late lactation (day 15-

Table 3.1 The daily food consumption during pregnancy and lactation. The average of the daily food consumption from 7 consecutive days for each rat was calculated and the means±SEM for each group is given. Statistical comparison with the control group : ** $p<0.01$; *** $p<0.001$.

| | Daily food consumption (g/day) |
|---|---|
| Control | 21.7±1.0 |
| Pregnant | |
| Day 1-7 | 22.0±0.2 |
| 8-14 | 28.3±1.2 ** |
| 15-21 | 27.7±1.3 ** |
| Lactating | |
| Day 1-7 | 34.2±0.1 *** |
| 8-14 | 56.9±1.6 *** |
| 15-20 | 73.0±2.8 *** |

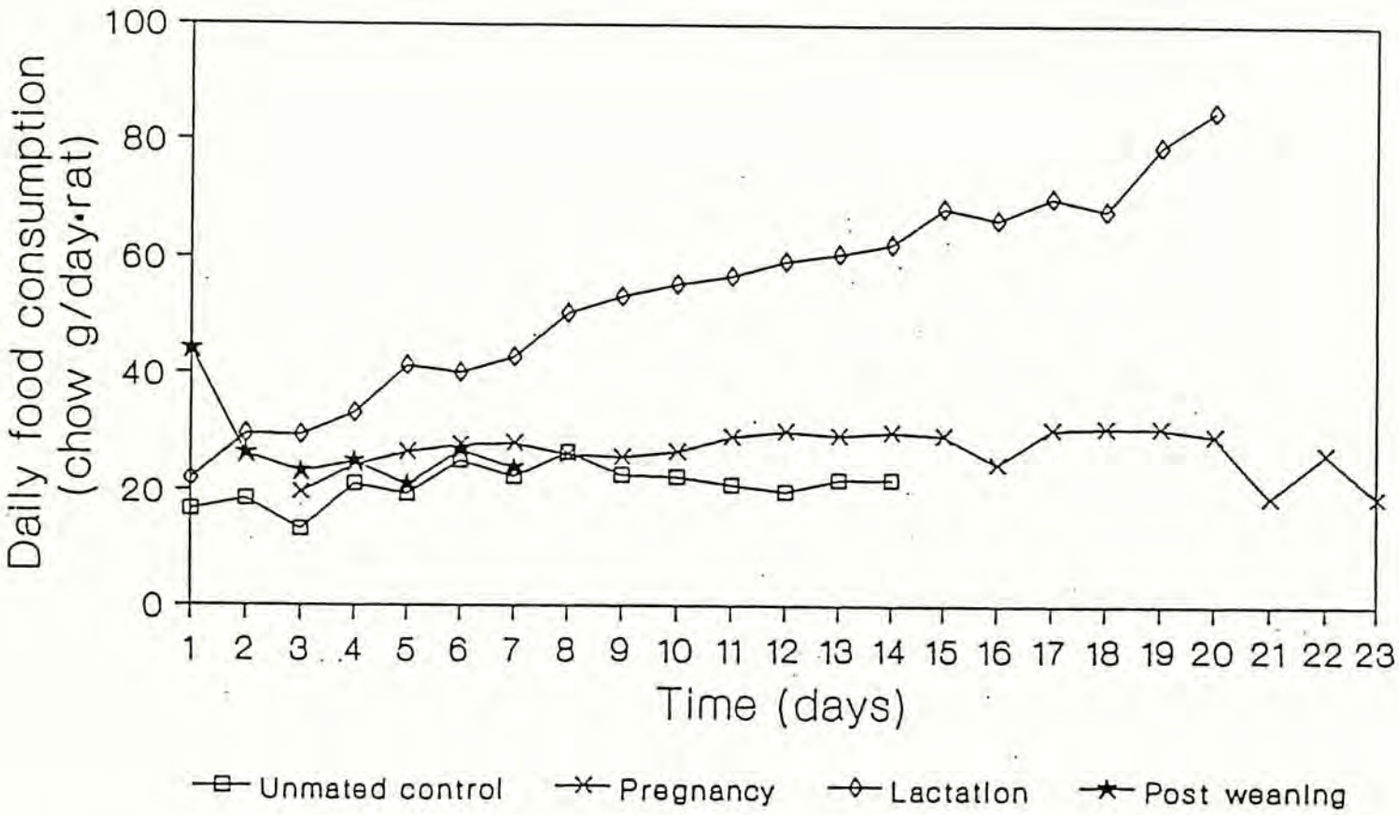



Fig. 3.3 Daily food consumption in pregnant, lactating and control rats. Food consumption was measured daily in 5 rats from each group. Mean values are given.

20). Food consumption throughout lactation was significantly higher than the control group ($p<0.001$). After weaning there was a fall of daily food consumption and the values for rats 4 days after weaning were similar to that of the controls.

Body weight during pregnancy and lactation are shown in Fig. 3.4. There was a gradual rise in body weight during pregnancy which became significant at mid and late pregnancy. Though there was a significant drop in body weight at parturition, the body weight remained slightly higher than the unmated controls during lactation (day 3 and day 10 of lactation) (Table 3.2). The body weight was similar to the control value on day 16 of lactation. In the late lactating group (day 20) and post weaning group, the body weight was significantly higher than that of the controls.

### 3.1.2. BAT

BAT weight at day 10 of pregnancy was higher than in the control group (Fig.3.5). In late pregnancy the mean BAT weight was higher than in the unmated control, but the difference was not statistically significant. During the whole period of lactation BAT weight was lower than in unmated controls; the lowest value was found on the day 3 of lactation. BAT weight tended to be higher towards the end of lactation, and 7 days after weaning was similar to that of the unmated control group.

Table 3.2 : Effects of pregnancy and lactation on body weight and BAT data.

| | Unmated | Days of pregnancy | | Days of Lactation | | | | 7 days after weaning |
|---|---|---|---|---|---|---|---|---|
| | control | 10 | 20 | 3 | 10 | 16 | 20 | |
| Rat number | 17 | 8 | 8 | 8 | 10 | 9 | 8 | 7 |
| Body weight (g) | 289±5 | 314±9** | 364±10*** | 312±3* | 317±6** | 284±6 | 353±9*** | 322±7** |
| BAT weight (mg) | 425±25 | 513±37* | 491±34 | 220±8*** | 270±9*** | 274±23** | 301±21** | 443±23 |
| Protein in Mitochondria (mg) | 5.07±0.47 | 5.61±0.28 | 2.44±0.21*** | 4.76±0.63 | 3.62±0.27* | 2.42±0.26*** | 2.71±0.21*** | 6.47±0.61 |
| Total UCP in BAT ($\mu$g) | 364±34 | 541±43** | 221±23** | 420±55 | 197±22** | 47±17*** | 32±9*** | 275±17 |

Values are means±SEM.

Significantly different (* $p<0.05$; ** $p<0.01$; *** $p<0.001$) compared with the unmated control group by analysis of variance and Duncan's multiple range test.

