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ION TRANSPORT AND BROWN ADIPOSE TISSUE ACTIVITY IN ENERGY BALANCE

by

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Abstract

The objective of this work was to study the activity of the Na^+,K^+ pump and the thermogenic activity and capacity of brown adipose tissue (BAT) under conditions of dietary energy deficit and surfeit in both human subjects and laboratory animals. While controversial, it had earlier been hypothesized that Na^+,K^+ pump activity 1) acts as a "metabolic pacemaker" in the control of cellular energy expenditure and 2) "adapts" during undernutrition so that overall energy expenditure is decreased. That BAT thermogenic activity increases during overfeeding and decreases during undernutrition is well recognised; BAT thermogenesis has hence been proposed as an "energy buffer" mechanism. However, at the outset of this work the effectiveness of dietary saturated fat in the induction of BAT thermogenesis was controversial.

The first sections of this thesis describe the effects of undernutrition and nutritional rehabilitation upon Na^+,K^+ pump activity in erythrocytes of children with cerebral palsy (CP). The results show that, unlike results from developing world undernourished children, erythrocyte Na^+,K^+ pump activity was not lower in cells from the undernourished children with CP.

The later sections describe the simultaneous study of both Na^+,K^+ pump and BAT activities during diet-induced obesity (DIO) and dietary restriction (DR) in rats. I hypothesized that both mechanisms might act as "energy buffers" and contribute to metabolic adaptation during DIO and DR. The effects of lard- and tallow- based

diets were studied. The extent of DIO was assessed using total body electrical conductivity (TOBEC), carcass analysis and fat pad weights; it was concluded that presently available TOBEC equipment is unsuitable for serial determinations of rat adiposity, and that fat pad weights are good estimates of adiposity. Na^+,K^+ pump activity increased in thymocytes of rats fed the high saturated fat diets; activity decreased in erythrocytes and hepatocytes in some groups following DR. BAT activity and thermogenic capacity were enhanced by high saturated fat diet-feeding; activity decreased following DR. Lard- and tallow- based diets had differing effects upon the extent and the timing of changes in the activities of the two mechanisms. Despite increases in Na^+,K^+ pump and BAT activities, basal metabolic rate (BMR) was not increased in the obese rats; decreases did however occur following DR. Diet-induced alterations in both mechanisms could occur through common pathways such as sympathetic neural activity and/or thyroid hormones. Increases in plasma T_3 concentrations were associated with high saturated fat diet feeding as were decreases with DR. It is concluded that the alterations in both mechanisms are compatible with the hypothesis that these mechanisms function as energy buffer mechanisms during dietary energy surfeit and deficit.

Dedication

This work is dedicated to Jon for his incessant support and interest, and to my parents, brother and sister for being the wonderful family that they are.

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I am especially grateful to my thesis supervisors, Dr. John Patrick and Dr. Jean Himms-Hagen. I have appreciated, and will continue to appreciate Dr. Patrick's constant encouragement to critically question, and to consider issues from a wider perspective. Dr. Himms-Hagen's expert guidance and her support have truly been invaluable.

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List of Abbreviations

ADP	adenosine diphosphate
ANOVA	analysis of variance
ATP	adenosine triphosphate
BAT	brown adipose tissue
BMR	basal metabolic rate
BSA	bovine serum albumin
BWt	body weight
CP	cerebral palsy
DIO	diet-induced obesity
DIT	diet-induced thermogenesis
DNA	deoxyribonucleic acid
EDTA	ethylenediamine tetraacetic acid
fl	femtoliter
g	centrifugal field
GDP	guanosine 5'-diphosphate
HANES I	Health and Nutrition Education Survey I
HEPES	N-2-hydroxyethylpiperazine-N'-ethane sulfonic acid
HF1	high fat diet one (lard-based)
HF2	high fat diet two (tallow-based)
HSF	high saturated fat diet
kBq	kilobecquerel
kcal	kilocalorie
kD	kilodalton
kJ	kilojoule
LBM	lean body mass
μCi	microcurie
MAMC	mid arm muscle circumference
MPF	moderately high polyunsaturated fat diet
M_r	relative molecular weight
NAD	nicotinamide adenine dinucleotide
NCHS	National Center for Health Statistics
NST	non-shivering thermogenesis
PBS	phosphate-buffered saline
PEM	protein energy malnutrition
PIP_2	phosphatidylinositol bisphosphate
PPO	2,5-diphenyloxazole
TOBEC	total body electrical conductance

Q	ubiquinone, or coenzyme Q
RBC	red blood cell, or erythrocyte
RIA	radioimmunoassay
SEM	standard error of the mean
SNS	sympathetic nervous system
TES	Tris(hydroxymethyl)-2-aminomethane sulfonic acid
Tricine	Tris(hydroxymethyl) methyl glycine
TEF	thermic effect of food
TSF	triceps skinfold thickness
T_3	3,3',5'-triiodothyronine
T_4	thyroxine
T5'D	thyroxine 5'-deiodinase
UCP	uncoupling protein
VMH	ventromedial hypothalamus
WAT	white adipose tissue

Chapter 1: General Introduction

I) Thermodynamics

a) Energy Balance: Energy intake versus energy expenditure

Research in the area of energy balance is extensive. At one end of the spectrum it involves studies in whole animals of the efficiency of various dietary energy sources under numerous physiological, environmental and pharmacological conditions. In such whole animal energy balance studies dietary energy is carefully measured against the energies lost in excreta, the energy used in metabolism, the energy used in work done on the environment and against the energy which is stored in the animal. On a less comprehensive and more specialized scale this research area also includes the study of bioenergetics. The latter involves the study of the many aspects of energy metabolism at the cell organelle and molecular levels as well as how those aspects relate to the energetics of individual animals or of populations of animals (Blaxter, 1989).

Energy balance is often described in equations such as the following (Blaxter, 1989):

Intake of energy	Energy of - feces	Energy of urine	Energy of combustible - gas	Heat produced =	Energy retained or secreted
I	- F	- U	- G	- H_p =	R

or,

Metabolisable energy - Heat produced = Energy retained

$$M_E - H_p = R$$

The first law of thermodynamics which states that energy is conserved applies to energy balance studies in animals and humans. Numerous calorimetric studies have demonstrated that under laboratory conditions the retention of energy in the body can be indirectly approximated as the difference between measurements of the intake of energy as food and the sum of excreted energy sources and heat produced (reviewed by Blaxter, 1989).

The research described in this thesis concerns the role of heat production, or thermogenesis, in the regulation of energy balance.

b) Obligatory and facultative thermogenesis

The many different processes contributing to thermogenesis can be classified into two major groups, obligatory thermogenic and facultative thermogenic processes.

Obligatory thermogenesis comprises heat production from all tissues that occurs as a result of maintaining essential cellular metabolic processes; this heat production is necessary to sustain the state of endothermy (warm bloodedness). The thermic effect of food (TEF), which occurs in the intestine, liver and white adipose tissue, also contributes to obligatory thermogenesis and is defined as the energy expenditure necessary for the processing of food during ingestion, digestion, absorption and storage (Himms-Hagen, 1989). The increased energy expended during growth, pregnancy, lactation and during tumor growth would also be classified as obligatory thermogenesis (Himms-Hagen, 1989).

Obligatory thermogenesis, except for the periodic increases due to TEF, remains fairly constant over time, and its regulation is primarily mediated by thyroid hormone (Guernsey and Edelman, 1983; Danforth and Burger, 1981; Oppenheimer, Schwartz, Mariash *et al*, 1987). Therefore in the absence of TEF obligatory thermogenesis is equal to basal metabolic rate (BMR) and it is measured as total oxygen consumption in a resting post-absorptive state at thermoneutrality.

Facultative thermogenesis, unlike obligatory thermogenesis, occurs only in skeletal muscle and brown adipose tissue (BAT). It can be rapidly induced and terminated. Exercise-induced thermogenesis in skeletal muscle is one component of facultative thermogenesis. Facultative thermogenesis also includes heat production occurring as a result of thermoregulatory processes such as cold-induced shivering in skeletal muscle and cold-induced non-shivering thermogenesis in BAT. Another component of facultative thermogenesis is diet-induced thermogenesis (DIT) which occurs in BAT and possibly also in other tissues; this component can be further subdivided into cephalic phase DIT and postprandial phase DIT. The former is associated with the sensory qualities of the diet and is mediated through increased sympathetic activity and neurally-mediated insulin secretion (Diamond and LeBlanc, 1987a; Diamond and LeBlanc, 1987b; Steffens, Van Der Gugten, Godeke *et al*, 1986). Postprandial phase thermogenesis refers to the adaptive diet-induced thermogenic activity in BAT which is mediated by sympathetic activity in response to a fed, and especially an overfed, state (Himms-Hagen, 1989).

The idea of adaptive DIT is not new; Neumann (1902) and Gulick (1922) both proposed the theory that humans and some animals after consuming excessive quantities of food can increase their heat production such that there is no storage of energy in the body. This process, referred to as *luxuskonsumption*, was later described by Rothwell and Stock (1979) who showed that rats when given a highly palatable "cafeteria" diet increased their energy intake by 80% but gained only 27% more weight than control rats. They proposed that the function of this mechanism which they termed "diet-induced thermogenesis" was to maintain zero energy balance in response to overfeeding. They further proposed that this mechanism occurred through sympathetic neural activation of BAT thermogenesis, similar to the mechanism responsible for cold-induced non-shivering thermogenesis in BAT.

The measurement of the various individual components comprising obligatory and facultative thermogenesis in intact animals is not usually feasible (Himms-Hagen, 1989). This is mainly because several of the mechanisms occur simultaneously and in different organs. An additional complicating factor is that facultative mechanisms which are superimposed on the obligatory mechanisms occur only in conscious unrestrained animals. (However non-shivering thermogenesis can be artificially induced when anesthetized rodents are given ß-adrenergic agonists.) Therefore, for example, the consumption of a palatable diet will induce TEF in many organs as well as inducing DIT in BAT (Himms-Hagen, 1989); thus the specific quantification of DIT in terms of the increases in thermogenesis for the whole animal is not possible.

II) Cellular respiration

a) General Aspects:

As discussed by Blaxter (1989), the sources of basal heat production at the level of the whole organism can be categorized into those sources which arise due to whole organism "service" functions and cellular "maintenance" functions. The first category includes those functions such as the provision of oxygen through the work of the heart and lungs, the removal of waste products by the kidney and the functional integration accomplished by the central nervous system. The second category includes cellular functions such as ion transport and the turnover of protein, lipid and other constituents. Service functions are estimated to account for 36-50%, and cellular maintenance functions for 40-56% of total basal heat production.

At the cellular level, regardless of the details of the oxidation processes or the reactions that hydrolyze the ATP produced in a cell, the free energy released during the oxidation of substrates must all be released as heat. Free energy, by definition, is that energy which is available for work. Moreover, regardless of the efficiency of mitochondrial energy transduction, heat production is proportional to oxygen consumption as oxygen is the final electron acceptor in the electron transfer system.

Although most of the important cellular metabolic reactions have been identified, those that are prominent in terms of heat production have not been unanimously acknowledged (see Brand and Murphy, 1987; Brand, 1990; Himms-Hagen, 1976; Milligan and Summers, 1986). The distribution of the heat production

between mechanisms involved in ATP production in mitochondria and those involved in its use is unknown, although recent estimates of the thermodynamic efficiency of oxidative phosphorylation imply that ATP consumption is responsible for a slightly larger proportion of heat production than ATP production (Brand, 1990).

Several specific ATP-consuming reactions have been proposed as important contributors to basal heat production, but their quantitative contributions to total ATP turnover are unknown (see Himms-Hagen, 1989; Milligan and Summers,1986; Blaxter, 1989; Brand, 1990). It is however generally agreed that the ubiquitous Na^+,K^+-ATPase is an important ATP-consumer. But again estimates of its quantitative contribution to overall basal respiration or heat production are widely disparate. The view that the Na^+,K^+-ATPase might account for as much as 35-50% of cellular respiration is strongly supported by some researchers (see Milligan and Summers, 1986; Ismail-Beigi, 1988) while much more conservative estimates are given by others (see Himms-Hagen, 1976; Clark, Brinkman, Filsell *et al*, 1982; Nobes, Lakin-Thomas and Brand, 1989; Brand, 1990).

b) Control of cellular respiration:

A model of the control of cellular respiration, and therefore of heat production, is necessary for a more comprehensive understanding of cellular energy metabolism and its efficiency. Various important aspects of the control of cellular respiration have been addressed in several reviews (Himms-Hagen, 1976; Williamson, 1979; Hansford, 1980; Erecinska and Wilson, 1982; Brown, 1985; Brand and Murphy,

1987). As discussed by Brand and Murphy (1987) it appears that cellular respiration is controlled mainly by the supply of reducing equivalents and by the concentrations of cytosolic adenine nucleotides. The supply of reducing equivalents is controlled by the availability of substrates for oxidation and by the activity of the metabolic pathways which provide electrons to oxidized nicotinamide-adenine dinucleotide (NAD) and ubiquinone (Q), which are themselves controlled by diet, by hormones and by Ca^{++} and adenine nucleotides. The concentration of cytosolic adenine nucleotides, which reflects the cellular demand for ATP, controls respiration via its effects on the mitochondrial protonmotive force (*ie.* electrical potential across the inner mitochondrial membrane). Increases in cytosolic ADP, which itself is a substrate of the proton-translocating ATP synthetase, increases the inner mitochondrial membrane permeability to protons. The increased permeability to protons in turn lowers the protonmotive force and this is thought to stimulate cellular respiration by increasing the rate of proton pumps and hence increasing the rate of electron transport.

Normally fuel is always available to cells and substrates are available for oxidation (Himms-Hagen, 1976). Therefore when cellular respiration is tightly coupled to ATP synthesis, as occurs in most cells for most of the time, respiration is controlled to a large extent by the phosphorylation state ratio (*ie.* $[ATP]/[ADP][P_i]$). However, in loosely coupled, or in uncoupled, states where respiration proceeds but oxidative phosphorylation slows or ceases, respectively, substrate availability appears

to be the major controlling factor (Himms-Hagen, 1976).

In one cell type, namely, the brown adipocyte, the control of cellular respiration can be physiologically uncoupled from ATP synthesis. The mitochondria in BAT can operate in a coupled mode and respiration is regulated by ADP *etc.* as described above. The mitochondria can, however, become uncoupled. In this uncoupled state ADP production does not regulate respiration and heat production occurs primarily during the unrestrained oxidation of reduced coenzymes.

In each of the following described studies the cell types available for analysis were limited, particularly in the research projects involving humans. The erythrocyte is among the cell types used in the study of the potential effects of nutritional status upon Na^+,K^+ transport. Although erythrocytes do not respire, they were included in the studies because they provide a readily available, and more easily managed, substitute for other cell types in the assessment of ion transport in intact cells. Therefore if effects were seen in ion transport in this cell model then erythrocytes could be used further in the elucidation of the involved control mechanisms.

The research described in this thesis involves the study of alterations in Na^+K^+ pump activity and brown adipose tissue activity and capacity in relation to energy balance. Accordingly the following sections provide a short review on the function, structure and regulation of these two mechanisms which, through their induction and suppression, could be capable of altering energy expenditure.

III) The Na^+K^+ pump

a) Historical aspects:

The Na^+K^+ pump was first identified half a century ago during the second World War when medical personnel recognized an unknown mechanism which caused erythrocytes in cold-stored blood to accumulate Na^+ and lose K^+ but which could restore ion gradients if the erythrocytes were incubated at body temperature in the presence of glucose (Danowski, 1941). The initial proposal for a membrane-associated active transport system was made by Dean (1941). Based on his own experimental results and on those of Steinbach (1940), Dean demonstrated that the uphill movements of Na^+ and K^+ were obligatorily coupled to one another by the activity of a Na^+,K^+ "pump". That the immediate source of energy for ion pumping is ATP was first shown by Gárdos (1954). Skou (1957), using a membrane preparation from crab nerve, identified this enzyme which was activated by Na^+ and K^+ and which required ATP hydrolysis. The coupling ratio in red blood cells was found by Post and Jolly (1957) to be close to 3 Na^+ transported out of, for 2 K^+ transported into the cells. This stoichiometry has been confirmed for many other cell types (Clarke, Apell and Läuger, 1989), but whether this stoichiometry is the same in all tissues under all conditions is not known. It is generally thought that more sodium is extruded from cells than potasssium is transported into cells in each pump cycle (Skou, 1990).