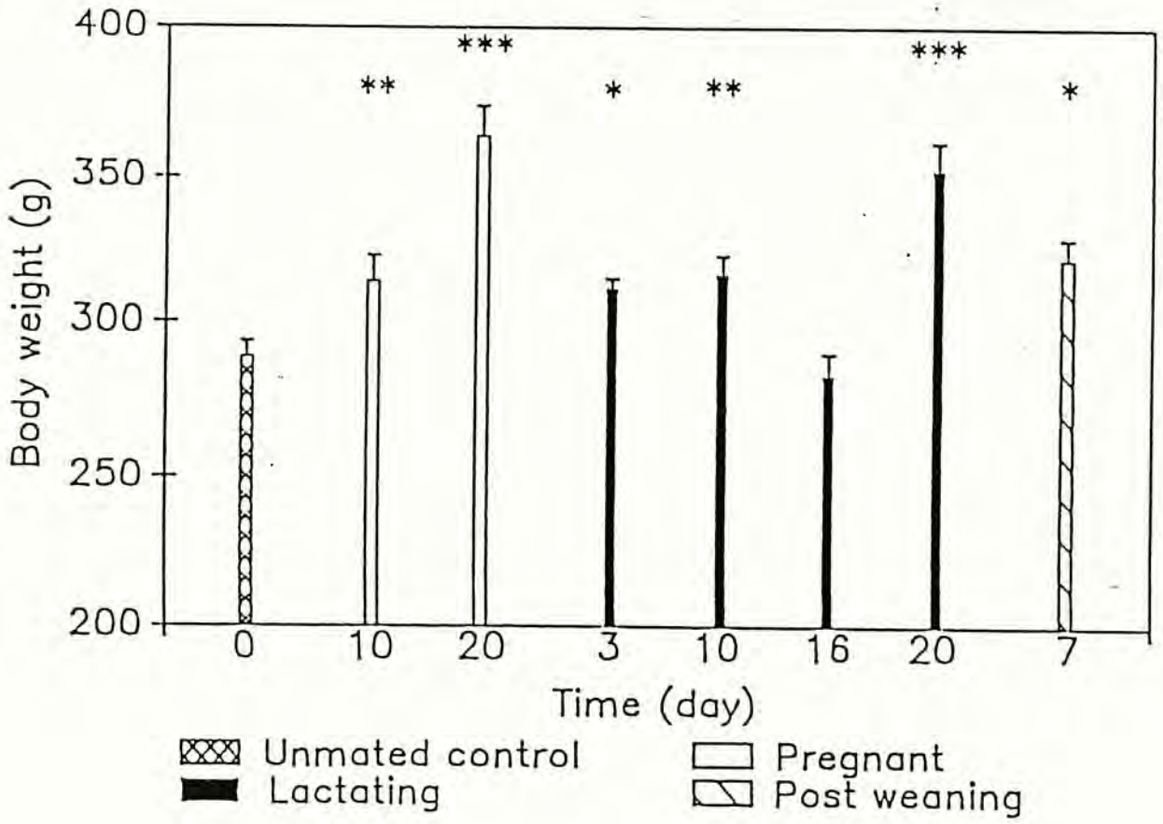



Fig. 3.4 Changes in body weight of control, pregnant, lactating, and post weaning rats. The number of rats in each group is given in Table 3.2. Values are means±SEM. Groups were compared with the unmated control group by ANOVA and Duncan's multiple-range test. The statistical differences are denoted: * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

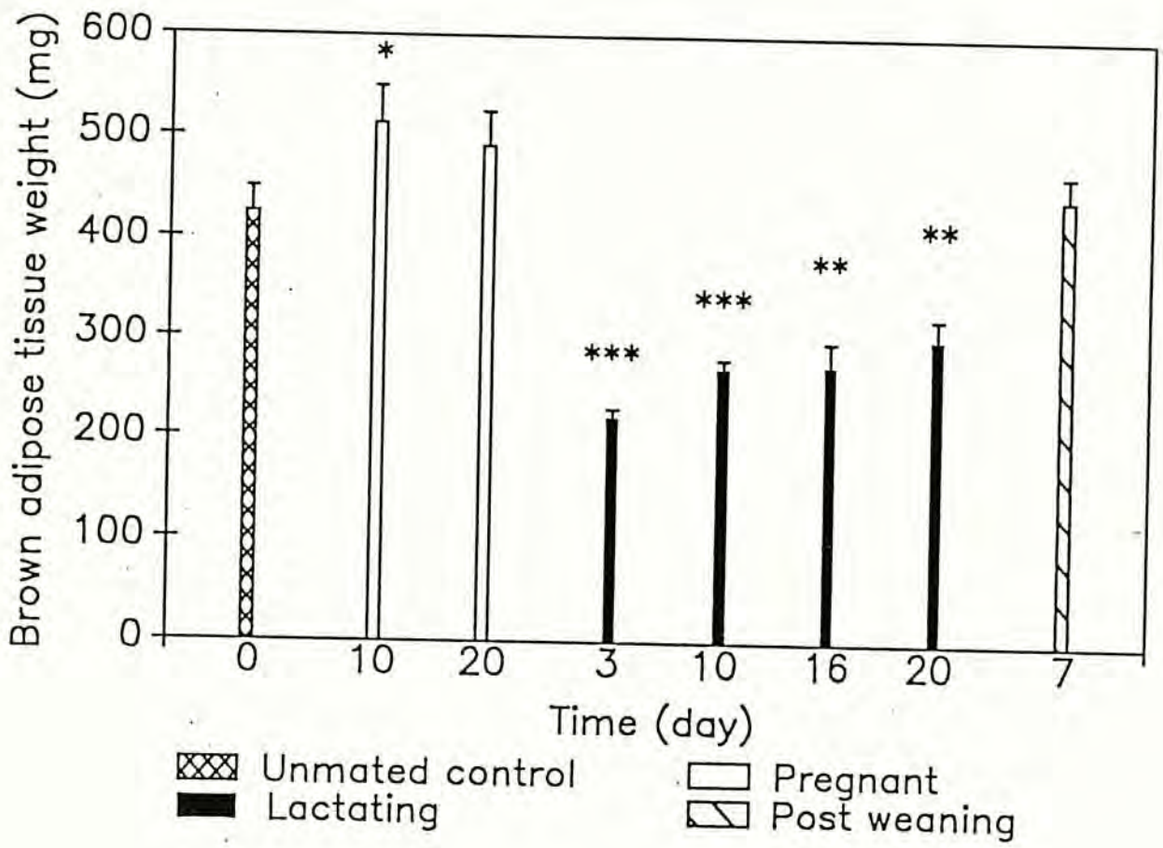



Fig. 3.5 Changes in interscapular brown adipose tissue weight during the breeding cycle in rats. Values are means±SEM. Groups were compared with the control group. Statistically significant differences are shown: * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

The protein content of BAT mitochondrial pellet started to decline in late pregnancy and remained low throughout lactation (Fig. 3.6) except in early lactation (day 3). The lowest value was observed on day 16 of lactation (Table 3.2). The protein content returned to the control value at 7 days after weaning.

Total UCP content in BAT increased in mid pregnancy (day 10) but decreased in late pregnancy and continued to decrease during lactation (Fig. 3.7). At day 10 of pregnancy UCP content was 541±43 μg and had decreased to 221±23 μg at day 20 of pregnancy (Table 3.2). The lowest value was found on day 20 of lactation (31.6 ± 9.3 μg/BAT) when it was 11 times less than that of the unmated control group (363.9 ± 34.1 μg/BAT). However 7 days after weaning the total UCP content had returned back to normal.