A better understanding of this transport system was gained through the

extensive experiments of Garrahan and Glynn (1967 a,b,c,d,e). They altered intra- and extra- cellular concentrations of Na^+, K^+, ATP and P_i and showed that the Na^+,K^+ pump can also perform in three different modes: 1) as a Na^+:Na^+ exchanger, 2) in a reversed mode with the subsequent incorporation of Pi into ATP, and 3) as a means of uncoupled Na^+ efflux. A fourth mode, K^+:K^+ exchange, which was later described by Glynn, Lew and Lüthi (1970), is thought to occur under physiological conditions and involve about one entering K^+ in five being exchanged for an internal K^+ instead of an internal Na^+. Similar to Na^+:Na^+ exchange, K^+:K^+ exchange is not associated with the hydrolysis of ATP.

b) <u>Cellular functions of the Na^+,K^+-ATPase:</u>

The literature describing Na^+,K^+-ATPase is vast and has been frequently reviewed (Glynn and Karlish, 1975; Sweadner and Goldin, 1980; Karin and Cook,1983; Gick, Ismail-Beigi and Edelman, 1988; Fambrough, 1988; Sweadner, 1989; Lingrel, Orlowski, Shull *et al*, 1990; Skou, 1990). It is now generally understood that the Na,$^+$$K^+$-ATPase functions as an energy transducer that transforms the chemical energy of ATP hydrolysis into gradients for sodium and potassium, and that these gradients are then used as potential energy sources for many other processes including the maintenance of membrane potential, the restoration of membrane potential after depolarization, cell volume regulation, the uphill transport of some amino acids and glucose into certain cells, ion co- and counter- transport across cell

membranes, and transepithelial transport of ions and molecules in the kidney, intestine and secretory glands (Skou, 1990). Thus the Na^+,K^+-pump indirectly provides energy for all of these processes through the transduction of energy from the hydrolysis of ATP.

c) **Molecular aspects of the Na^+,K^+-ATPase:**

Structure: The Na^+,K^+-ATPase has been purified from many different tissues, and in all instances it has been shown to be a heterodimer (see Sweadner, 1980). The catalytic subunit, referred to as α, contains both the active site for ATP hydrolysis and the binding site for the plant glycoside, ouabain, a specific inhibitor of Na^+,K^+-ATPase activity. It has a molecular weight of approximately 112 kD. The smaller subunit, referred to as ß, is a glycoprotein and has a molecular weight of about 35 kD. This subunit has not been directly associated with the enzymatic or transport functions of the sodium pump, but it appears to be necessary for pump activity as the separation of the two subunits leads to irreversible inactivation of pump activity. The ß subunit appears to function in the regulation of the number of sodium pumps which are translocated to the plasma membrane during the process of αß heterodimer assembly (McDonough, Geering and Farley, 1990).

The primary sequences of the α and ß subunits have been resolved from cDNA (Shull, Young, Greeb *et al*, 1988). The α subunit contains a total of 1016 amino acids, and has 8 hydrophobic regions. The hydrophilic region at the N-terminal

which is located on the cytoplasmic side of the membrane contains 92 amino acids. This is followed by an uncertain total number of transmembrane segments, 6, 7, or 8; the location of the C-terminal is also still uncertain. The ATP binding site and a phosphorylation site (Asp-369) are located in the large cytoplasmic loop of about 439 amino acids between transmembrane segments 4 and 5. The ouabain binding site is located in the first extracellular domain (residues 111-122) (Shull, Greeb and Lingrel, 1986).

The protein portion of the ß subunit consists of 302 amino acids and it spans the membrane once near the N-terminal of the α subunit. The hydrophilic extracellular domain contains three N-linked carbohydrate groups and seven cysteine residues which participate in three disulphide bonds (see McDonough *et al*, 1990).

Multiple isoforms: The existence of multiple isoforms of the α subunit that are coded by different genes was shown by cDNA cloning and sequencing (Shull *et al*, 1986). At present there are three known α isoforms, α1, α2 and α3. The isoforms are found in varying proportions in different tissues. For example, kidney and heart contain predominantly α1, brain contains approximately an equal mixture of the isoforms, white adipose tissue contains predominantly α1 and skeletal muscle contains predominantly α2; in liver all three isoforms have been reported, but it is not known which isoform(s) predominate(s) (Sweadner, 1989). The isoforms differ in their sensitivities towards cardiac glycosides, α2 and α3 being more sensitive than α1 in a given tissue. Moreover, cardiac glycoside sensitivity also differs between

species; rats and mice show the most extreme differences between tissues (Sweadner, 1989). The differences in cardiac glycoside sensitivity between rodents and other species are thought to be due to amino acid sequence variation in the first extracellular domain (Lingrel *et al*, 1990).

cDNA cloning and sequencing studies have also been used in the attempt to identify different isoforms of the ß subunit; the results, however, do not suggest that there are multiple isoforms of the ß subunit (Lingrel *et al*, 1990). The molecular heterogeneity in these subunits in different tissues apparently arises from varying subunit glycosylation in different cell types (Takeyasu, Tamkun, Siegel *et al*, 1987).

d) <u>Regulation of Na^+,K^+-ATPase:</u>

As a result of the existence of multiple α isoforms the analysis of Na^+,K^+-ATPase regulation is extremely complex. It is likely that each cell type has its own distinctive combination of regulatory controls which may include regulated isoform selection, levels of gene expression, sensitivities to various modulators and spatial distribution (Rossier, Geering and Kraehenbuhl, 1987). However, Lingrel and colleagues have suggested that because the α1 and ß isoforms have been found in all cell types studied it may be that the α1 isoform functions in a "housekeeping" capacity to maintain osmotic balance and cell volume regulation, while the other α isoforms execute more specialized ion transport functions necessary for differentiated cell-specific functioning (Lingrel *et al*, 1990).

As well as the tissue and cell-specific regulation of Na^+,K^+-ATPase through the expression of different α subunit isoforms, isoform changes must be integrally involved in regulation during development because different adult cell types express various subsets of isoforms. For instance, in cardiac muscle there is a switch in α subunit isoforms during the time between fetal and postnatal life (Schneider, Mercer, Gilmore-Herbert *et al*, 1988).

Na^+,K^+-ATPase activity is regulated by numerous hormones including vasopressin, epidermal growth factor, nerve growth factor, insulin, glucagon, catecholamines, glucocorticoids, mineralocorticoids, and triiodothyronine (see Karin and Cook, 1983; Clausen, 1986; Rossier *et al*, 1987; Gick *et al*, 1988; Lingrel *et al*, 1990). As described by Lingrel *et al* (1990), these hormones can be classified in two general categories, 1) hormones that alter the level of Na^+,K^+-ATPase gene expression. This group includes steroid and thyroid hormones and alterations occur over a period of hours to days; and 2) hormones which alter the activity of already existing Na^+,K^+ pumps. This group include peptide hormones and catecholamines; the effects of these hormones occur rapidly (within seconds or minutes) by altering membrane ion permeabilities and thereby changing cation substrate concentrations. These hormones may also alter activity indirectly through specific membrane receptors which modify Na^+,K^+ pump activity possibly through the post-translational modification of the enzyme.

The mechanism by which steroid and thyroid hormones exert their control of

the Na^+,K^+ pump is thought to occur through a high affinity hormone-receptor/ DNA binding complex that directly or indirectly alters the rates of Na^+,K^+-ATPase α and ß subunit gene transcription. This hormone-receptor complex may also alter nuclear processing of the primary transcripts, mRNA stability, and mRNA transport from the nucleus to the cytoplasm (Lingrel *et al*, 1990). Thus these mechanisms appear to modulate the *de novo* synthesis of Na^+,K^+ pump molecules.

The catecholamines and peptide hormones are thought to modulate Na^+,K^+ pump activity through plasma membrane receptor interaction, the subsequent stimulation of second messengers, including cyclic nucleotides and diacylglycerol, and through their subsequent activation of the protein kinases (Lynch, Wilson, Blackmore *et al*, 1986; Kennedy and Lever, 1984; Stewart and Sen, 1981).

e) <u>The energetics of Na^+,K^+ pump activity:</u>

The hypothesis that the Na^+,K^+ pump is a significant contributor to cellular reactions which consume ATP and thereby drive respiration was first proposed by Whittam (1961). Based on his work in kidney cortex and brain slices he referred to the Na^+,K^+ pump as a "pacemaker" of respiration (Whittam, 1961; Whittam and Willis, 1963; Whittam and Blond, 1964). The hypothesis that the Na^+,K^+ pump acts as a pacemaker of respiration is based on the idea that by stimulating Na^+,K^+ pump activity ADP is generated and the phosphorylation state ratio is decreased which in turn stimulates respiration.

The idea that a significant proportion of cellular energy expenditure is attributable to Na^+,K^+-ATPase activity is indeed controversial. The estimated proportion of the basal respiration rate attributable to the activity of this enzyme extends from none in muscle (Wardlaw, 1986), liver slices (Israel, Videla, MacDonald *et al*, 1973) and thymocytes (Lakin-Thomas and Brand, 1988) to 5-6% in perfused liver (Folke and Sestoft, 1977), 2-8% in isolated hepatocytes (Clark *et al*, 1982), and 20-46% in isolated hepatocytes, muscle, liver and kidney slices (Gregg and Milligan, 1980a,b; McBride and Milligan, 1985; Ismail-Beigi and Edelman, 1970; 1971; Ismail-Beigi, Bissell and Edelman, 1979). When respiration is stimulated by thyroid hormone there is an even greater range of the estimated increased proportions of oxygen consumption resulting from increased Na^+,K^+ pump activity. These estimates in thyroid hormone-treated, normal and hypothyroid rats range from less than 10% (Folke and Sestoft, 1977) to 85-90% (Israel *et al*, 1973; Wardlaw, 1986; Ismail-Beigi and Edelman, 1970; 1971; Ismail-Beigi *et al*, 1979; Asano, Liberman and Edelman, 1976). At least some of the variation in these estimates would be due to differences in the methods of cell preparation which would result in differences in cell permeability to ions; for example, estimates for liver tissue were carried out using hepatocyte suspensions (Nobes *et al*, 1989), liver slices (Ismail-Beigi and Edelman, 1971), and perfused liver (Folke and Sestoft, 1977). Considering the variation in the density of Na^+,K^+ pumps between different cell types (erythrocytes have approximately 200 pumps per cell, while cells in the thick ascending loop of Henle

have several million pumps per cell (see Skou, 1990)) some of the overall variation would also be due to the cell types studied.

Moreover, in most of these studies ouabain is used to inhibit Na^+,K^+ ATPase activity, and in many of the studies it is not acknowledged that ouabain quickly alters Na^+ and K^+ fluxes (Folke and Sestoft, 1977) and that ouabain will cause K^+ depletion after prolonged incubation periods (*eg.* one hour) which coincides with decreased oxygen consumption (Whittam and Willis, 1963). The effects of ouabain are discussed in more detail by Himms-Hagen (1976) and Brand (1990). Brand (1990) describes the biphasic time course of ouabain inhibition; within seconds it abolishes Na^+,K^+ pump activity while only marginally inhibiting oxygen consumption of hepatocytes. However, after prolonged incubation periods ouabain no longer abolishes Na^+,K^+ pump activity but inhibits oxygen consumption. Ouabain therefore has some secondary effects on respiration (see Himms-Hagen, 1976; Brand, 1990). When Nobes and her colleagues (1989) partially controlled for these effects of ouabain by studying hepatocyte gramicidin-induced ATP turnover by the Na^+,K^+-ATPase which leads to the stimulation of respiration, they found that ouabain rapidly inhibits respiration of palmitate or glucose by only 6-10%. This suggests that Na^+,K^+-ATPase plays a smaller role in cellular ATP turnover than many of the earlier estimates. When they used the same technique with isolated hepatocytes from hyperthyroid rats they showed that 29% of the thyroid hormone-induced stimulation of respiration was attributable to Na^+,K^+-ATPase activity.

f) Measurements of Na^+,K^+ ATPase and Na^+,K^+ pump activities:

It is important when comparing Na^+,K^+ pump and Na^+,K^+-ATPase data to be aware of what these assays actually measure. The Na^+,K^+-ATPase assay measures ouabain-sensitive inorganic phosphate release in cell or plasma membrane homogenates. This *in vitro* assay, although it may provide an estimate of the total potential activity of the Na^+,K^+-ATPase, does not estimate the *in vivo* activity of the pump under physiological conditions. Moreover, Na^+,K^+-ATPase assays are usually carried out using saturating concentrations of the ion substrates that are considerably different from physiological ion concentrations. Conversely, Na^+,K^+ pump assays are carried out using intact cells and physiological concentrations of substrates. Thus, Na^+,K^+ pump measurements provide an estimate of the *in vivo* activity of the protein, and if any correlation between the activity of this protein and energy expenditure is to be detected it is the measurement of Na^+,K^+ pump activity which is more relevant.

IV) Brown adipose tissue

a) Functional aspects of BAT activity:

The specific function of brown adipose tissue (BAT) is facultative thermogenesis. BAT is the only mammalian tissue in which heat is the *primary* product of metabolism (Trayhurn, 1990). The generation of heat in BAT can be stimulated either through exposure to the cold, and this is referred to as cold-induced

non-shivering thermogenesis (NST), or through the diet and this is referred to as diet-induced thermogenesis (DIT) (Himms-Hagen, 1989). The apparently adaptive changes in BAT activity that coincide with changes in energy intake in laboratory animals has led to the concept that BAT thermogenesis functions as an "energy buffer" in the regulation of energy balance and obesity prevention (Himms-Hagen, 1984).

BAT is found in distinct locations in the bodies of mammals (Néchad, 1986). The total amount of BAT and the proportions of BAT located in the various depots in the body varies between species. A major BAT depot in rats and mice is found in the interscapular region whereas in primates, the depots in the axillary regions are predominant (Néchad, 1986; Lean and James, 1986; Lean, James, Jennings *et al*, 1986).

The mechanism by which heat is produced in BAT, which differs from the ATPase or ADP-controlled mechanisms present in all other mammalian tissues, depends on the unique composition of brown adipocyte inner mitochondrial membranes. Brown adipocyte mitochondria, which are found in abundance in these adipocytes, contain a 32 kD protein referred to as uncoupling protein (UCP); UCP has not been identified in any tissue other than BAT (Cannon, Hedin and Nedergaard, 1982). The mitochondrial proton conductance pathway mediated by UCP causes a proton short circuit across the inner mitochondrial membrane which allows the proton gradient produced by the oxidation of substrate to be dissipated as

heat instead of being used for ATP synthesis by mitochondrial ATP synthetase (Nicholls and Locke, 1984; Nicholls, Cunningham and Rial, 1986). BAT thermogenesis is fueled predominantly by fatty acids which are derived from BAT or white adipose tissue (WAT) lipid stores or from the diet (Himms-Hagen, 1989).