### 3.1.3. Serum PRL level

Serum PRL level was monitored during pregnancy, lactation and post weaning (Fig. 3.8). During pregnancy serum PRL concentrations fluctuated but were not significantly different from the control values. There was a dramatic increase in serum PRL level during lactation. At day 2 of lactation the serum PRL level was 94±49 ng/ml

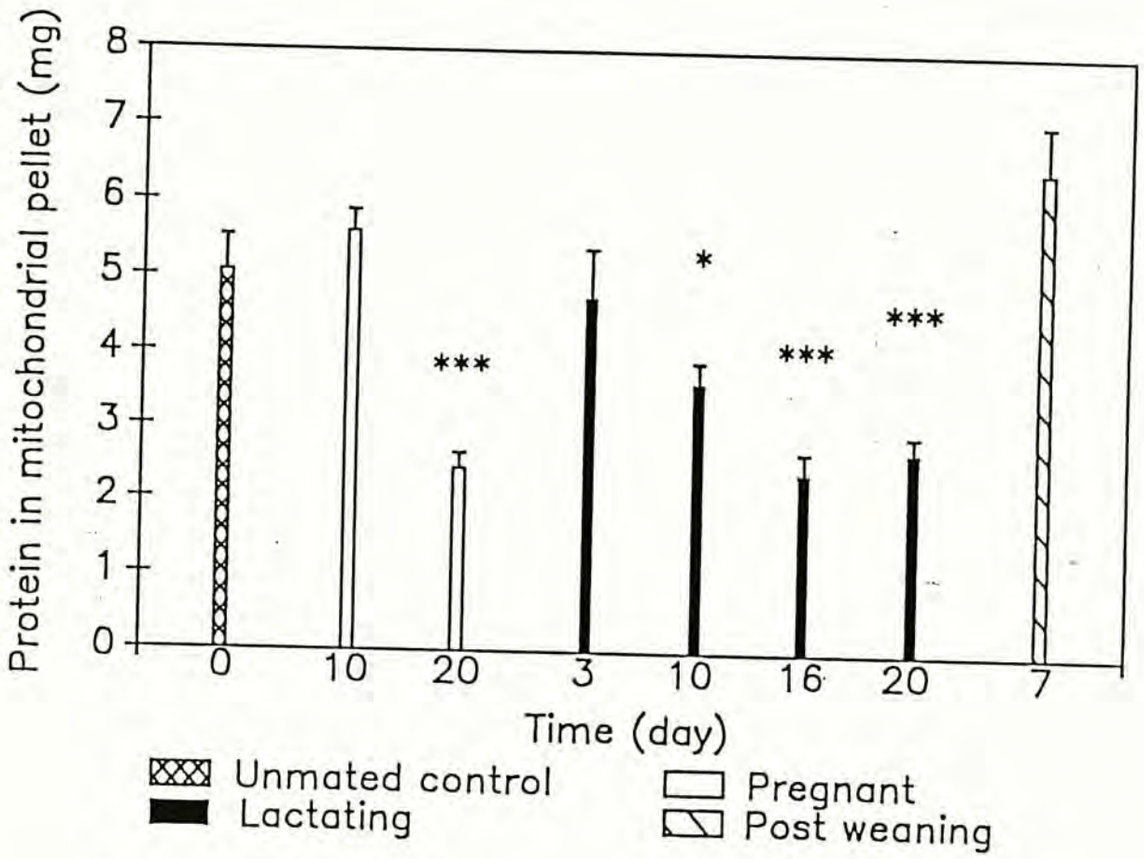



Fig. 3.6 Changes in protein content of BAT mitochondrial pellet in unmated control, pregnant, lactating or post weaning rats. Values are means±SEM. Significant differences compared with the control group are represented: * $p<0.05$; *** $p<0.001$.

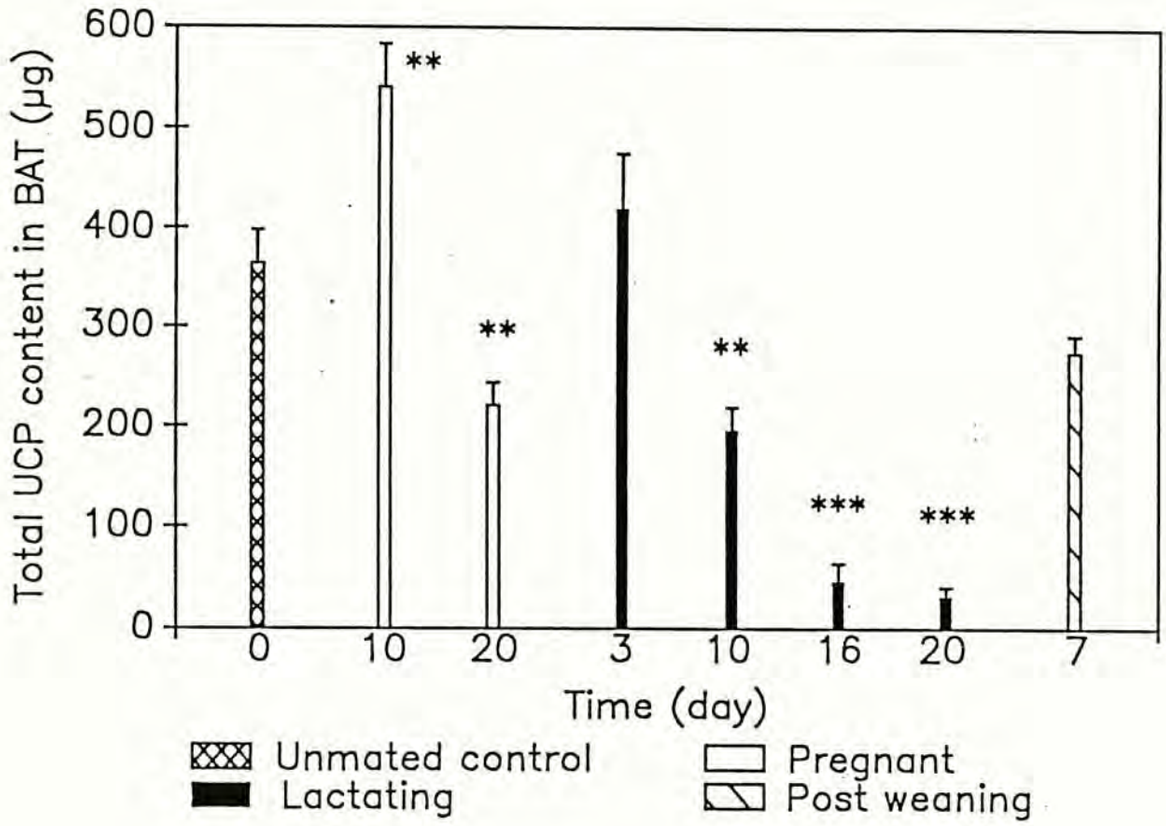



Fig. 3.7 The total UCP content of interscapular brown adipose tissue from unmated control, pregnant, lactating and post weaning rats. UCP content was measured by ELISA method as decribed in chapter 2, section 2.11. Values are means±SEM; Statistically significant results compared with the unmated control group are represented: ** $p<0.01$; *** $p<0.001$.