As well as brown adipocytes, BAT also contains interstitial cells, preadipocytes, endothelial cells, and mast cells (Bukowiecki, Collet, Folléa *et al*, 1982; Néchad, 1986). Approximately 40% of interscapular BAT cells are mature brown adipocytes (Bukowiecki *et al*, 1982). BAT is highly vascularized and it includes arteriovenous anastomoses (Nnodim and Lever, 1988); it therefore can receive high blood flow rates during stimulation (Foster, 1986) and the heat generated in BAT can be quickly transferred throughout the body.

b) <u>Regulation of thermogenic activity:</u>

i) <u>Neural regulation:</u> BAT thermogenesis is primarily induced by noradrenaline from the sympathetic nervous system (SNS) (Girardier and Seydoux, 1986). Both α- and ß- adrenergic receptors are found in BAT. The subtype(s) of ß-adrenoceptor(s) which are found in brown adipocyte plasma membranes is still unresolved; it may be that there are various different subtypes including $ß_1$, $ß_2$ and a unique third subtype (see Himms-Hagen, 1989; Arch, Ainsworth, Cawthorne *et al*, 1984). The predominant mechanism by which noradrenaline activates BAT is through ß-adrenergic stimulation of cyclic AMP-dependent hormone-sensitive lipase and the

subsequent activation of the proton conductance pathway by the free fatty acids generated through lipolysis (Nicholls and Locke, 1984). ß-adrenergic stimulation also activates BAT thermogenesis indirectly by increasing the substrate supply in BAT through stimulating glucose uptake (Cooney, Caterson and Newsholme, 1985) and increasing lipoprotein lipase activity which results in an increased supply of fatty acids (Carneheim, Nedergaard and Cannon, 1984). In order to respond thermogenically to norepinephrine, BAT requires permissive amounts of thyroid hormone (Triandafillou, Gwilliam and Himms-Hagen, 1982). The conversion of T_4 to T_3 in BAT by endogenous iodothyronine 5'-deiodinase is also under sympathetic nervous system control (Silva and Larsen, 1983).

It has been reported that approximately 20% of thermogenesis in isolated BAT cells that is stimulated by noradrenaline is due to α_1-adrenergic receptor activation (Horwitz and Hamilton, 1984; Mohell, Connolly and Nedergaard, 1987; Schimmel, McCarthy and McMahon, 1986). The primary acute effect of α_1-adrenergic receptor activation and the subsequent activation of the phosphatidylinositol bisphosphate (PIP_2) cycle is Ca^{++} release from intracellular stores and the translocation of protein kinase C to the plasma membrane. The thermogenesis associated with α_1-adrenergic stimulation is related to the increased utilization of ATP for ion pumping, primarily by Na^+K^+-ATPase, which is necesssary for re-establishing ion gradients that are dissipated through the opening of ion channels following PIP_2 cycle activation (see Himms-Hagen, 1989).

The recent discovery that nerves on blood vessels, but not those on the adipocytes, also contain neuropeptide Y has raised the idea that noradrenaline is not the only neurotransmitter involved in the regulation of BAT activity (see Himms-Hagen, 1990a). Additional control may be exerted by sensory nerves which contain substance P and calcitonin gene-related peptide and which directly innervate BAT cells and blood vessels in BAT (Norman, Mukherjee, Symons *et al*, 1988; Lever, Mukherjee, Norman *et al*, 1988).

The control of DIT in BAT, which is exerted through the central nervous system, is not well understood (see Rothwell and Stock, 1982b). However the ventromedial hypothalamus (VMH) appears to be an important regulatory center since the signals which are stimulated through food ingestion and perhaps through specific components of the diet are apparently recognized there (see Himms-Hagen, 1990b). Moreover, lesions in the VMH will inhibit diet-induced thermogenesis, but not cold-induced NST (Hogan, Coscina and Himms-Hagen, 1982).

ii) **Endocrine regulation:** BAT activity is also modified by several hormones including corticosteroids, insulin and thyroid hormones. Corticosteroids inhibit BAT activity indirectly through SNS suppression (York, 1987). While corticosteroid receptors have been found in BAT (Feldman, 1978) no direct effects of corticosteroids are known. Insulin appears to stimulate thermogenesis through several direct and indirect mechanisms (see Himms-Hagen, 1989). Directly, insulin has been shown to stimulate specific mechanisms in BAT including lipogenesis (McCormack

and Denton, 1977; Saggerson, McAllister and Baht, 1988) and glucose uptake (Cooney *et al,* 1985; Smith, Young and Cawthorne, 1986). Insulin also stimulates the SNS (Rothwell and Stock, 1988; Landsberg and Young, 1984) and therefore it can indirectly stimulate BAT. Moreover, in response to dietary carbohydrate, insulin is also thought to stimulate diet-induced thermogenesis via the hypothalamus (Rothwell and Stock, 1988).

As earlier mentioned, BAT possesses its own supply of thyroxine 5' deiodinase (T5'D) which converts thyroxine (T_4) to the thermogenically active 3,5,3'-triiodothyronine (T_3). It thus can provide the tissue with endogenous T_3 and also contribute to the systemic supply of T_3 (Bianco and Silva, 1987b). Thyroid hormone contributes to the hypertrophy of BAT through both acute and chronic (trophic) activation mechanisms. While T_3 has no direct thermogenic effect on BAT, it is thought to exert a permissive effect in BAT at receptor and post-receptor levels (Sundin, Mills, and Fain, 1984). During the acute activation of BAT by ß-adrenergic stimulation T_3 carries out a permissive role. T_3 is also necessary for the induction of the trophic response which includes substantial selective increases in UCP synthesis (Bianco and Silva, 1987a,b).

c) <u>Physiological induction and suppression of BAT growth:</u>

i) <u>Induction of BAT growth:</u> The most potent stimulus for BAT growth is chronic exposure to cold (Himms-Hagen, 1990a). Hyperphagia and/or the

consumption of a palatable and varied cafeteria diet will also induce growth; however, the mechanisms involved differ from those involved in the activation of BAT through cold exposure (Himms-Hagen, 1990a). For the purposes of this thesis the discussion here will be limited mainly to diet-induced growth.

Feeding a cafeteria diet to rats will result in hypertrophied BAT, increased thermogenic capacity and enhanced blood flow within BAT following noradrenaline infusion (Rothwell and Stock, 1979, 1981, 1982a; Brooks, Rothwell, Stock *et al*, 1980). (A cafeteria diet consists of palatable high energy foods that are varied frequently.) Both high fat diets and high carbohydrate diets will induce BAT hypertrophy, and they apparently are equally effective in doing so (Rothwell, Stock and Warwick, 1983; Vander Tuig and Romsos, 1984). During diet-induced growth of BAT the alterations in $mRNA_{UCP}$ and in UCP levels are much smaller than those induced by cold exposure (see Himms-Hagen, 1990a). Also, in contrast to the changes occurring in BAT due to cold exposure, only small changes in BAT lipoprotein lipase occur in response to diet, and no changes in thyroxine 5'-deiodinase occur (see Himms-Hagen, 1990a). Therefore, although diet-induced growth of BAT, similar to cold-induced growth of BAT, is stimulated by SNS activity, the pattern of induction differs. It is probable that other mechanisms mediate the trophic responses of BAT to diet; these mechanisms may include insulin and/or specific diet-derived signals.

ii) **Atrophy of BAT:** Atrophy of BAT occurs when sympathetic tone is decreased or removed (for example, following surgical denervation) (see Himms-Hagen, 1989). When a heat load is imposed on the body, such as that during exercise, lactation or late pregnancy, atrophy occurs and it is thought to be induced by

thermoregulatory sensors and mediated by decreased SNS activity in BAT under control of the hypothalamus (Himms-Hagen, 1990a). Similar to the effects of denervation, fasting in rats and mice will also induce atrophy which is associated with losses of total BAT protein and total mitochondrial protein (Rothwell, Saville and Stock, 1984; Desautels, Dulos and Mozaffari, 1986; Trayhurn and Jennings, 1986, 1988). Fasting may (Trayhurn and Jennings, 1986, 1988) or may not (Desautels, 1985; Desautels and Dulos, 1988) produce selective decreases in mitochondrial UCP concentrations. No decreases in BAT DNA content occur during fasting (Desautels, 1985; Desautels *et al*, 1986; Desautels and Dulos, 1988). Decreases in cellular mitochondrial content and/or protein content also occur in several different types of obese rodents, including the genetically obese Zucker fa/fa rat, ob/ob mouse and db/db mouse. The defective BAT thermogenesis in these rodents is thought to contribute to their positive energy balance and obesity development (Himms-Hagen, 1989).

d) <u>Role of diet composition in BAT activity:</u>

The induction of BAT activity through the diet is related both to the composition of the diet and to the amount eaten. A low protein diet will activate BAT through the induction of sympathetic nervous system activity in BAT (Vander Tuig and Romsos, 1984; Young, Kaufman, Saville *et al*, 1985). This activation of BAT activity occurs even if hyperphagia is not observed. There is also some interaction

between dietary protein level and the response of BAT to sucrose feeding (Vander Tuig and Romsos, 1984; Kanarek, Aprille, Hirsch *et al*, 1987). When cafeteria diets are fed, hyperphagia is necessary for the stimulation of BAT activity (Himms-Hagen, 1990b).

The lipid composition of the diet appears to play an important role in DIT. Dietary lipid effectively stimulates sympathetic nervous system activity in BAT (Schwartz, Young and Landsberg, 1983) and stimulates BAT thermogenesis (Rothwell, Stock and Warwick, 1983; Vander Tuig and Romsos, 1984; Mercer and Trayhurn, 1984, 1987; Kim and Romsos, 1987). There are reports that the type of dietary fat is important in the activation of BAT (see Himms-Hagen, 1989). Dietary unsaturated fatty acids (from corn oil, 54% linoleic acid) have been reported to be more effective in the stimulation of BAT thermogenesis and SNS activity than the more saturated fatty acids of beef tallow (Mercer and Trayhurn, 1987). However in other studies in which rats were fed diets supplemented with linoleic acid (10% of energy) the activation of BAT thermogenesis was observed in one study (in which linoleic acid supplemented beef tallow) (Nedergaard *et al*, 1983) but not in another study (in which linoleic acid supplemented coconut oil) (Rafael, Patzelt and Elmadfa, 1988).

However, there is some evidence supporting the idea that diets containing high amounts of fat will suppress many aspects of BAT activity (see Himms-Hagen, 1989). High fat diets have been shown to decrease insulin responsiveness in BAT

(Kraegen, James, Storlien *et al*, 1986; Storlien, James, Burleigh *et al*, 1986), and suppress the synthesis of both fatty acids (Van Den Brandt and Trayhurn, 1981; Rothwell and Stock, 1983; Mercer and Trayhurn, 1984; Storlien *et al*, 1986) and triacylglycerol-glycerol (Storlien *et al*, 1986). The mechanisms involved in these effects are as yet unknown. A direct effect of the lipid on BAT cannot be ruled out as the administration of a lipid emulsion (Intralipid, 54% linoleic acid and including heparin), which greatly increases plasma levels of free fatty acids, results in a rapid decrease in BAT insulin-responsiveness (Jenkins, Storlien, Chisholm *et al*, 1988).

V) Metabolic adaptation to energy deficit and surfeit:

Adaptation can be defined as the process or processes by which an organism appropriately adjusts to its environment. As will be discussed in the following chapters, it has been shown in humans that during states of severe energy deficit, or protein energy malnutrition, the BMR is often decreased by up to 10% (Waterlow, 1986). A decrease in energy requirements in the face of decreased available energy resources would be considered as adaptation. During energy surfeit such as that occurring during the excessive consumption of dietary energy sources, a simultaneous increase in energy expenditure would similarly be considered as adaptation. This type of adaptation was referred to earlier as *luxuskonsumption* and BAT activity is thought to be one potentially contributing energy buffer mechanism.

The two mechanisms of energy expenditure which were selected for study

were Na^+,K^+ pump activity and BAT activity. The former is an ATPase-type of thermogenic mechanism in the control of substrate oxidation rates. The cell types studied included erythrocytes, thymocytes and hepatocytes. The latter mechanism is an uncoupled, ATPase-independent, type of thermogenic mechanism in the control of substrate oxidations. Various aspects of brown adipose tissue activity were studied; these included estimates of BAT cellularity, activation, total mitochondrial content and potential thermogenic capacity.

Chapter 2: General objectives

The objective of this research was to study the activity of the Na^+,K^+ pump and the thermogenic activity and capacity of brown adipose tissue (BAT) under conditions of energy deficit and surfeit in both human subjects and laboratory animals.

When this research was begun in 1986 there was evidence that blood cell Na^+,K^+ pump activity decreased during energy deficit in undernourished children in developing countries. It was hypothesized that this decrease in Na^+,K^+ pump activity contributed to "metabolic adaptation" - specifically, to the overall decrease in BMR observed during protein energy malnutrition. My first aim was to determine whether erythrocyte Na^+,K^+ pump activity was 1) decreased during the undernutrition observed in severe cerebral palsy and 2) altered following nutritional rehabilitation. This research is described in Chapter 3 of this thesis.

My second aim was to simultaneously study the two mechanisms of altered energy expenditure, Na^+,K^+ pump activity and BAT activity, in laboratory rats during diet-induced obesity and food restriction. As compared to the earlier human studies, the use of laboratory rats allowed greater control over potentially confounding variables (*eg.* drug treatments), as well as access to cell types other than erythrocytes. At the time that the study was started the idea that during diet-induced obesity and during food restriction Na^+,K^+ pump activity may act as an energy buffer similar to the role proposed for BAT, was controversial. It was hoped that the findings from the

simultaneous study of both mechanisms in rats might help to clarify some of the existing controversy. This research is described in Chapter 4 of this thesis.

Chapter 3: Undernutrition associated with severe cerebral palsy (CP) in children: Erythrocyte Na^+ and K^+ transport.

Introduction

Research in the area of Na^+ and K^+ transport during protein energy malnutrition (PEM) has focussed on the distinctly different alterations in ion transport observed in the two major forms of PEM, marasmus and kwashiorkor. According to the Wellcome classification system which utilizes the Boston weight/age standards (Hamill, Drizd, Johnson *et al*, 1977), marasmus is diagnosed when a child's weight/age is less than 60% of the standard weight of a normal child at the same age. Using the same classification system, kwashiorkor is diagnosed when a child is edematous and their weight/age is between 60-80% of the standard weight of a normal child at the same age (Jackson and Golden, 1983).

In developed countries PEM occurs much less frequently than in developing countries, and is usually secondary to disease which impairs intake and/or increases energy needs (*eg.* cystic fibrosis, cancer). Severe cerebral palsy (CP) is one such condition and malnutrition is commonly observed. Cerebral palsy is a descriptive term for a collection of nonprogressive neurological disorders of central origin that become apparent in early life, and are not the result of a recognized cerebral malformation (Paneth, 1986). In contrast to the origins of malnutrition in developing countries, where infection and defects in quality and quantity of food are important,

in CP the problem is with eating sufficient food because feeding efficiency is often reduced to less than 10% of normal (Gisel and Patrick, 1988).

In severe undernutrition the basal metabolic rate (BMR) can be reduced by up to 10% (Waterlow, 1986). In marasmus, it is thought that lower rates of active Na^+ and K^+ transport could play a significant role in the metabolic adaptation to the reduced nutrient availability (Jackson and Golden, 1983; Kaplay, 1984; Willis and Golden, 1988). In kwashiorkor, increased rates of pump-mediated ion transport have been implicated in contributing to the observed Na^+ retention and edema formation (Patrick, 1979; Golden and Ramdath, 1987). Low pump rates have been observed in erythrocytes (Willis and Golden, 1988) and leukocytes (Patrick and Golden, 1977) of marasmic children. After two to five weeks of nutritional rehabilitation, these transport rates increase (Patrick and Golden, 1977; Willis and Golden, 1988). In kwashiorkor, however, increased Na^+,K^+ pump activity has been observed in both cell types (Patrick and Golden, 1977; Willis and Golden, 1988); following nutritional rehabilitation Na^+,K^+ pump activity decreases and edema dissipates.

However, in both forms of PEM, Na^+ and K^+ fluxes which are mediated through mechanisms other than the Na^+,K^+ pump (*ie.* ouabain-insensitive ion fluxes) are increased in both blood cell types (Patrick and Golden, 1977; Willis and Golden, 1988). Because the maintenance of ion gradients requires a balance between ATP-requiring pumps and passive permeation (*ie.* ATP-independent), the estimation of changes in passive permeation of ions is at least as important as the estimation of

isolated pump activity (Zhao and Willis, 1988). The results of these studies involving children with PEM therefore indicate that there are alterations in both active and "passive" mechanisms of Na^+ and K^+ transport.