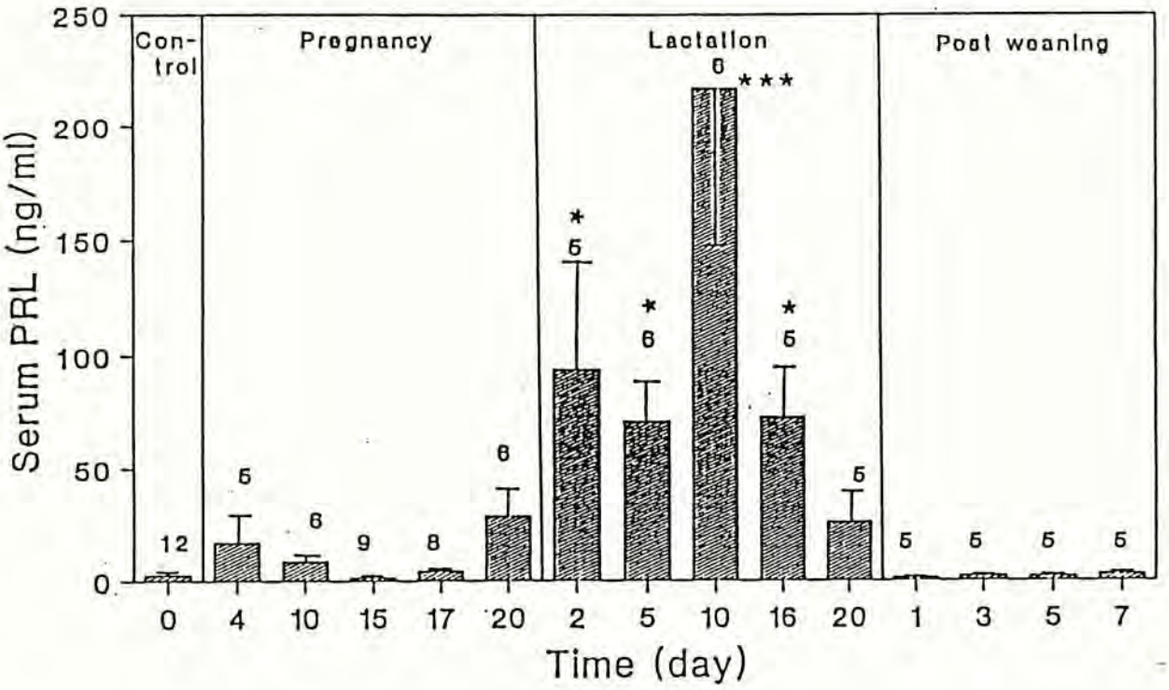



Fig. 3.8 Serum PRL concentration in rats during pregnancy, lactation and post weaning. Values are means±SEM. Number of animals in each group is given at the top of each error bar. Statistically significant differences from the unmated control group are shown: * $p<0.05$; *** $p<0.001$.

and reached a peak value of 217±70 ng/ml by mid lactation (day 10). Thereafter serum PRL began to fall and towards the end of lactation was 26±15 ng/ml which was not significantly different from the controls. Serum PRL concentration one day after weaning was similar to that of the unmated controls.

### 3.2. Effects of PRL Injection

Rat-PRL was injected into rats subcutaneously twice daily for 5 days and the rats were sacrificed 12 h after the last dose. The results are shown in Table 3.3. There was no change in body weight in either the PRL or vehicle injected groups and BAT weight was not significantly different between the two groups. Although the mean value of total UCP content in the PRL injected group was lower than the control group, the difference was not statistically significant.

### 3.3. Effects of Continuous Infusion of PRL on BAT

When rats underwent continuous infusion of PRL by implantation of a mini-osmotic pump, there was no significant change in body weight. BAT weight, total UCP content, mitochondrial protein, and serum PRL levels in the PRL infused group were not significantly different from the control group (Table 3.4).

Table 3.3 The effect of PRL injection in rats. PRL (0.104 mg/rat) was injected subcutaneously twice daily for 5 consecutive days and rats were sacrified on day 6 morning. UCP was assayed by ELISA. Each group had 8 rats. Values are means±SEM.

| Treatment | Body weight (g) | | BAT weight | Total UCP |
|---|---|---|---|---|
| | Before | After | (mg) | content/BAT (μg) |
| Saline | 290.0±5.5 | 282.3±6.2 | 300.5±23.0 | 171.2±39.6 |
| PRL | 274.3±6.5 | 268.6±7.9 | 350.7±29.5 | 133.6±29.4 |

Table 3.4: Effects of continuous infusion of PRL by implantantion of mini-osmotic pump for 5 consecutive days on body weight and BAT data.

| Treatment | Saline | PRL |
|---|---|---|
| Body weight (g) | | |
| Before | 285.4±10.3 | 273.6±12.2 |
| After | 291.4±10.0 | 278.6±11.0 |
| BAT weight (mg) | 337.7±15.8 | 307.7±15.6 |
| Total UCP μg/BAT pad | 546±94 | 558±92 |
| Mitochondrial protein (mg) | 4.15±0.25 | 4.52±0.18 |
| Serum PRL (ng/ml) | 20.8±15.6 | 10.1±2.9 |

### 3.4. Effects of Metoclopramide on BAT

Metoclopramide did not cause any change in body weight or BAT weight regardless of the dose injected (Fig. 3.9). No change in UCP content could be observed in the low dose group (0.2 mg/kg BW) whose content was 230±44 μg, similar to that of the control group (273±50 μg). In rats given the high dose of metoclopramide (1.67 mg/kg BW) UCP content of BAT (108±18 μg/BAT) was significantly lower than that of the control group ($p<0.005$).

### 3.5. Effects of Bromocriptine and Metoclopramide on BAT

Effects of bromocriptine and metoclopramide on body weight, BAT weight, UCP content and serum PRL concentration are shown in Fig 3.10. There were no significant changes in body weight after administration of either metoclopramide, bromocriptine or a combination of metoclopramide and bromocriptine for 5 days. There was a significant decrease in BAT weight in the group which received bromocriptine and metoclopramide treatment. In this group BAT weight was 252±12 mg compared with 360±26 mg in the control group. There was a significant drop in UCP content in the rats injected with metoclopramide (1.67 mg/kg BW). However this fall was abolished by concomitant treatment with bromocriptine. Bromocriptine treatment alone did not alter UCP content. In rats given metoclopramide the serum PRL level was significantly

Fig. 3.9 Effects of metoclopramide (1.67 mg/kg or 0.2 mg/kg body weight) and saline (control) injection on body weight, BAT weight and UCP content. Values are means±SEM. Each group had 7 rats. Statistically significant difference from the control group is denoted: ** $p<0.01$.

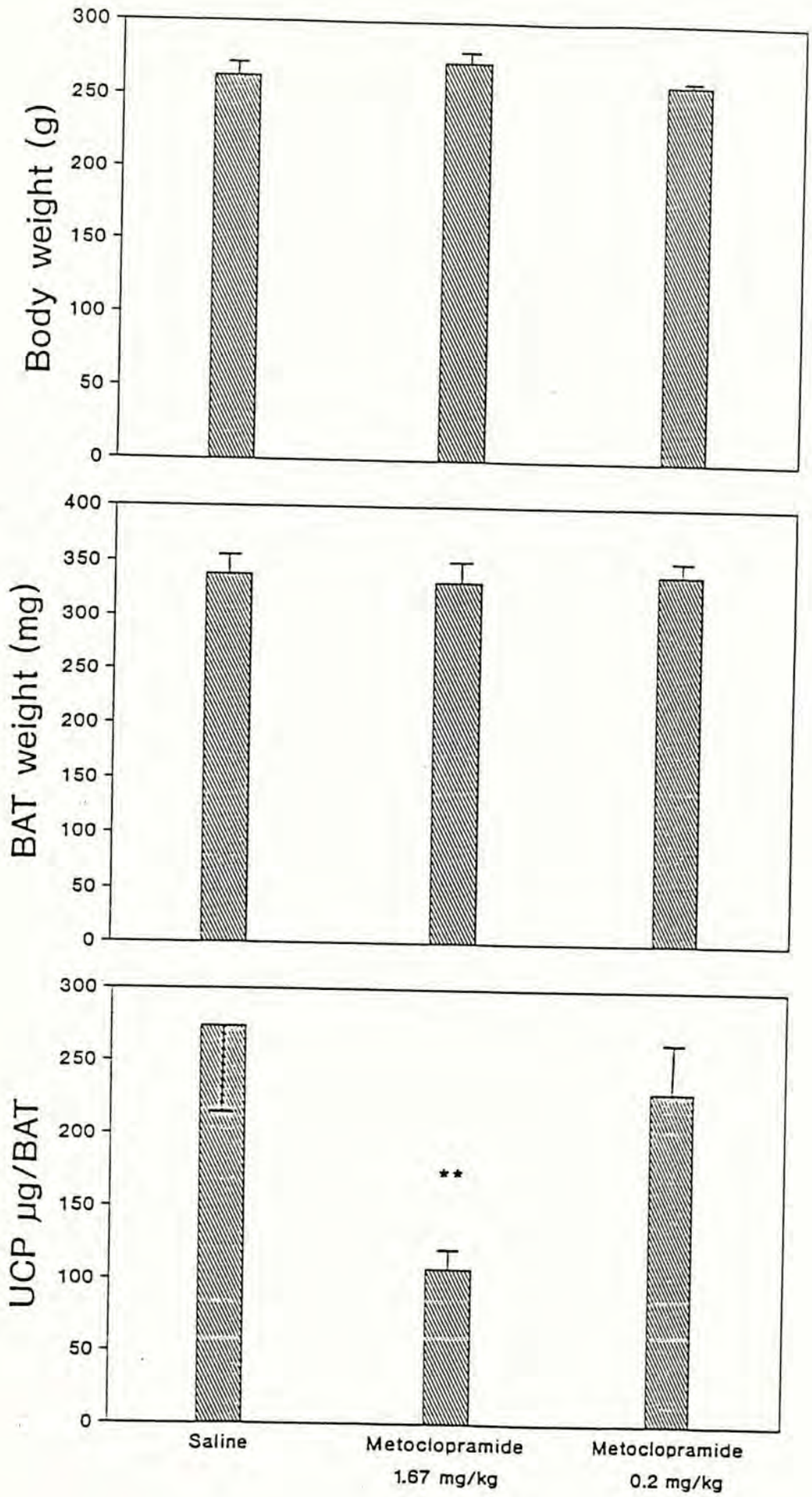
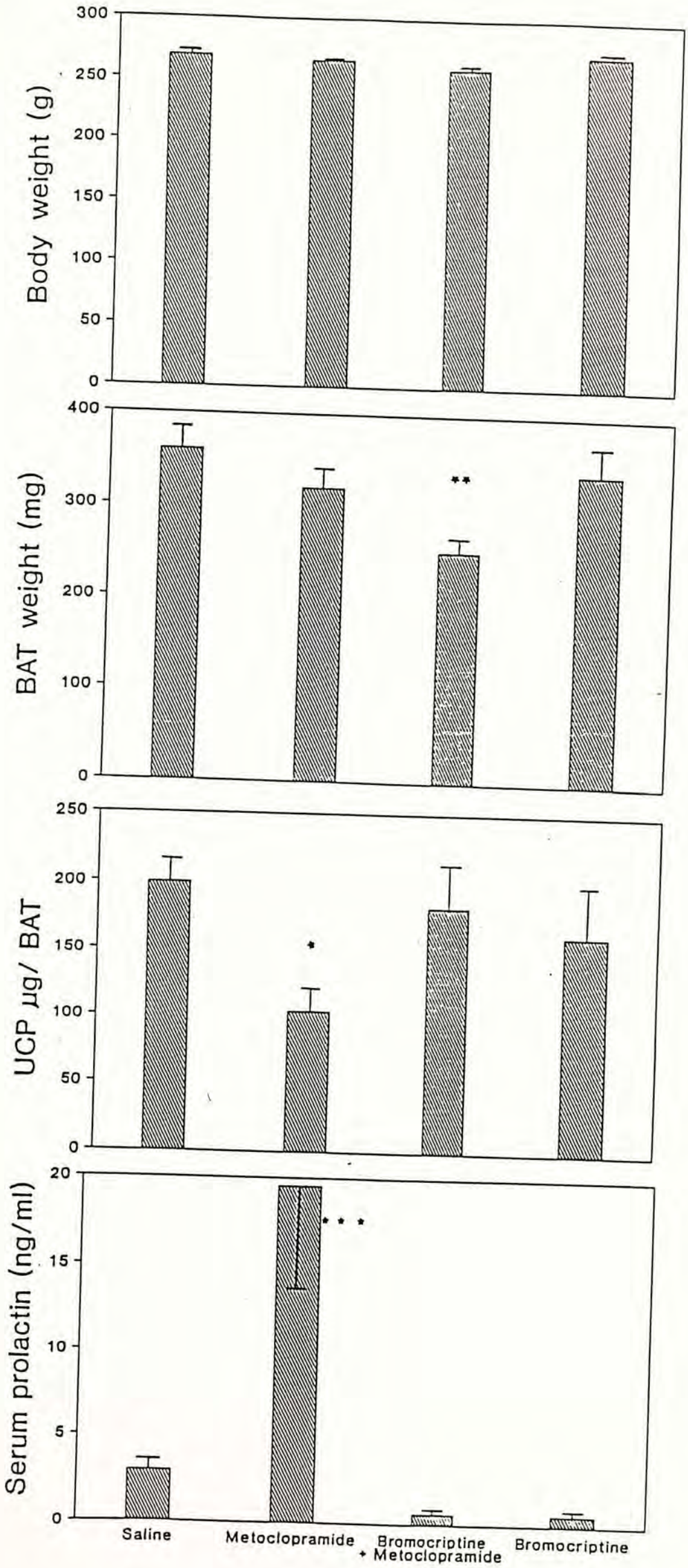

Body weight (g)
300
250
200
150
100
50
0
BAT weight (mg)
400
350
300
250
200
150
100
50
0
UCP µg/BAT
300
250
200
150
100
50
0
**
Saline
Metoclopramide
1.67 mg/kg
Metoclopramide
0.2 mg/kg

Fig. 3.10 Effects of saline (control), metoclopramide (1.67 mg/kg), bromocriptine (2 mg/kg) and metoclopramide (1.67 mg/kg) and bromocriptine (2 mg/kg) treatments on body weight, BAT weight, total UCP content and serum PRL concentration. Values are means±SEM. Each group had 7 rats. Statistically Significant differences from the control group are denoted: * $p<0.05$; ** $p<0.01$; *** $p<0.001$.


Body weight (g)
300
250
200
150
100
50
0
BAT weight (mg)
400
300
200
100
0
**
UCP µg/ BAT
250
200
150
100
50
0
*
Serum prolactin (ng/ml)
20
15
10
5
0
* * *
Saline
Metoclopramide
Bromocriptine + Metoclopramide
Bromocriptine

higher than in the controls, and this was suppressed by concomitant treatment with bromocriptine to a value similar to that the control group. Serum PRL concentration in the bromocriptine treated group was not significantly different from the control group.