The biochemical mechanisms of metabolic adaptation during severe undernutrition are not well understood. However, the selective induction and repression of enzymes (Waterlow, 1968; 1986) and changes in hormonal profiles (Gardner and Amacher, 1973; Waterlow, Garlick and Millward, 1978; Torun and Viteri, 1988) are clearly involved. Although the amount of cellular energy used to fuel the Na^+,K^+ pump has not been established, as earlier discussed, it is thought that even a small but widespread reduction in cellular Na^+,K^+ pump activity in undernutrition could have a significant overall effect on basal energy requirements.

Objectives

The purposes of my work were twofold: 1) to determine if active and passive Na^+,K^+ transport differ between malnourished children with CP, well nourished children with CP and healthy control children. Since this form of secondary malnutrition is clinically more similar to marasmus than kwashiorkor, we expected that ion transport characteristics might also be similar to those during marasmus. 2) to treat the malnutrition using the rapid refeeding protocol developed at the Children's Hospital of Eastern Ontario (Patrick, Boland, Murray *et al*, 1986) and to determine whether the treatment reversed any changes in ion transport detected in

malnourished children.

Materials and methods

Patients: The children with CP were among those admitted to the Children's Hospital of Eastern Ontario for nutritional rehabilitation or those in a regional nursing home. The control children were those having routine blood tests prior to minor surgery. Informed consent was obtained from parents or guardians and the study was approved by the hospital's ethics committee.

Identification of malnutrition: On the basis that reduced subcutaneous fat stores are indicative of reduced energy reserves, triceps skinfold thicknesses less than the fifth percentile (HANES I %iles; Frisancho, 1974), combined with the failure to gain weight over the previous year were used to identify severe malnutrition in the children with CP. Anthropometric methods included a variety of circumferential and skinfold measurements (Lange calipers, Cambridge Scientific Institute) which were carried out using standard techniques, and reported as the mean of triplicate determinations. Conventional stature-based anthropometric standards cannot be used to identify PEM in children with CP as the children usually have skeletal abnormalities.

Clinical nutritional rehabilitation: Continuous nasogastric feeding was carried out using Biosearch enteral feeding pumps and Entriflex tubing (both from Biosearch Medical Products). The essential points of the feeding protocol which is

described elsewhere (Patrick *et al*, 1986) are as follows: (1) Initial Phase: The goal of this phase is the re-establishment of normal metabolism, without inducing growth. For practical purposes growth is equated with weight gain in the absence of Na^+ and water retention. (2) Second Phase: In this phase energy intake is gradually increased to the maximum tolerated and continued until weight gain ceases or the high energy feeding is no longer tolerated. Since the volume tolerated becomes the factor which limits intake, a feed with high energy density is an advantage. Thus, enteral feeds such as Isocal (Mead Johnson) or Ensure (Ross) are appropriate; infant formulae will not support very rapid growth rates. In these children maximal rates of 'catch up' growth require energy intakes that can only be achieved by nasogastric, gastrostomy, or jejunostomy tube feeding. A patient's intolerance of a previously accepted intake, as indicated by vomiting, bloating, or no further gain in weight, indicated that energy intake should be returned to maintanence levels and phase three of the program commenced. (3) Third Phase: During this phase the child gradually returns to normal feeding. After returning to maintenance levels of intake, tube feeding is withdrawn gradually (approximately 15-25% daily) so that the tube can be left *in situ* while normal feeding is gradually established.

Na^+ Efflux Analysis: Methods used to assess Na^+ efflux were those established by Garrahan and Glynn (1967a). Briefly, whole blood samples (approximately 5 ml) were taken from each child into Na^+-heparinized tubes. Samples were also collected from laboratory personnel as procedural controls.

Erythrocytes were isolated through centrifugation (3500*g*; 4 min; 4°C), and washed with three centrifugation/resuspension cycles using four volumes of buffered Ringer's medium composed of, mM: 145 NaCl, 5 KCl, 10 glucose, 2.5 Tris(hydroxymethyl)-2-aminoethane (TRIS), 1 $MgCl_2$, and 2 $CaCl_2$ in double deionized water. Hematocrits were determined in triplicate using autocrit centrifugation (Clay Adams Autocrit centrifuge).

To a 3 ml aliquot of the 10% hematocrit suspension 370 kBq (10 μCi) of ^{22}Na was added. Cells in the suspension were brought to a steady state during a three hour equilibration period at 37°C in a shaking water bath. Following equilibration, cells were washed three times with Ringer's solution (4°C) to remove extracellular ^{22}Na, and resuspended in Ringer's solution with or without 2.5 mM ouabain and with or without 0.1 mM bumetanide. The suspensions were then incubated at 37°C in a shaking water bath and aliquots were removed at 0, 15, 30, and 45 minute intervals. To halt fluxes the suspensions were centrifuged at 4°C (3500*g*; 4 min). After each time interval, aliquots of supernatant were removed for counting (Beckman Gamma 5500 Counter). These counts were then subtracted from baseline cell pellet counts (*ie.* determined for 0 min). The natural logarithms of these differences, plotted against time, represent the amount of ^{22}Na remaining inside the cells over time, and allow the calculation of Na^+ efflux rates (mmol/l cells/h) and rate constants (h^{-1}) (Garrahan and Glynn, 1967a).

The ion transport pathways studied are depicted and defined in Figure 3.1.

The Na^+ efflux components were identified as follows. The difference between total Na^+ efflux (efflux through all pathways) and that occurring in the presence of ouabain is the ouabain-sensitive, or Na^+,K^+ pump-mediated, efflux. The difference between efflux in the presence of ouabain and that in the presence of both ouabain and bumetanide is the bumetanide-sensitive, or Na^+,K^+,Cl^- cotransporter-mediated, efflux. Finally, the efflux occurring in the presence of ouabain and bumetanide is referred to as residual, or "leak", Na^+ efflux.

K^+ Influx Analysis: Over the time during which these studies were conducted I also began to use methods for measuring erythrocyte K^+ influx; these methods are described by Young and Ellory (1982) and by Hall and Willis (1984). Briefly, approximately 2 ml of whole blood was collected into heparinized glass tubes. The erythrocytes were washed three times by centrifugation (3500*g*; 4 min), followed by aspiration of the buffy coat and resuspension in an ice-cold incubation medium (mM): 145 NaCl, 1.25 $MgCl_2$, 10 N-2-hydroxyethylpiperazine-N'-ethane sulfonic acid (HEPES), 7.5 KCl, 10 glucose, 5 adenosine and 5 NaOH (pH, 7.4). After exactly twenty minutes incubation (37°C) with ^{86}Rb (74.1 kBq/ml or 2 μCi/ml), cells were cooled for five minutes (0°C), separated from incubation medium by centrifugation (15000*g*, 15 seconds) in an Eppendorf microcentrifuge, and washed four times in ice-cold 107 mM $MgCl_2$, TRIS-buffered (pH 7.4) wash solution. Cells were lysed in 5% trichloroacetic acid, and the radioactivity measured as Cerenkov radiation detected in a liquid scintillation counter (Beckman LS 1801). The rate of K^+ influx (mmol/l

cells/hr) was calculated from the specific activity of ^{86}Rb, and cell volume determinations. Previous studies have revealed that ^{86}Rb is a suitable K^+ analogue for human erythrocytes (Willis and Golden, 1988). However, ^{86}Rb may produce a larger apparent bumetanide-sensitive K^+ influx (Canessa, Brugnara, Cusi *et al*, 1984; Wolowyk and Willis, 1984). The ion transport pathways studied are depicted and defined in Figure 3.1.

The K^+ influx components were identified as follows. The difference between total K^+ influx (influx through all pathways) and that occurring in the presence of ouabain is the ouabain-sensitive, or Na^+,K^+ pump-mediated, influx. The difference between influx in the presence of ouabain and that in the presence of both ouabain and bumetanide is the bumetanide-sensitive, or Na^+,K^+,Cl^- cotransporter-mediated, influx. Finally, the influx occurring in the presence of ouabain and bumetanide is referred to as residual, or "leak", K^+ influx.

Plasma and intracellular Na^+ and K^+ concentrations: Plasma and intracellular Na^+ and K^+ concentrations were measured by flame photometry (Instrumentation Laboratory, Model 443) using Li^+ as the internal standard. Intracellular ion concentrations were determined using cells that had been equilibrated and washed in parallel with the isotope-containing cell suspension tubes. During the analysis of each set of samples several standards which were prepared by serial dilution of Instrumentation Laboratory $[Na^+]$ and $[K^+]$ standards were run.

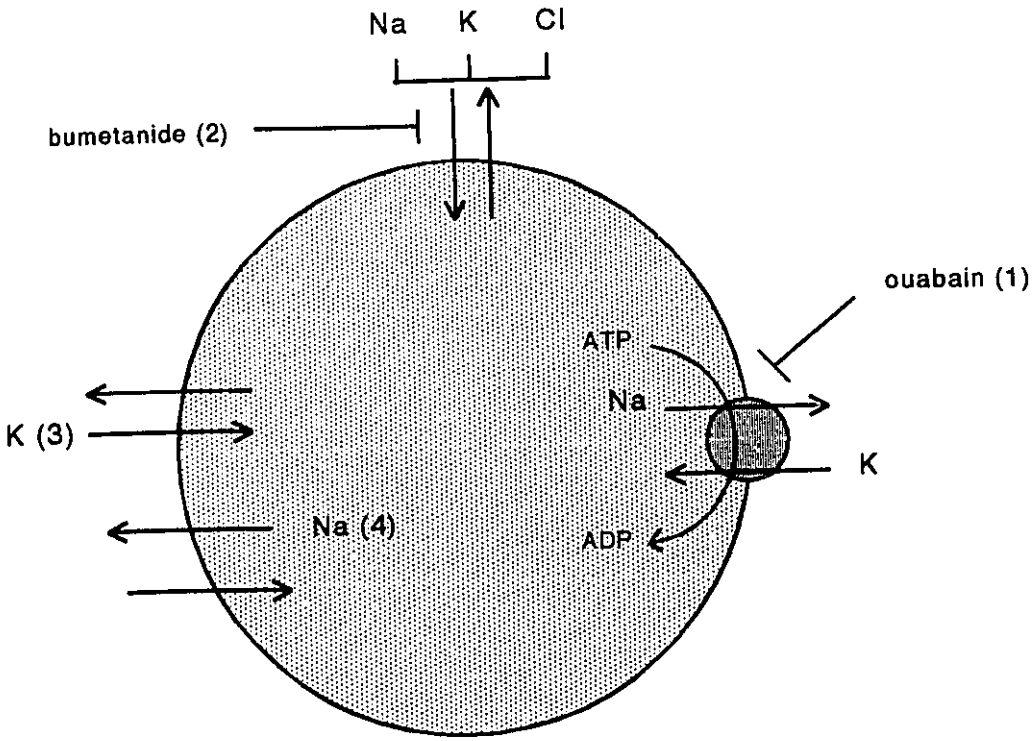

Figure 3.1: Cellular Na^+ and K^+ transport pathways studied. Total flux: the unidirectional flux of Na^+ or K^+ through all pathways in the absence of any inhibitors. 1) Na^+,K^+ pump-mediated flux: the component of total ion flux which is ouabain-inhibitable. 2) Na^+,K^+,Cl^- cotransporter-mediated flux: the component of total ion flux which is bumetanide-inhibitable. 3) and 4) Residual flux: the unidirectional ion flux which remains in the presence of ouabain and bumetanide.

Data presentation and analysis: Each of the results is presented as a mean (±SEM). Statistical analyses were carried out using True Epistat software on a personal computer. In studies 1 and 2 both parametric and non-parametric methods were used; the results obtained were not materially different between the two methods. However, due to the small sample sizes and some population distributions which differed from normal (*eg.* Fig. 3.5), the results from the more appropriate non-parametric tests are reported here. Therefore Wilcoxon rank sum (Mann-Whitney U) tests and sign rank tests were used for non-paired and paired comparisons, respectively. In study 3 the results between the three groups were compared using ANOVA which was followed by Newman-Keuls' tests. Bartlett's tests for homogeneity of variance revealed that ANOVA was appropriate for the analysis of these data.

Results

The groups of children which were involved in these studies are briefly described in Table 3.1. Body weights with reference to standards of weight-for-age are included to provide an idea of the abnormality and variability of anthropometrics in children with severe CP. Because of stunting and deformity, the data give no more than a general idea of nutritional status. In malnutrition associated with severe CP, triceps skinfold data are the best available indices of undernutrition and response to refeeding. With regard to the Na^+,K^+ flux measurements, paired comparisons were carried out before and after 3-5 weeks of clinical nutritional rehabilitation in study

2 only. Non-paired comparisons in studies 1 and 3 were carried out with patients that were classified on the basis of triceps skinfold data either as being malnourished or well nourished. It should be noted that in study 3 the K^+ influx data from the children with CP were also compared to results from the 20 control children having a mean age of 5.4 years (SEM = $\pm$0.805).

Na^+ efflux analyses: The comparison of erythrocyte Na^+,K^+ pump-mediated Na^+ efflux results from a group of malnourished CP children with results from a group of well nourished CP children (Table 3.1, study 1) revealed that Na^+ efflux was not significantly lower in the malnourished children (Table 3.2; Figure 3.2). Residual Na^+ efflux also was not significantly different between the two groups (Table 3.2; Figure 3.3). Bumetanide-sensitive Na^+ efflux and intracellular Na^+ and K^+ concentrations were not significantly different between the groups (Table 3.2).

Erythrocyte Na^+ efflux was also studied in a group of malnourished CP children before and after 3-5 weeks of clinical nutritional rehabilitation (Table 3.1, study 2). Anthropometric data collected from the children before and after nutritional rehabilitation are shown in Table 3.3. The erythrocyte Na^+ efflux results are provided in Table 3.4 and in Figures 3.4 and 3.5. The results also do not support lower Na^+,K^+ pump activity during undernutrition associated with CP. When the data were analysed using sign rank tests no statistically significant differences were identified in erythrocyte Na^+,K^+ pump activity ($P < 0.18$) and residual Na^+ efflux

($P < 0.08$) between the children before and after the nutritional treatment. Again, bumetanide-sensitive Na^+ efflux and intracellular Na^+ and K^+ concentrations were not significantly different between the groups.

Table 3.1: Overview of Na^+,K^+ flux studies using erythrocytes from children with cerebral palsy.*

Study	RBC Na^+,K^+ flux assay	Patient Group	Age (yr)	Wt/Age (%)[a]	TSF (%)[b]
1	Na^+ efflux	MN (n=16)	10.7 (1.30)	49 (2.5)	40 (1.4)
		WN (n=10)	10.1 (1.49)	57 (4.7)	72 (5.9)
2	Na^+ efflux	Pre (n=7)	6.9 (1.73)	48 (4.4)	38 (2.1)
		Post (n=7)	7.0 (1.73)	62 (5.4)	71 (5.0)
3	K^+ influx	MN (n=11)	16.6 (2.20)	41 (2.4)	43 (2.4)
		WN (n=11)	15.4 (2.20)	54 (4.1)	74 (3.7)

* Information is organized into groupings which correspond to the Na^+,K^+ flux analyses conducted. MN: malnourished; WN: well nourished; Pre: before clinical nutritional rehabilitation; Post: after clinical nutritional rehabilitation. Each of the data is presented as a mean (±SEM).

[a] Data are expressed as a percentage of the National Center for Health Statistics (NCHS) mean for age (Hamil, Drizd, Johnson *et al*, 1977).

[b] Triceps skinfold data (mean of three measurements) are expressed as a percentage of the 1971-74 Health and Nutrition Examination Survey mean for age (Frisancho, 1974).