### 3.6. PRL Receptor in BAT

Rat-PRL and ovine-PRL were used to detect PRL receptor in BAT membrane. Membranes from both rat prostate gland and liver were used as positive controls. The results are presented in Table 3.5. Using rat-PRL as ligand, radioreceptor binding in liver membrane showed a positive linear relationship with the amount of membrane protein used (Fig. 3.11). Rat prostate gland membrane had a weaker dose response than liver. However, BAT had the lowest (close to nil) binding to rat-PRL . Using ovine-PRL as ligand, liver again had a very high binding activity, greater than prostate or BAT. BAT membrane had a slightly higher binding to ovine-PRL than rat-PRL, but it was still very low. Liver membrane had higher PRL binding than prostate and BAT regardless of whether rat- or ovine-PRL was used as ligand and its binding to rat- or ovine-PRL was similar being in the range of 0.995 to 1.26 pg/$\mu$g of protein for rat and 1.139 pg/$\mu$g of protein for ovine. The binding of prostate membrane to rat-PRL and ovine-PRL was 0.0604 to 0.1042 pg/$\mu$g of protein and 0.1310 pg/$\mu$g of protein respectively. The binding of BAT membrane to rat-

Table 3.5 Radioreceptor assay of PRL for BAT, liver and prostate gland membrane. Both rat- and ovine-PRL were used in the receptor assay. Values are means of triplicate samples.

Using rat-PRL as ligand :

| | Protein (μg) | Total bound (cpm) | Non-specific binding (cpm) | Specific binding (cpm) | PRL bound (pg/μg protein) |
|---|---|---|---|---|---|
| BAT | 100 | 2024 | 1934 | 90 | 0.0215 |
| | 200 | 2992 | 2827 | 166 | 0.0198 |
| | 500 | 5445 | 5597 | -152 | 0.0000 |
| | 1000 | 8531 | 8270 | 261 | 0.0062 |
| | 2000 | 12176 | 12497 | -321 | 0.0000 |
| Liver | 50 | 3279 | 639 | 2640 | 1.2600 |
| | 100 | 3997 | 1380 | 4617 | 1.1014 |
| | 200 | 10238 | 1891 | 8347 | 0.9956 |
| Prostate gland | 100 | 358 | 121 | 437 | 0.1042 |
| | 200 | 825 | 318 | 507 | 0.0604 |

Using ovine-PRL as ligand :

| | Protein (μg) | Total bound (cpm) | Non-specific binding (cpm) | Specific binding (cpm) | PRL bound (pg/μg protein) |
|---|---|---|---|---|---|
| BAT | 100 | 496 | 541 | -55 | 0.0000 |
| | 500 | 2540 | 2035 | 505 | 0.0031 |
| | 2000 | 9636 | 7469 | 2167 | 0.0033 |
| Liver | 100 | 37598 | 407 | 37191 | 1.1390 |
| Prostate gland | 100 | 4405 | 178 | 4277 | 0.1310 |

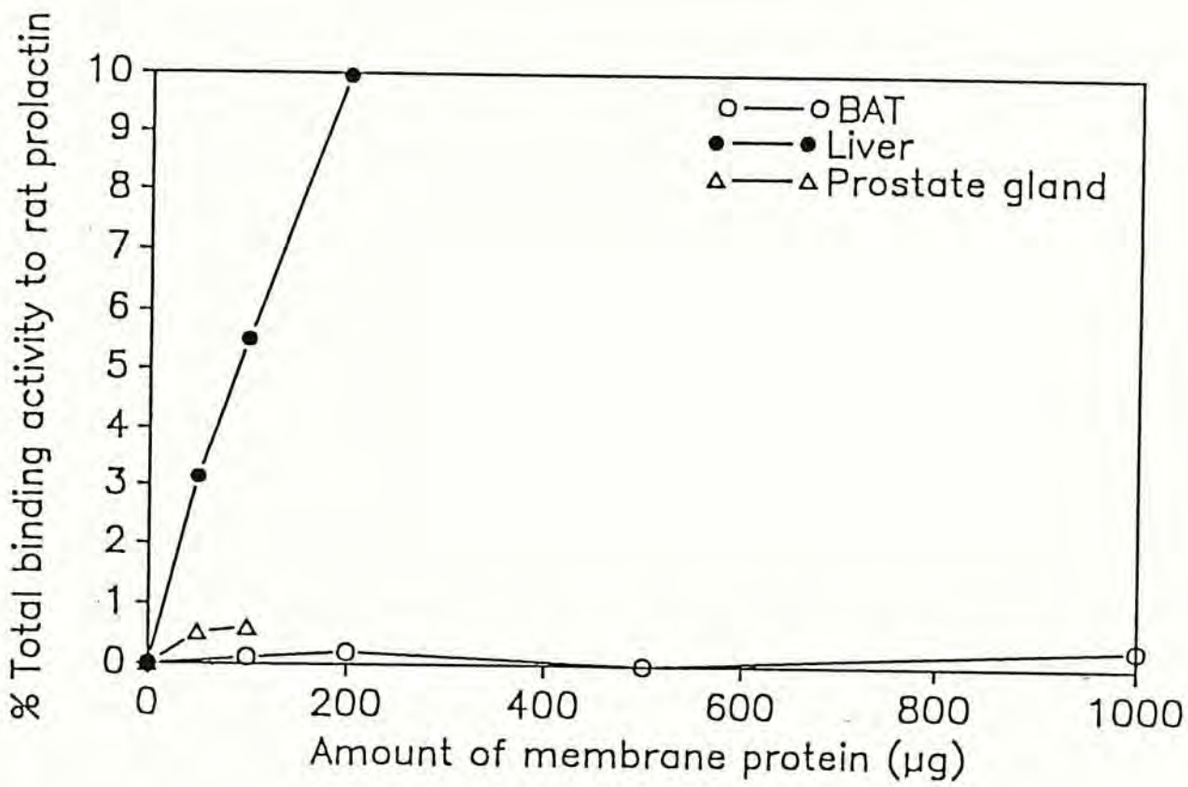



Fig. 3.11 Binding of prolactin to the membrane preparations from interscapular BAT, liver and prostate gland. The method is decribed in section 2.8.

or ovine-PRL varied from 0.0000 to 0.0215 pg/μg of protein. The study therefore shows that liver membrane had a higher number of PRL receptors than prostate gland or BAT and BAT was found to have extremely low binding activity to PRL indicating the absence of PRL receptors in this tissue.

## 4. DISCUSSION

The nutrient requirements for pregnancy are substantial and must take into account not only the demands of the developing foetus, but also the maintenance of the mother's tissues and her preparation for lactation. In the present study, food intake was found to be increased during pregnancy as was found for Sprague-Dawley rats (Jen, Juuhl and Lin 1988), Wister rats (Abelenda and Pureta 1987), mice (Richard and Tryhurn 1985), and Djungarian hamsters (Schneider and Wade 1987). It has been reported that no change in food consumption during pregnancy occurs in Syrian hamsters (Wade et al. 1986) perhaps because of this animal's habit of hoarding food (Wade et al. 1986). In pregnant mice there was no increase in overall energy efficiency when their food was restricted to the energy intake of normal non-pregnant animals (Richard and Trayhurn 1985) suggesting that modification of energy efficiency does not occur in this species.

In the present study a dramatic increase in food intake was observed in rats during lactation. A significant increase during lactation was described previously in rats and mice (Trayhurn 1985) when nutrient is primarily shunted to milk production.

Body weight gradually increased during pregnancy from 289±5 to 364±10 g and was mainly due to increases in the mass of the uterus, foetuses and placenta. Others have reported similar findings in pregnant animals. In Djungarian hamsters, there were significant increases in body weight and food consumption in the latter half of pregnancy (Schneider and Wade 1987) but there was a loss of nearly 50% of their body lipid stores: the water and fat-free dry content of the maternal carcases did not differ from those of unmated controls (Schneider and Wade 1987). An increase in protein deposition and a loss of body lipid in pregnant Syrian hamsters was also reported (Wade et al. 1986). The body weight decreased remarkably after delivery of the foetus.