Table 3.2: **Na^+ efflux from erythrocytes of well and malnourished children with cerebral palsy.***

Patient Group	Total (mmol/l/h)	Na^+,K^+ pump (mmol/l/h)	Na^+,K^+,Cl^- Cotransporter (mmol/l/h)	Residual (mmol/l/h)	$[Na^+]_i$ (mmol/l)
Mal-nourished n = 16	2.44 (0.208)	1.57 (0.188)	0.26 (0.030)	0.61 (0.073)	7.5 (0.38)
Well nourished n = 10	2.04 (0.114)	1.27 (0.221)	0.31 (0.076)	0.46 (0.0632)	7.1 (0.44)

* Each of the data is expressed as a mean (±SEM).

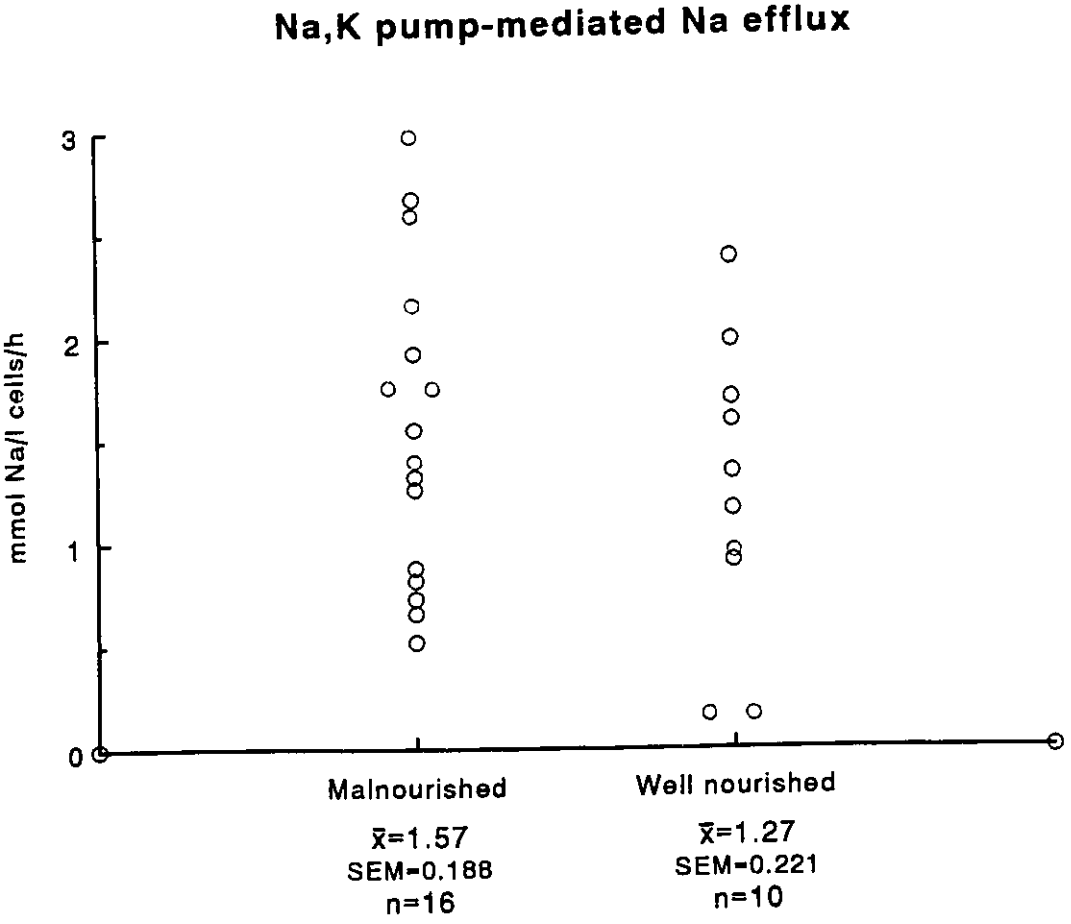

Figure 3.2: Na^+,K^+ pump-mediated Na^+ efflux from erythrocytes of well and malnourished children with cerebral palsy. A statistical comparison of the means using a Wilcoxon rank sum test revealed that the means are not significantly different.

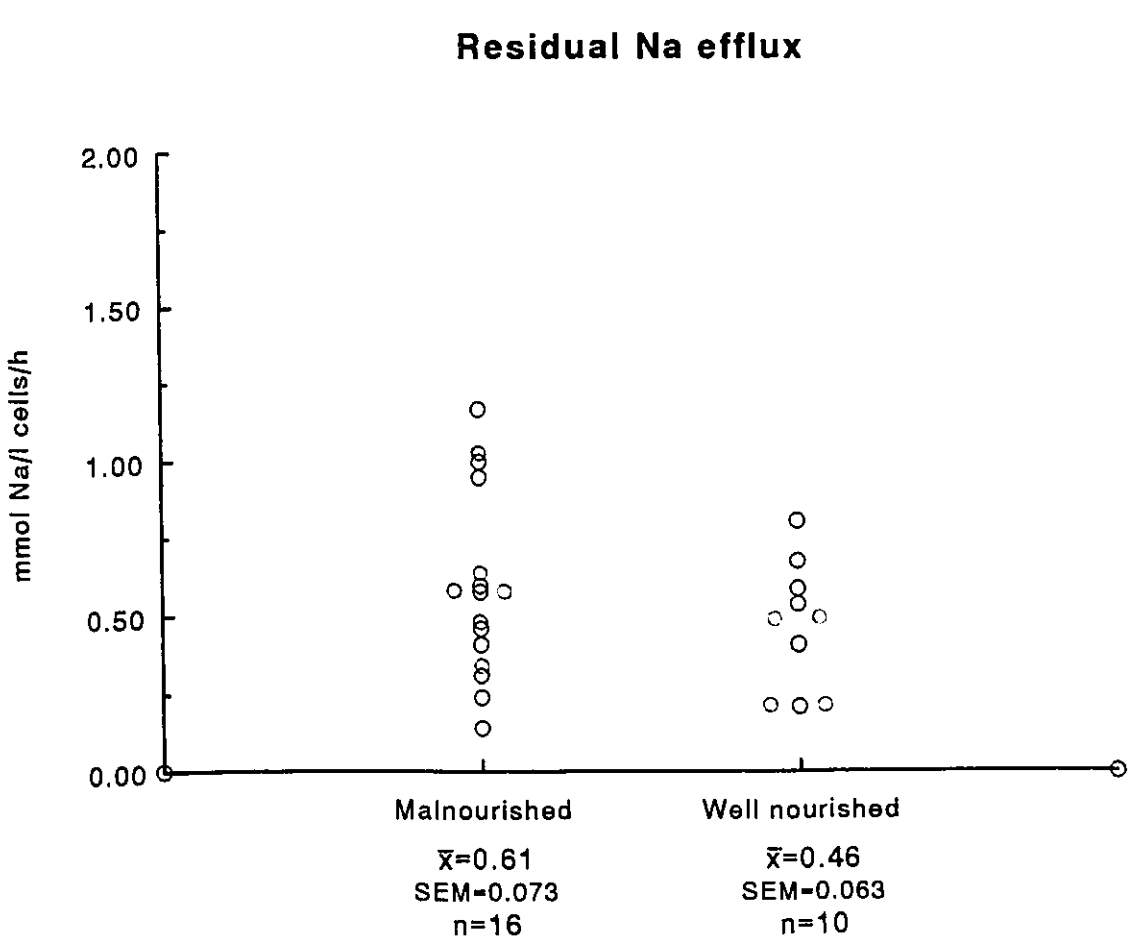

Figure 3.3: Residual Na^+ efflux from erythrocytes of well and malnourished children with cerebral palsy. A statistical comparison of the means using a Wilcoxon rank sum test revealed that the means are not significantly different.

Table 3.3: Anthropometric profiles of children with cerebral palsy before and after clinical nutritional rehabilitation.

Patient; sex	Age (yr)	BWt (kg)	BWt (% mean)[a]	TSF (cm)[b]	TSF (%ile)[c]	MAMC (cm)[d]
Pre 1; F	2.0	7.7	63.1	6.8	<5	11.0
2; F	14.5	18.2	34.1	4.4	<5	13.9
3; M	11.1	13.4	35.8	2.0	<5	11.4
4; F	1.9	7.6	62.2	6.0	<5	11.2
5; M	3.0	6.2	43.0	2.0	<5	10.4
6; F	6.3	11.0	52.1	1.5	<5	12.0
7; M	9.5	12.1	39.8	2.2	<5	11.8
Post 1; F	2.1	9.7	79.4	8.2	<25	12.9
2; F	14.6	--[e]	--	7.8	<10	14.6
3; M	11.2	18.9	50.4	6.0	<10	14.5
4; F	2.0	9.2	75.4	7.5	<25	12.9
5; M	3.1	9.4	64.5	7.0	<25	12.9
6; F	6.4	13.8	65.5	6.5	<10	13.7
7; M	9.6	16.6	54.6	6.4	<10	13.4

[a] Data are expressed as percentages of the National Center for Health Statistics (NCHS) mean-for-age (Hamil, Drizd, Johnson, *et al*, 1977).
[b] Triceps skinfold thickness (mean of three measurements).
[c] Percentiles are based on the 1971-74 Health and Nutrition Examination Survey (Frisancho, 1974).
[d] Mid arm muscle circumference: calculated using the nomogram method of Gurney and Jelliffe (1973).
[e] Data not available; the child was given a leg cast during the feeding period.

Table 3.4: **Na^+ efflux from erythrocytes of children with cerebral palsy: The effects of three to five weeks of nutritional rehabilitation.***

Patient Group	Total (mmol/l/h)	Na^+,K^+ pump (mmol/l/h)	Na^+,K^+,Cl^- Cotransporter (mmol/l/h)	Residual (mmol/l/h)	$[Na^+]_i$ (mmol/l)
Pre n = 7	2.63 (0.438)	1.87 (0.382)	0.20 (0.034)	0.56 (0.113)	7.1 (0.49)
Post n = 7	2.37 (0.125)	1.56 (0.159)	0.21 (0.038)	0.40 (0.057)	6.8 (0.57)

* Each of the data is expressed as a mean (±SEM).

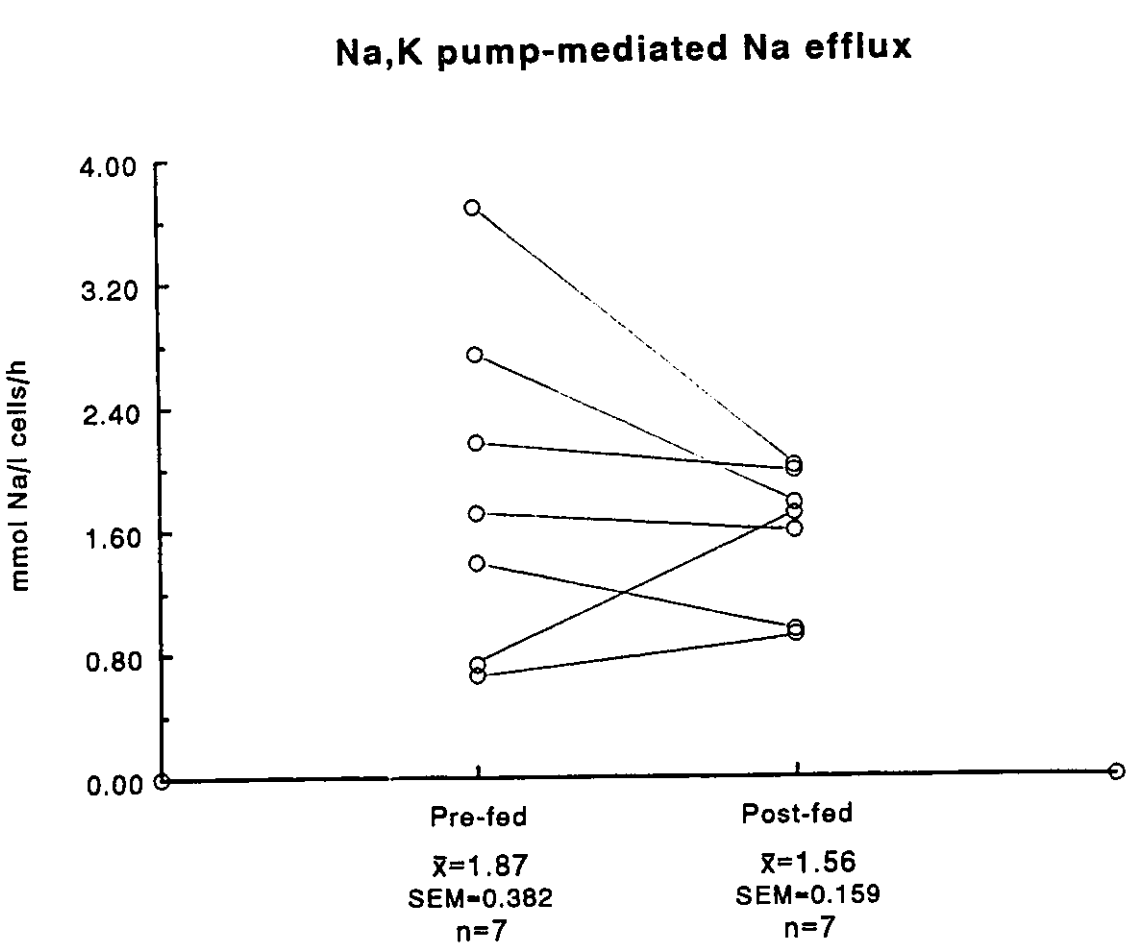

Figure 3.4: Na^+,K^+ pump-mediated Na^+ efflux from erythrocytes of children with CP before and after clinical nutritional rehabilitation. A statistical comparison of the means using a sign rank test revealed that the means are not significantly different.

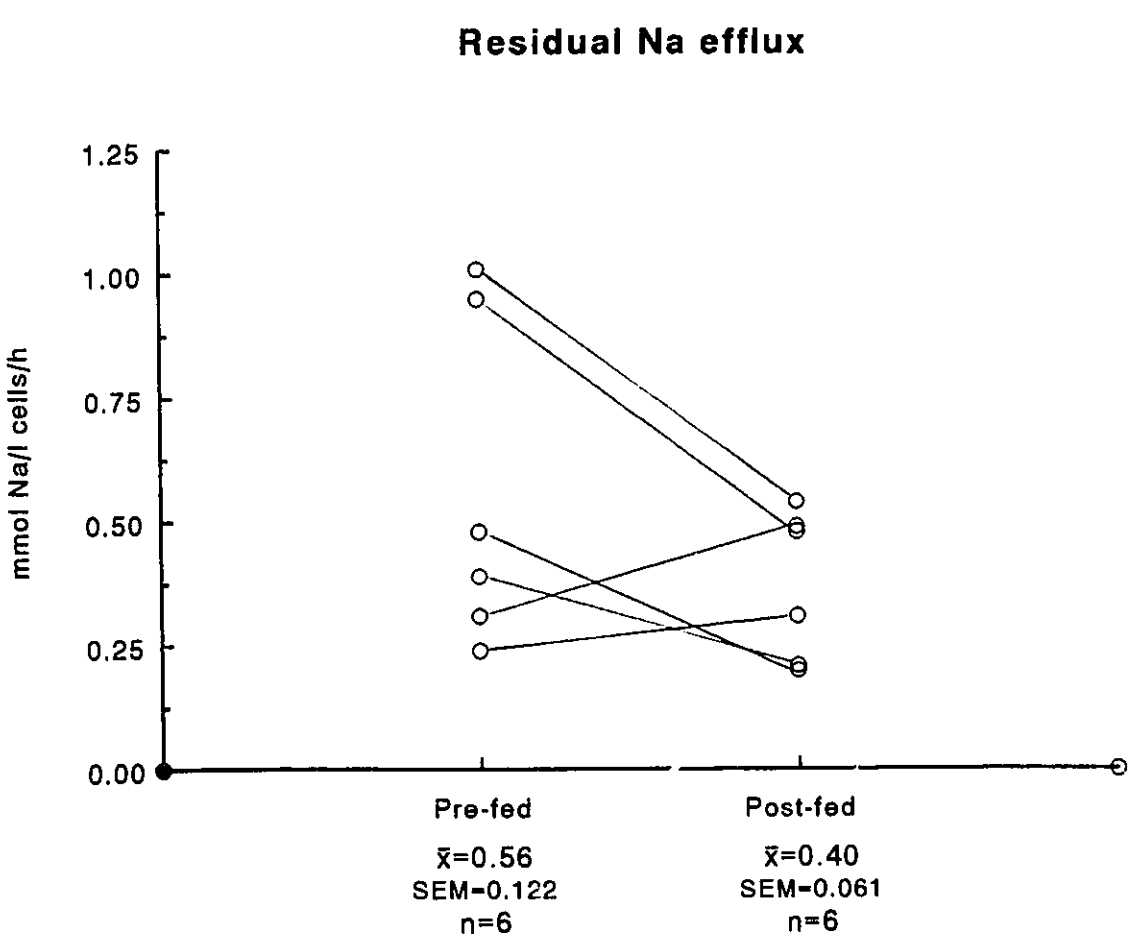

Figure 3.5: Residual Na^+ efflux from erythrocytes of children with CP before and after clinical nutritional rehabilitation. A statistical comparison of the means using a sign rank test revealed that the means are not significantly different.

K^+ influx analyses: K^+ influx data from 11 malnourished CP children, 11 well nourished CP children, and 20 well nourished control children are shown in Table 3.5 and in Figures 3.6 and 3.7. Total K^+ influx was greater in malnourished CP children than in control children. Na^+,K^+ pump-mediated K^+ influx was greater in malnourished CP than in well nourished CP children and greater in malnourished CP children than in control children (Table 3.5; Figure 3.6). However, no significant difference was found between results from well nourished CP children and controls. Na^+,K^+ pump-mediated K^+ influx was thus greatest in malnourished CP, followed by well nourished CP and control children. Analyses of residual K^+ influx results did not reveal statistically significant differences between the means of the three groups (Table 3.5; Figure 3.7). There were no differences in intracellular Na^+ concentrations, $[Na^+]_i$, between groups and thus the greater Na^+,K^+ pump activity observed in the malnourished CP group cannot be accounted for by an elevated $[Na^+]_i$ which would tend to stimulate pump activity. The results, therefore, again do not support the original hypothesis that Na^+K^+ pump activity is lower in this form of secondary PEM.

Table 3.5: K^+ influx in erythrocytes from well and malnourished children with cerebral palsy and control children.*

Patient Group	Total Influx (mmol/l/h)	Na^+,K^+ pump (mmol/l/h)	Na^+,K^+,Cl^-, cotransporter (mmol/l/h)	Residual (mmol/l/h)	$[Na^+]_i$ (mmol/l)
Mal-nourished (n=11)	3.57 (0.280)[a]	2.46 (0.181)[bc]	0.42 (0.069)	0.70 (0.048)	8.6 (0.32)
Well nourished (n=11)	2.90 (0.208)	1.86 (0.130)[c]	0.38 (0.069)	0.66 (0.039)	9.2 (0.28)
Controls (n=20)	2.53 (0.101)[a]	1.63 (0.081)[b]	0.27 (0.029)	0.62 (0.060)	9.6 (0.29)

* Each of the data is expressed as a mean (±SEM). Values having the same superscript letters are significantly different ($P < 0.01$). Analysis of data between patient groups (within columns) was carried out using ANOVA which was followed by Newman-Keuls' tests. Bartlett's tests for homogeneity of variance revealed that ANOVA was appropriate for the analysis of these data.

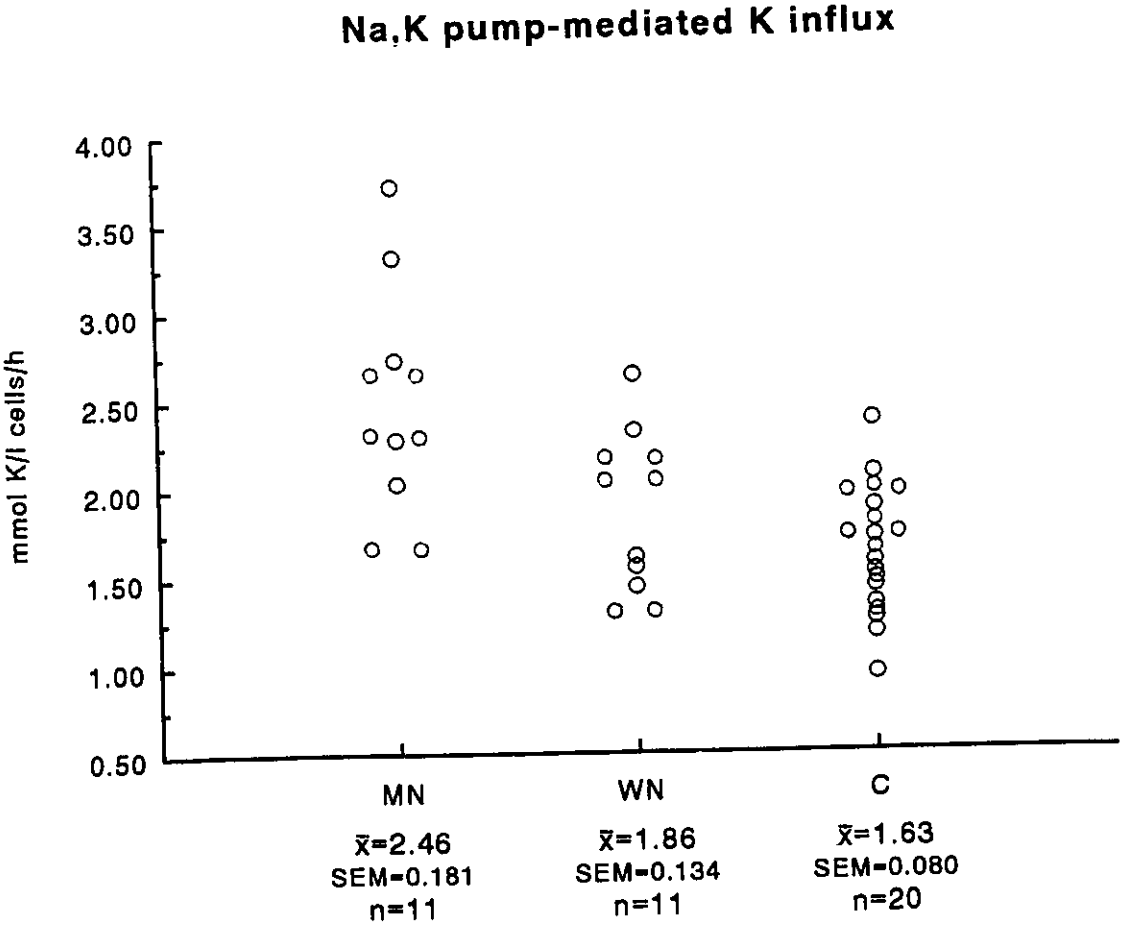

Figure 3.6: Na^+,K^+ pump-mediated K^+ influx in erythrocytes of malnourished and well nourished children with CP and of control children. A statistical comparison of the means using Newman-Keuls' tests showed that Na^+,K^+ pump-mediated K^+ influx is significantly ($P < 0.01$) greater in the malnourished group than in the well nourished or control groups.

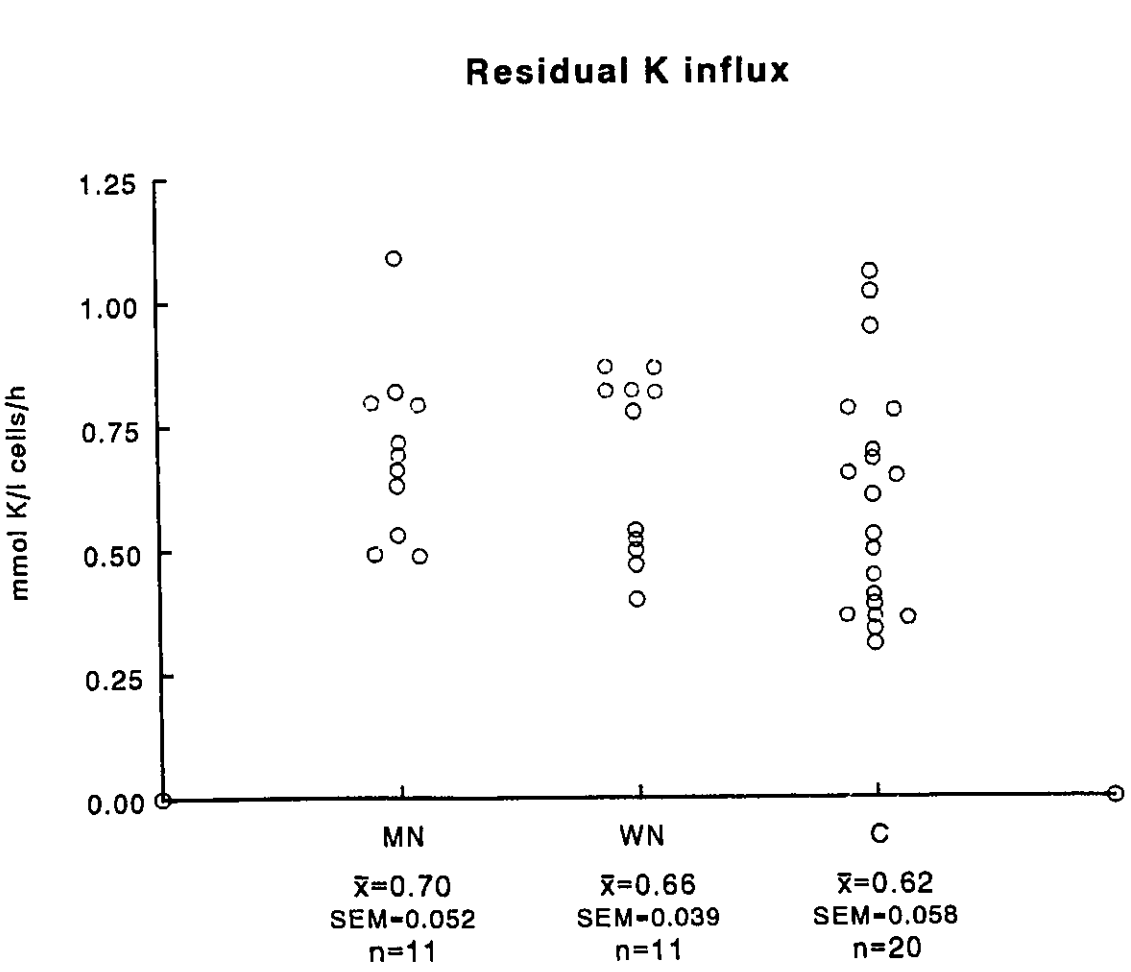

Figure 3.7: Residual K^+ influx in erythrocytes of malnourished and well nourished children with CP and of control children. A statistical comparison of the means using Newman-Keuls' tests revealed that the means are not significantly different ($P > 0.05$).

Discussion

The results of studies 1 and 2 demonstrate no effects of nutritional status on erythrocyte Na^+ efflux. In study 3 it was shown that Na^+,K^+ pump-mediated K^+ influx was increased in malnourished CP children as compared to well nourished CP children and to control children. In all three studies no effect of nutritional status was observed on Na^+,K^+,Cl^-cotransporter-mediated, or on "passive" residual Na^+ and K^+ transport. Why differences in Na^+,K^+ pump activity were observed when ouabain-sensitive K^+ influx was assayed, but not when ouabain-sensitive Na^+ efflux was assayed is unknown. However, the precision of the K^+ influx techniques was consistently greater than that of the Na^+ efflux techniques; the coefficient of variation (*ie.* (standard deviation/mean) x 100) of the former was approximately 23% while that of the latter was approximately twice the value. One of the main reasons why I began to use the K^+ influx techniques instead of the Na^+ efflux techniques was the increased precision associated with the K^+ influx techniques. The possibility that there were changes in pump-mediated K^+ influx and not in pump-mediated Na^+ efflux (*ie.* altered stoichiometry of the Na^+,K^+ pump) however cannot be ruled out.

Previous studies of Na^+,K^+ pump activity in erythrocytes and leukocytes of marasmic children in developing countries have revealed distinct transport differences between cells of marasmic children before and after nutritional rehabilitation. Patrick and Golden (1977) revealed lower Na^+,K^+ pump-mediated Na^+ effluxes balancing a lower Na^+ influx in leukocytes of marasmic children, when compared to efflux

determinations in the same children following nutritional rehabilitation. In children with kwashiorkor leukocyte Na^+,K^+ pump activity was increased but decreased during nutritional rehabilitation.

The results of Willis and Golden (1988) obtained from erythrocytes of marasmic children revealed lower Na^+,K^+ pump activities (when differences in cell $[Na^+]_i$ were controlled for) during marasmus as compared with activities in cells both from the same children following nutritional rehabilitation, and from well nourished control children. Since changes in erythrocyte activity occurred within two to five weeks, a small fraction of the red cell lifetime of 120 days, the authors concluded that the alterations in activity must have been mediated via a circulating factor (Willis and Golden, 1988). The *de novo* synthesis of erythrocyte pump molecules could not have been involved as this would involve cell renewal via the stem line. Indirect support for a circulating factor in the modulation of Na^+,K^+ pump activity during PEM and its treatment may be obtained from the results of earlier studies. Kaplay (1978), who studied purified erythrocyte membrane Na^+,K^+-ATPase activities (*ie.* inorganic phosphate release), found no differences between marasmic and well nourished children. Patrick and Golden (1979) who not only studied Na^+,K^+ pump activity in intact leukocytes but also incubated the cells in their own plasma, found that there were abnormalities in pump activity during marasmus and kwashiorkor which were normalized during nutritional rehabilitation. It is possible that during the preparation of the purified erythrocyte membranes (Kaplay, 1978) any effect of an

circulating factor was lost.

In both forms of PEM ouabain-insensitive ion fluxes are increased (Patrick and Golden, 1977; Willis and Golden, 1988). Patrick and Golden (1977) observed that in kwashiorkor the total amount of leukocyte Na^+ efflux which remained in the presence of ouabain was increased. Similar to the K^+ transport assays which I conducted, Willis and Golden (1988), as well as assaying the total amount of Na^+ efflux and K^+ influx which remained in the presence of ouabain, also assessed the proportion of ouabain-insensitive ion flux which was attributable to Na^+,K^+,Cl^- cotransporter activity, and the residual ion fluxes which remained in the presence of both ouabain and bumetanide. Willis and Golden (1988) found the residual ion fluxes to be increased in both marasmus and kwashiorkor. Similar to my results, they too found no changes in Na^+,K^+,Cl^- cotransporter activity. While Patrick and Golden found that the increased ouabain-insensitive Na^+ efflux was corrected in most cases within 5 to 7 days of dietary treatment, Willis and Golden found no changes in either Na^+,K^+,Cl^- cotransporter activity or residual ion fluxes following nutritional rehabilitation.

The mechanistic aspects of altered ouabain-insensitive Na^+ efflux and K^+ influx during undernutrition are unknown. The physiological functions and regulation of Na^+,K^+,Cl^- cotransport are complex (Haas, 1989). Ouabain-insensitive fluxes of both Na^+ and K^+ have, however, been shown to be raised by oxidative stress (Mardones-neau, Braquet and Garay, 1988; Hynes and Willis, 1986), which is believed

to occur during severe PEM (Golden and Ramdath, 1987).

The results of the one study in laboratory animals of intact cell Na^+,K^+ transport during starvation (Zhao and Willis, 1988) are consistent with an adaptive decrease in Na^+,K^+ pump activity during severe undernutrition. Erythrocyte Na^+,K^+ pump activity, Na^+,K^+,Cl^- cotransporter activity and residual (*ie.* ouabain- and bumetanide-insensitive) pathways of K^+ influx were assessed in Sprague Dawley rats before and after 7 - 14 days of starvation. Na^+,K^+ pump activity decreased 31% during this period, and decreases were correlated to the degree of weight loss. As in marasmus, Na^+,K^+,Cl^- cotransporter activity was unaffected by the undernutrition. In contrast to the earlier human studies, residual K^+ influx decreased significantly.