The body weight in early lactating rats was higher than controls and the gain was even greater in late lactation and after weaning reflecting energy deposited when food consumption was high.

The PRL level did not change during pregnancy but it increased more than 100-fold by mid lactation. This confirms the results reported by Amenomori et al. (1970).

During mid pregnancy (day 10) hypertrophy of BAT was observed (Table 3.2) and others have reported similar findings in mid pregnancy (Agius and Williamson 1980), and in late pregnancy in rats (Abelenda and Pureta 1987; Villarroya and Mampel 1986), and in late pregnancy in mice

(Andrews et al. 1986). This hypertrophy has been shown to be due to deposition of lipid (Agius and Williamson 1980; Andrews et al. 1986).

In the present study the BAT weight significantly increased in mid pregnancy in rats. However the data from studies in hamsters are very much different. Teodure and Grishman (1961) have reported a progressive decrease of weight and cell size in interscapular brown fat throughout pregnancy in hamsters. In hamsters in late pregnancy, there are substantial decreases in interscapular BAT weight, protein content, cytochrome C oxidase activity (Wade et al. 1986). In Djungarian hamsters there are decreases in BAT protein and cytochrome C oxidase activity but no change in GDP binding activity (Schneider and Wade (1987). In rats lipoprotein lipase activity and fatty acid synthesis in BAT decrease in late pregnancy (Villarroya and Mampel 1986). There has been no previous report of the UCP content during late pregnancy. The present findings shows that there were reductions in both UCP content and protein of BAT mitochondria in late pregnancy although these were not accompanied by changes in BAT weight.

The changes in UCP content during lactation confirm the report of Ashwell et al. (1983b). UCP content began to fall at late pregnancy, and continued to drop to the lowest level in late lactation, in a manner similar to thermogenic activity measured by GDP binding (Villarroya et al. 1986; Andrews et al. 1986; Trayhurn et al. 1982a;

Zamora and Arola 1989). The atrophy of BAT during lactation which was found in the present study confirms that of Agius and Williamson (1980). The thermogenic index as shown by UCP content gradually fell during lactation and returned to normal after weaning thus confirming previous findings (Ashwell et al. 1983b; Trayhurn and Jennings 1987). Thermogenic activity measured by GDP binding has also been reported to be low during lactation (Andrews et al. 1986; Trayhurn and Jennings 1987; Trayhurn, Douglas and McGuckin 1982a; Villarroya et al. 1986; Villarroya et al. 1987; Zamora and Arola 1989). Trayhurn and Jennings (1987) reported major functional atrophy of BAT during lactation in mice. Although neither mitochondrial mass nor UCP decreased in early lactation, the decreased GDP binding found in their study led these authors to conclude that the decrease in thermogenic activity in early lactation was predominantly due to 'inactivation' of existing UCP.

The observed increase in UCP content in mid pregnancy has not been reported previously. Although the UCP content is increased, it may be functionally inactive, as during early lactation and in the post weaning period (Trayhurn and Jennings 1987). Another possible explanation of the increase is that it parallels increased thermogenic function in response to the increase in food consumption during mid pregnancy. Further work is required to confirm this.

In the present study there was depletion of only about half of the mitochondrial protein in late lactation compared with the unmated control (Table 3.2), whereas, UCP content dropped to about one tenth. Similar findings of a remarkable decline in GDP binding (to about one sixth of the unmated control) and only a moderate loss in mitochondrial protein in late lactation (day 15) have been reported (Villarroya et al. 1986). These results suggest that there is a specific loss of thermogenic protein and suppression of thermogenic function. The dramatic decreases in both GDP binding and UCP content in late lactation indicate that the fall in GDP binding is due to the decrease in the amount of UCP rather than due to 'inactivation' of the existing UCP as has been suggested for early lactation (Trayhurn and Jennings 1987).

Results of Scatchard analysis of GDP binding show no difference in the dissociation constants of virgin and lactating mice (see review Trayhurn 1985) and rat (Villarroya et al. 1986). Thus the decrease in GDP binding found in lactating rats appears to be due to a reduction in the binding sites without any change in affinity.

This impaired thermogenic capacity in late pregnancy, and lactation in the presence of high food intake indicates an inability to show DIT. The impairment was reversible and recovered in post weaning.

Besides changes in BAT described above during pregnancy and lactation, other enzyme activities were also reported to be altered. For example, BAT 5'-deiodinase activity was significantly lower in day 17 pregnant rats compared with virgin controls and remained low during late pregnancy and lactation but fully recovered after weaning (Vinas et al. 1988). There were decreases in BAT Na-K-ATPase activity and glucose uptake in BAT in late pregnancy and lactation but no change in uptake of amino acid (2-aminoisobutyric acid) was found (Zamora and Arola 1989). BAT from rats in late pregnancy showed an intrinsic reduction in glucose utilization for the oxidase pathway as well as for fatty acid synthesis.

The increased energy demand of lactation can be met by one or more of the following: increase in food intake, utilization of maternal fat stores, and adaptations or economies in the non-lactational component of maternal energy expenditure (Trayhurn and Richard 1985). Of these, increase in food intake has been suggested to be more important than the others (Trayhurn and Richard 1985). However the present finding of a profound decline in UCP content (to only one tenth of the value of the unmated control at late lactation) indicates that BAT may be more important in the energy balance during lactation than previously suggested.

PRL level started to increase in late pregnancy and continued to rise in lactation. The correlation in the

changes of PRL level and UCP content suggested that PRL may play a role in the suppression of thermogenic function of BAT during lactation. This hypothesis is supported by study of Gerardo et al. (1985) and study the photoperiod by Kott et al. (1989). In seasonal animals eg. hamsters, photoperiod affects not only PRL concentration but also BAT. Reiter (1975) observed a fall in PRL and hypertrophy of BAT but did not relate these two events. In golden (Syrian) hamsters, 10 weeks of short photoperiod (10:14 light:dark) induces hypertrophy of BAT (with a correspondingly low PRL level). Hyperprolactinaemia induced by pituitary implantation, prevents this change (Kott, et al. 1989). To substantiate this hypothesis a pharmacological approach was used. Injection of metoclopramide at a dose of 1.67 mg/kg decreased UCP content in rats. This suppression was again confirmed in the other experiment where the effects of metoclopramide and bromocriptine were studied (sections 3.4. and 3.5.). In this experiment elevation of endogenous PRL occurred concomitantly with a decrease in UCP content. Concomitant injection of bromocriptine abolished these changes. The treatment with bromocriptine alone did not alter thermogenic function perhaps because the basal level of PRL is not high enough to have an effect on thermogenic function. The present finding confirms that of Gerardo et al. (1985) who found that in Osborn-Mendel rats made hyperprolactinaemic by using ectopic pituitary transplants the GDP binding activity of BAT was suppressed. However these authors did not report the PRL level in these

animals. In the present study, increased endogenous PRL level induced by metoclopramide was shown to decrease UCP content in BAT and this indicates that PRL can suppress the thermogenic function of BAT.

Treatment with metoclopramide reduced the UCP content only by half. Gerardo et al. (1985) found a 50% reduction in GDP binding in their report. During lactation the UCP content decreased to about one tenth. The reason for this discrepancy between the experimental and physiological studies may be the different degree of elevation of PRL level. The PRL level in metoclopramide treated rats was only 19.5±6.7 ng/ml as compared with the highest value of 217±70 ng/ml found in mid lactation. In addition to PRL the energy stress of lactation may contribute to the suppression of thermogenic function in BAT by other mechanisms.