In the non-edematous undernutrition associated with anorexia nervosa, lower ouabain-sensitive K^+ influx and [^{3}H]ouabain binding were observed in leukocytes as compared to results following weight gain, and to results from healthy controls (Turaihi, D'Souza, Wakeling *et al*, 1988). These results are similar to those obtained during the refeeding of marasmic children (Patrick and Golden, 1977; Willis and Golden, 1988). Erythrocyte ion transport analyses have not, however, revealed significant differences in pump activity between anorexia nervosa patients and healthy controls (DeLuise, Izumo, Grace *et al*, 1983; Pasquali, Strocci, Malini *et al*, 1985). Thus, it appears that differences can exist among different cell types in response to some forms of undernutrition.

My results therefore are not consistent with the changes in erythrocyte Na^+,K^+

pump activity observed during marasmus and starvation. It was expected that if alterations were observed in Na^+ and K^+ transport during secondary PEM in children with severe CP, the alterations would be similar to those changes seen in marasmic PEM. My results are more similar to those obtained from children with kwashiorkor where increased Na^+,K^+ pump-mediated fluxes were reported in both leukocytes and erythrocytes (Patrick and Golden, 1977; Willis and Golden, 1988). However, unlike the results obtained from children with kwashiorkor, no increases in residual ion fluxes were observed in the results from these undernourished children with cerebral palsy. Why the characteristics of erythrocyte Na^+,K^+ pump-mediated ion transport in undernutrition during severe CP more closely resemble those during kwashiorkor is unknown. The proposed involvement of free radicals in the pathogenesis of kwashiorkor (Golden and Ramdath, 1987) is most likely not applicable to these children. The mechanisms responsible for altered Na^+,K^+ pump activity during secondary PEM in severe CP and kwashiorkor are probably distinct.

There are several possible explanations for my findings. The nutritional problem in CP is caused by oral-motor insufficiency with feeding function often as low as 10% of normal (Gisel and Patrick, 1988). The children are frequently in the process of feeding for 3 to 5 hours each day - much longer than normal children. Conceivably the sensory stimulation occurring as a result of food ingestion during these extended time periods induces an extended stimulation of sympathetic nervous system activity. As earlier discussed catecholamines are known to stimulate Na^+,K^+

pump activity. Perhaps this in part explains the higher erythrocyte Na^+,K^+ pump-mediated K^+ influx in the malnourished CP children as compared to the well nourished CP and control children. Many of these children also have substantial uncontrolled voluntary muscle activity; this is in marked contrast with the apathetic inactive marasmic child. Seizures and seizure medication also complicate the picture in most of these children with CP. In addition, these children are older than the third world children previously involved in blood cell ion transport studies. The malnutrition in these children with CP for example, has developed for up to 15 years.

Finally, erythrocyte membrane lipid alterations do occur during the feeding of the high polyunsaturated fat diet used in the nutritional rehabilitation of these undernourished children (Harper, Patrick, Kramer *et al*, 1990). Such local changes in membrane composition could possibly have contributed to the unaltered or decreased Na^+,K^+ pump activity during the rehabilitation of these children.

My results demonstrate that the effects of undernutrition on Na^+,K^+ transport in children with cerebral palsy are distinct from the effects observed in children with PEM in developing countries. Further studies are needed in order to further characterize the changes in ion transport in undernourished cerebral palsy children. It would be well to assess Na^+,K^+ transport in another cell type, such as the leukocyte, in these children. However, there are several technical problems to surmount, at least for this patient population, because of the large blood volumes required for leukocyte Na^+,K^+ transport analyses.

Chapter 4: Diet-induced obesity and undernutrition in laboratory animals: metabolic adaptation and mechanisms of altered energy expenditure.

Introduction

The physiological relevance of mechanisms of altered energy expenditure (such as altered Na^+,K^+pump and BAT activities) in the energy balance equation remains controversial. In laboratory animals deficient diet-induced activation of BAT has been shown in several models of obesity (see Himms-Hagen, 1989). In these animals it is thought that deficient activation of BAT contributes to the development of obesity (Himms-Hagen, 1989). Included in these animal models of obesity are the genetically obese fa/fa rat and the ob/ob mouse; the A^{vy} mouse; the GTG mouse; the aging rat and rats having lesions in the VMH. In contrast when obesity is induced in laboratory animals through the provision of a palatable diet *increased* BAT activity is also thought to play mitigate the development of this diet-induced obesity (DIO). In this case the diet-induced thermogenesis in BAT is thought to attenuate the degree of the obesity which otherwise might result (Rothwell and Stock, 1979; Himms-Hagen, 1989).

Other studies involving both humans and laboratory animals have focused on the hypothesis that decreased energy expenditure attributable to decreased Na^+,K^+ pump activity also contributes to the development of some forms of obesity (DeLuise, Blackburn and Flier, 1980; Klimes, Nagulesparan, Unger *et al*, 1982; Bray, York and

Yukimura, 1978). This hypothesis is based on the idea that even a small generalized decrease in Na^+,K^+ pump activity could have a significant effect on basal energy requirements, considering the ubiquity of the Na^+,K^+ pump. However support for this hypothesis has not been unanimous. The results of several studies describe unaltered or increased Na^+,K^+ pump activity in obesity (Bray, Kral and Björntorp, 1981; Clausen and Hansen, 1982; Charalambous, Morgan, Spurlock *et al*, 1984a; Charalambous, Webster and Mir, 1984b; Turaihi, Baron and Dandona, 1987).

Diet-induced obesity (DIO): The model of obesity which I chose for study is that induced in Sprague Dawley rats by high fat diets. The Sprague Dawley rat is appropriate for such studies as this strain is known to be susceptible to diet-induced obesity (Levin, Triscari and Sullivan, 1986).

In the development of DIO there are three potential components, as discussed by Himms-Hagen (1989). The first component relates to the lower metabolic cost of storing dietary lipid in the form of adipose triacylglycerol as compared to the cost of converting dietary protein or carbohydrate into adipose triacylglycerol. Therefore, with a high fat diet animals would have to decrease their dietary energy intake to maintain energy balance, unless energy expenditure was simultaneously increased. Amplifying this enhanced efficiency is the fact that high fat diets are often found to be more palatable by the animals and increased, not decreased, dietary energy intakes result. Thirdly, after obesity has been established additional metabolic changes occur which contribute to the obesity; such changes include the development

of insulin resistance and hyperinsulinemia. As a result, the energy intake side of the energy balance equation continues to outweigh energy expenditure and the propensity for the development of obesity increases.

Na^+,K^+ pump activity and obesity: In recent years a minor literature on Na^+,K^+ pump activity in obese humans and animals has emerged (DeLuise *et al*, 1980; 1982; Klimes *et al*, 1982; Bray *et al*, 1978; 1981; Clausen and Hansen, 1982; Charalambous *et al*, 1984a; Charalambous *et al*, 1984b; Turaihi *et al*, 1987; Mir, *et al*, 1981; Beutler, Sacks and Kuhl, 1982; Beutler, Kuhl and Sacks, 1983; El Mallakh, 1986; Pasquali, Cesari, Melchionda *et al*, 1988). The overall findings of these studies have not consistently provided support for the original hypothesis that subnormal activity of the Na^+,K^+ pump contributes to an energy imbalance which would favour the development of obesity.

The first of these publications was from DeLuise *et al* (1980) who measured ouabain binding and Na^+,K^+ pump-mediated K^+ influx in human erythrocytes of obese and control subjects. They concluded that there was an inverse relationship between body weight and the membrane concentration of Na^+,K^+ ATPase. They also found that ouabain-sensitive K^+ influx was reduced in the obese subjects. Several reports then followed which confirmed these results (Klimes *et al*, 1982; Beutler *et al*, 1982; DeLuise *et al*, 1982), one found the opposite result (Mir *et al*, 1981) and two found no differences between obese and normal subjects (Pasquali *et al*, 1988; Charalambous *et al*, 1984a).

Results from further studies using other human tissues have generally not supported the original hypothesis. Charalambous *et al* (1984b) found elevated Na^+,K^+ ATPase activity in skeletal muscle from obese subjects. A study of hepatic Na^+,K^+ ATPase activity revealed that activity was increased in obese subjects as compared to normal controls (Bray *et al*, 1981). Turaihi *et al* (1987) found increased leukocyte Na^+,K^+ pump-mediated K^+ influx and [^{3}H]ouabain binding during obesity; following two weeks of dietary restriction which resulted in a 4-5% weight reduction there were significant decreases in both pump activity and [^{3}H]ouabain binding.

In laboratory animals decreased Na^+,K^+ ATPase or Na^+,K^+ pump activity has been observed in various tissues from ob/ob mice (Bray *et al*, 1978; Lin, Romsos, Akera *et al*, 1978; Guernsey and Morishige, 1979). While decreased hepatic Na^+,K^+ ATPase activity has been observed in db/db mice, no decreases in hepatic Na^+,K^+ ATPase activity were observed in the Zucker fa/fa rat (Bray *et al*, 1978). In rats fed a cafeteria diet the changes in erythrocyte, renal, and hepatic Na^+,K^+ ATPase activities were found to be sex- and organ- specific (Davis and Bernardis, 1984). In the female Sprague Dawley rats which, unlike the male rats, showed significant increases in carcass lipid, erythrocyte Na^+,K^+-ATPase activity was significantly correlated with carcass lipid content. No correlations between Na^+,K^+-ATPase activity and carcass lipid content were observed in kidney and liver crude membrane preparations from the males and females, and in erythrocyte membrane preparations from the males (Davis and Bernardis, 1984).

BAT activity and diet-induced obesity: Based on the many biochemical studies of BAT in laboratory animals it is now generally accepted that this tissue is the primary site of DIT (Trayhurn, 1990). However, different tissues, such as the liver, have also been proposed as major effectors of DIT (Ma, Nadeau and Foster, 1987). The quantitative importance of BAT activity in the development of DIO has not been established. In a series of studies conducted by Levin and colleagues, and others, it has been shown that diets which are moderately high in fat will, at least initially, increase BAT thermogenesis (Hogan, Sullivan and Triscari, 1985a; 1985b) and sympathetic nervous system activity (Levin, Triscari, Hogan *et al*, 1987; Levin, Triscari and Sullivan, 1983a; 1986). The diet-induced stimulation of BAT activity has been proposed as an energy buffering mechanism acting to deter obesity (Rothwell and Stock, 1979; Himms-Hagen, 1984; 1989). However, it appears that after DIO has developed the animals become insulin-resistant and atrophy of BAT occurs (Cunningham, Calles, Eisikowitz *et al*, 1983; Levin, Finnegan, Triscari *et al*, 1985; Levin, Triscari and Sullivan 1983a; 1983b). It is unknown whether the loss of this energy buffering mechanism contributes to the resistance of long-standing obesity to reversal which has been shown to occur (Rolls, Rowe and Turner, 1980; Hill, Dorton, Sykes *et al*, 1989).

Objectives

The overall objective of the research described in this chapter was to study the Na^+,K^+ pump activity and BAT thermogenic activity, two potential mechanisms of altered energy expenditure, during diet-induced obesity and undernutrition. In some types of experimental obesity the development of obesity is associated with reduced energy expenditure and increased metabolic efficiency while in others it is not. Conversely, dietary restriction and weight loss are also associated with adaptive reductions in energy expenditure which then results in energy conservation, thus slowing weight loss. An outline of the two animal studies described in this chapter is provided in Table 4.0.1.

Na^+,K^+ pump activity and related Na^+,K^+ transport pathways were studied in rat erythrocytes, thymocytes, and hepatocytes. Erythrocytes were selected for study since they have been used in several earlier investigations (DeLuise *et al*, 1980; Klimes *et al*, 1982) and since erythrocyte ion transport analyses from individual animals can be repeated and the results compared over time. Thymocytes provide a nucleated cell model for the study of ion transport and are easily isolated as a suspension of cells having high viabilty (> 95% viability; Jones, Patrick and Hilton, 1981a,b). However, some investigators have criticized the idea that thymocytes are a superior cell model to erythrocytes based on their findings of no essential differences between the two cell types in terms of cell Na^+ regulation (Senn and Garay, 1989). Hepatocytes were studied because the liver has also been proposed as

Table 4.0.1: Outline of the diet-induced obesity (DIO) study protocols*.

CHARACTERISTIC	STUDY	
	DIO 1	DIO 2
Diets and number of SD rats per diet group at baseline	HSF, 22 MPF, 22 C, 22	HF1, 40 HF2, 40 C, 40
Length of dietary treatment before dietary restriction (DR)	no DR	12 and 24 wk.
Length of dietary restriction	no DR	12 wk.
Total length of rat study	9 wk.	36 wk.
Daily energy intake analyses	yes	yes
Metabolic efficiency analyses	yes	yes
Whole animal oxygen consumption analyses	no	yes
Total body fat analyses: (TOBEC) (carcass analysis)	TOBEC	TOBEC, carcass analysis
Fat pad weights	yes	yes
Na^+,K^+ transport analyses: (E) (T) (H)	E, T	E, T, H
BAT analyses: (GDP) (UCP) (COX) (DNA)	UCP, COX, DNA	GDP, UCP, COX, DNA

* Abbreviations: C: control diet, COX: BAT cytochrome c oxidase activity, DNA: BAT DNA content, DR: dietary restriction, E: erythrocyte, H: hepatocyte, HF1 and HF2: high fat diets containing lard and tallow, respectively; HSF: high saturated fat (lard) diet, GDP: BAT mitochondrial GDP binding assay, MPF: moderately-high polyunsaturated fat (corn and safflower oils) diet, SD: Sprague Dawley, T: thymocyte, TOBEC: total body electrical conductance, UCP: BAT uncoupling protein content.

a major effector of DIT (Ma *et al*, 1987) and because of the importance of the liver in whole-body tissue ATP expenditure (Gill, France, Summers *et al*, 1989). Thus I aimed to study Na^+,K^+ pump activity in each of these cell types to determine 1) whether activity was consistently affected by DIO and whether undernutrition (induced by food restriction) reversed the observed changes, and, 2) if altered activities were observed, whether these alterations were consistent with the idea that the Na^+,K^+ pump activity, similar to BAT thermogenesis, could function as an energy buffer mechanism.

Several aspects of BAT function were examined since this tissue is well known as an important site of diet-induced thermogenesis (Himms-Hagen, Triandafillou and Gwilliam, 1981) and since its stimulation may act as an energy buffering system towards the prevention of some types of DIO (Himms-Hagen, 1984). As discussed in the General Introduction in Chapter 1, there exists some debate in the literature as to the relative effectiveness of a high fat diet which is comprised largely of saturated fats to induce BAT activity, as compared to a high fat diet which is comprised mainly of polyunsaturated fats. Moreover the long term effects of high fat diets on BAT activity and growth were not well understood prior to my studies. Less well understood was the effect of food restriction following high fat diet feeding. Thus I aimed to assess BAT activity and growth during relatively long term studies in rats which were fed various high fat diets or a control diet, and in rats which were subsequently food-restricted.

Materials and methods

Animal care: Both of the following studies involved weanling (21 days old) male Sprague Dawley rats which were received from Charles River Breeding Laboratories, Montreal. Following arrival in Animal Care Services at the University of Ottawa, the rats were housed in groups of 3 to 5 in plastic cages and were fed Prolab 4020 RMH rat chow (Agway™, 17.6 kJ/g). After 5 to 7 days the rats were then placed in hanging wire mesh cages at 24°C with lighting between 07:00-19:00 h. The rats continued to be fed chow. After a 2 to 3 week baseline period the experimental diets were begun. All rats were killed between 07:30 and 09:30 h.