Daily injection of PRL did not reduce UCP content in BAT. This may be due to the injection of an insufficient amount to produce an observable effect. During lactation PRL level increased more than 100 times than that of the control. It has been reported that PRL has a high metabolic clearance (0.65 ml/min) in the normal rat and, furthermore, the rate of clearance increases as the PRL infusion rate or the dose of PRL administered increases (Grosvenor, Mena and Whitworth 1976; van der Gugten and Kwa 1976). Daily injection of PRL might not be adequate to elevate PRL level sufficiently to produce an effect of UCP

content. Gerardo-Gettens, Moore, Stern and Horwitz (1989) performed a similar experiment by injecting PRL twice daily for 10 days at three dose levels. Despite the hyperphagia in PRL treated rats, no significant difference in BAT mitochondrial GDP binding was reported though there is a dose dependent increase in food intake and PRL level. They suggested that PRL induces either a dissociation of the relationship between hyperphagia and brown fat thermogenic capacity or a 'masking' of the expected DIT; i.e. the usual increase in thermogenic activity accompanying increased food intake is offset by a PRL-induced decrease in GDP binding.

Continuous infusion of hormone by implantation of a mini-osmotic pump has been suggested as an efficient method for providing a steady infusion for a long period of time. Stress to the animal is minimal in this method compared with daily injection or catheterization of a blood vessel. Disappointingly, PRL did not show a suppressive effect on the UCP content. This lack of response is most likely to be explained by a rapid deterioration of the exogenous PRL solution at body temperature of 37°C. It is clearly stated by NIDDK that, once solubilized, NIDDK-rat-PRL-B-6's biological activity begins to deteriorate.

From these studies, we conclude that in addition to PRL other factors may contribute to the changes in BAT thermogenic capacity during lactation. These factors include hormonal and/or nutritional factors.

Of all the methods for demonstrating the effect of PRL on UCP content in BAT, the pharmacological approach seems to be the best. The commercial availability of metoclopramide allows the possibility of repeating experiments and minimizes limitations in experimental design. The effect of metoclopramide on plasma PRL level was rather prolonged compared with PRL injection. The effect of metoclopramide on PRL level in rats has been reported (Scott et al. 1987). Ten min after metoclopramide injection (1.67 mg/kg BW) i.v. PRL level rapidly increased to 147.9 ng/ml from 3.5 ng/ml and then fell but remained twice as high as the control value at 3 h after treatment in rats (Scott et al. 1987). Intravenous or intramuscular injection or oral intake of 10 mg metoclopramide in men or women increases PRL levels 3 to 8-fold; these levels remain elevated for at least 8 h (McNeilly, Thorner, Volans and Besser 1974). Therefore in the present experiment twice daily subcutaneous injection of metoclopramide was expected to maintain a high PRL concentration through the entire day. Indeed, the stimulatory effect of metoclopramide on PRL one h after treatment was measured and showed a significantly increased serum PRL concentration from 2.9±0.7 to 19.5±6.7 ng/ml.

Metoclopramide is a non-selective dopamine receptor antagonist and acts by acceleration of dopamine (DA) turnover (Hassan et al. 1986). This drug has a profound stimulatory effect on PRL secretion by the anterior pituitary (Van der Niepen et al. 1987). The dopamine receptor involved in the regulation of the release of PRL from the anterior pituitary has been shown to be very similar pharmacologically to the peripheral $DA_2$-receptor (Stoof and Kebabian 1984). Although metoclopramide also enhances aldosterone secretion, the magnitude is far less (Van der Niepen et al. 1987). The diminished UCP content caused by metoclopramide is more likely to be due to the increased PRL rather than aldosterone lack because the suppression by metoclopramide is abolished by bromocriptine which inhibits PRL secretion. Bromocriptine interacts with post-synaptic DA receptors and hence mimicks the effect of dopamine (Judd 1978). The present result confirms previous work showing that a metoclopramide-induced increase in PRL level could be suppressed by bromocriptine in the rat (Lipham et al. 1987).

Although an effect of PRL on UCP content has been shown here, the mechanism of PRL action has not been elucidated. PRL may have either an indirect or a direct action on BAT. The indirect effect may be through an alteration in another mechanism eg. by modification of sympathetic activity. The direct effect may be through receptor action on BAT and this requires the presence of

PRL receptors on the BAT membrane. In the present study we investigated the PRL receptor binding of BAT membrane. Both rat and ovine-PRL were used as ligands in the receptor binding studies. Liver and prostate gland were used as positive controls and both were shown to have PRL receptors in their membranes but, on the contrary, no binding of PRL could be observed in BAT membrane. This clearly indicated that there was no PRL receptor in the BAT membrane. To the knowledge of the author, similar reports have not been published before. More studies should be performed to confirm the present finding.

The absence of PRL receptor in the BAT membrane rules out the possibility of PRL having a direct effect on BAT. The effect is more likely to be through modification of sympathetic activity thus altering the thermogenic function of BAT. During lactation, the activity of the SNS is markedly reduced in lactating rats (Villarroya et al. 1987) and mice (Trayhurn and Wusteman 1987b). A primary decrease in sympathetic activity is thought to be the cause of the reduction of thermogenesis of BAT in lactating mice, although the involvement of additional factors cannot be ruled out in these studies. Insulin has been demonstrated to affect the ventromedial nucleus of the hypothalamus and modulate the firing rate of sympathetic nerves in BAT, resulting in decreased BAT thermogenesis (Amir et al. 1989). It may be possible that PRL may act in a fashion similar to that of insulin to alter sympathetic activity and thus to change BAT

thermogenesis. This question remains open.

In summary, BAT is not only an important organ in NST and DIT, but also plays an important part in energy sparing during pregnancy and lactation.

## GENERAL CONCLUSION

The low UCP content measured by ELISA method during late pregnancy and lactation strongly suggests that there is a significant decline in thermogenic capacity of BAT during this period. The decrease in mitochondrial protein and weight of BAT also support the above conclusion.

During pregnancy and lactation, there is a remarkable increase in food consumption. However the hyperphagia induced increase in thermogenesis of BAT is suppressed, indicating the SNS associated with BAT is not activated by dietary signal. Measurement of sympathetic activity such as noradrenaline turnover or the electrical firing rate of sympathetic nerves may reveal the underlying mechanism.

The lack of response of thermogenesis to dietary stimulation in lactating animals leads to the suggestion that the cold stimulation may also be suppressed during lactation. It is probable that the high metabolic rate resulting from the synthesis of milk obviates the demand for thermoregulatory heat from BAT. However further investigation is necessary in order to confirm this.

The evidence from metoclopramide and bromocriptine experiments supports PRL as a possible inhibitor of BAT

thermogenesis during lactation. The absence of PRL receptors in BAT excludes the possibility of PRL acting directly on BAT therefore it is suggested that PRL alters sympathetic activity. Moreover other factors, such as decrease in responsiveness to sympathetic stimulation or other hormones, may also be involved in the regulation of thermogenesis of BAT during lactation. Therefore considerably more work is required before the emergence of a comprehensive picture of the mechanism of inhibition of thermogenesis in BAT during lactation.

In small rodents, it has been shown that BAT plays an important role in energy balance in NST, DIT and during lactation. A great decline in thermogenic capacity of BAT suggests a considerable contribution of BAT in energy regulation in lactating animals in order to conserve energy for milk production. However in large mammals, where only small amounts of BAT are found, the role of BAT may not be as important as in small rodents.

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