Body weight determinations: Body weights of all rats were assessed 3 days per week during DIO study 1, and weekly in DIO study 2. Within each study body weights were assessed within the same hour each day and on the same day(s) each week. During food intake and metabolic efficiency determinations body weights were assessed more frequently each week.

Food intake determinations: Daily food intake was calculated as the difference between the weight of the food given to each rat on the first day of the food intake study and the weight of the food that remained 24 hours later; the same procedure was used on the subsequent days during each food intake study. Food intake studies were conducted over 3 or 4 day intervals. The food intake was corrected for the weight of the food that had spilled through the floor of the wire mesh cage. All weights were assessed within the same hour each day.

The daily energy intake was then calculated by multiplying the weight of the food eaten by the energy content of the specific diet being fed. The energy content of the defined diets which were prepared on site for the rats was calculated using the Atwater (1899) energy coefficients of 4 (17), 4 (17) and 9 (38) kcal (kJ) per gram of dietary protein, carbohydrate, fat, respectively.

Metabolic efficiency determinations: Estimates of metabolic efficiency (mg BWt gained/kJ dietary energy) were derived during the food intake study periods. Body weights were assessed on each day of the food intake study period, and the body weight gained over each 24 hour period was divided by the dietary energy consumption over that time.

Body fat analysis:

1) Total body electrical conductance (TOBEC): Measurements of total body fat mass were carried out using principles of total body electrical conductivity with an EM-SCAN™ SA-1 analyser. Each rat was anesthetized with Na pentobarbital (35 mg/100g BWt), placed in the prone position within the calibrated analyser, and the displayed reading was used to calculate the lean body mass (Walsberg, 1988). The difference between the lean body mass and body weight provided an estimate of the total body fat mass. As the position of the rat's body within the analyser is critical for reproducible results, the rat was very carefully placed in the center of the plexiglass cylinder which is then placed in the analyser. The availability of several different sizes of rectangular plexiglass plates allows the vertical adjustment of the rat's

position within the cylinder.

This method is based on the changes in electrical conductivity which occur when the animal is placed in the electromagnetic field in the analyser. The technique depends on the differences in electrical conductivity and dielectric properties of body fat and fat-free body mass (Gibson, 1990). While fat is a poor conductor, the fat-free mass, composed essentially of electrolyte-containing water, readily conducts the applied current. During each measurement the rat is positioned within the uniform solenoid coil in the analyser. A current is then passed through the coil which induces an electromagnetic field in the space enclosed by the coil. This then produces a current in the rat and the magnitude of the current is proportional to the fat-free mass of the rat. As a result of this current a secondary magnetic field is produced which is then measured (see Gibson, 1990).

The equipment (EM-SCAN™ SA-1 analyser) for the assessment of total body fat content has only recently (1989) been commercially available for use with small laboratory animals. The benefits and drawbacks of this methodology in my use are discussed along with the results generated from its use in the following studies.

2) Carcass lipid extraction: After each rat was killed and those tissues that were studied removed, the carcass was frozen at -20°C. For analysis, each carcass was thawed and the tail, paws and fur were removed. The remaining carcass was sliced and then homogenised (Brinkman, Model PT45) in a known amount of distilled water; 500ml of the total homogenate volume was then frozen at -20°C before lipid

extraction. For lipid extraction, duplicate 30ml aliquots of this homogenate were poured into cellulose extraction thimbles (Whatman) and the non lipid-containing aqueous fraction was allowed to filter through each thimble overnight at 4°C. The thimbles were then dried overnight at 55°C and weighed. The total lipid content of each sample was subsequently extracted over a 3 hour period using petroleum ether in a Soxlet extraction system (Precision Scientific, GCA corporation). The extracted lipid was weighed, and the total weight of lipid in the total volume of carcass homogenate was calculated. To calculate the percentage of total body weight as fat, the total body fat weight was then divided by the body weight of the rat immediately before its death and the result was multiplied by 100.

3) Fat pad weights: Within one hour after each rat was killed the perirenal and epididymal fat pads were removed from the rat and weighed. After weighing, the fat pads were returned to the carcass so that these fat depots would be included in the carcass lipid determinations.

Whole animal resting oxygen consumption: The determinations were carried out using a calibrated Beckman Industrial™ Oxygen Analyser (Model 755) over time periods of approximately 1.5 h. Unanesthetized rats were placed in a water jacket-enclosed chamber (28°C) and oxygen consumption was monitored. Resting oxygen consumption was defined as that during periods over which oxygen consumption was consistently at a minimal rate. The data are expressed per unit surface area of each

rat (μl/min/cm^2). Body surface area was calculated using equations for the surface area of the rat (Diack, 1930). These equations were $S = 7.47W^{0.667}$ for normal rats and $S = 7.64W^{0.667}$ for emaciated rats (Diack starved his rats for 3 to 5 days), where S represents surface area (cm^2) and W represents body weight (g).

Na^+,K^+ transport analyses:

1) Erythrocyte Na^+,K^+ transport analyses: The methods used were those as described for human erythrocyte K^+ influx (Chapter 3). However, as the Na^+,K^+ pump in rodent erythrocytes is typically more ouabain-resistant than the Na^+,K^+ pump of human tissues, 10mM ouabain was used instead of 2.5 mM ouabain; this concentration is commonly used with rodent erythrocytes (Hall and Willis, 1984; Zhao and Willis, 1988). The K^+ influx pathways are depicted and defined as shown in Figure 3.1. Figure 4.0.1 describes the results from rat erythrocyte K^+ influx time course experiments and shows the time during which influx is linear. As with the human erythrocytes K^+ influxes were measured during 20 minute flux periods.

2) Thymocyte Na^+,K^+ transport analyses: The thymus was removed from each carcass within minutes after the rat was decapitated. The thymocytes were isolated from the thymus into an isolation medium comprised of (mM): 135 NaCl, 1 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 glucose, 20 N-2-hydroxyethylpiperazine-N'-ethane sulfonic acid (HEPES), 10 NaOH, 5 adenosine (pH 7.4) and kept at room temperature. Cells were

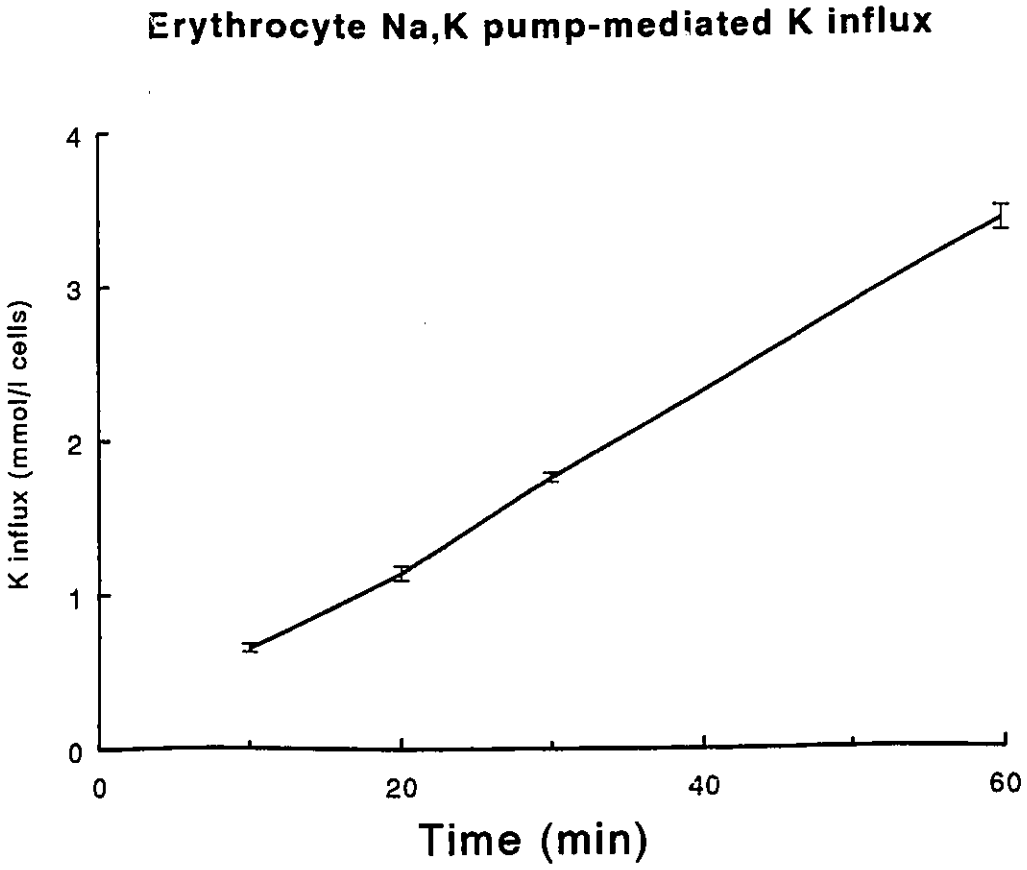

Figure 4.0.1: Time course of rat erythrocyte Na^+,K^+ pump-mediated K^+ influx. Each of the data points is a mean (±SEM) of the results from three control SD rats. The ouabain-sensitive K^+ influx rate between 10 and 60 minutes (during which time influx is linear) is 3.62 mmol/l cells/hr (SEM, ±0.117).

liberated from the thymus through mincing with scissors and flushing the suspension though a broken-ended Pasteur pipette. The suspension was filtered twice through six layers of woven surgical gauze and the thymocytes were washed using two centrifugation (2000g, 5 min.)/resuspension cycles.

The thymocyte K^+ influx techniques used are those described by Grinstein, Clarke, Dupre *et al* (1982) and Grinstein, Goetz and Rothstein (1984) and are briefly described as follows. For K^+ influx measurements aliquots of 20-30 x 10^6 cells/ml were suspended in the presence of ^{86}Rb (371 kBq/ml or 10 μCi/ml) with or without ouabain (2.5 mM), or with ouabain (2.5mM) and bumetanide (0.2 mM). K^+ influx was stopped after exactly 4 min. by the dilution of cell suspension aliquots in ice-cold isotonic choline chloride solution, and the immediate centrifugation (Eppendorf 3200 microcentrifuge; 15000g, 25 seconds) through a corn oil:dibutyl phthalate mixture (3:10; v/v). The radioactivity of the dissolved cell pellet was then measured using liquid scintillation counting. The K^+ influx data is expressed as pmol $K^+/10^6$ cells/ min. The K^+ influx pathways were identified as described for erythrocytes and are depicted in Fig. 3.1. Figure 4.0.2 describes the results from rat thymocyte K^+ influx time course experiments and shows the time during which influx is linear.

Thymocyte counting and sizing: Cell counting and sizing were performed electronically using a Coulter Counter Channelizer combination (Model ZM). In this system cells are counted as they pass through a fine orifice which alters the current flow through the orifice resulting in a series of electronic pulses which are sorted and

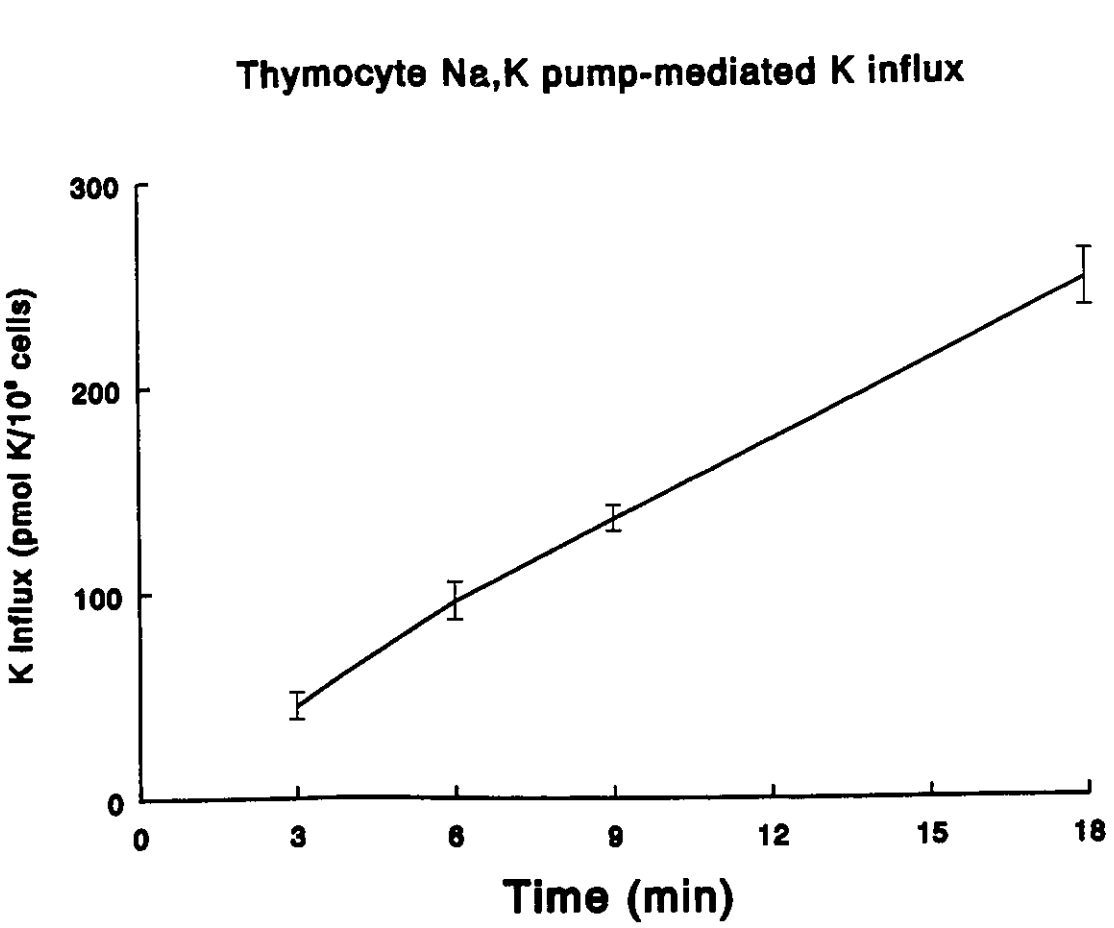

Figure 4.0.2: Time course of rat thymocyte Na^+,K^+ pump-mediated K^+ influx. Each of the data points is a mean (±SEM) of the results from eight control SD rats. The ouabain-sensitive K^+ influx rate between 3 and 18 minutes (during which time influx is linear) is 14.7 pmol/10^6 cells/min (SEM, ±0.34).

counted. The median cell size was usually in the range of 105 to 115 fl and no differences in cell size were observed between diet groups.

3) Hepatocyte Na^+,K^+ transport analyses:

Hepatocyte isolation: Immediately after each rat was decapitated and the interscapular BAT removed, the *in situ* techniques for the isolation of rat hepatocytes were begun. The techniques were adapted from those of Seglen (1976) and are described as follows. The compositions of the buffers used are shown in Table 4.0.2. The compositions are identical to those described by Seglen except that glucose was added to the final suspension buffer and 2% bovine serum albumin was added to all solutions. The inclusion of albumin using the techniques appeared to enhance cell yield and viability.

A loose ligature was placed around the portal vein near the site where the cannula was to be inserted. The 21 gauge cannula which contained the Ca^{2+}-free perfusion buffer was inserted into the portal vein, the ligature was tightly secured and the perfusion was begun. At this time the lower vena cava was cut to allow perfusate efflux. Perfusate flow was started slowly at first and then increased to approximately 35 ml/min. Holter peristaltic pumps (Model 911) were used to generate the perfusion flow. Approximately 450ml of Ca^{2+}-free buffer was perfused before beginning the collagenase buffer perfusion. Approximately 350 ml of the 0.05% collagenase buffer was then perfused. Both of these buffers were brought to 37°C before use.

Table 4.0.2: Composition of the buffers used in the isolation of hepatocytes*.

	Ca^{++}-free perfusion buffer (mM)	Collagenase (0.05%) buffer (mM)	Washing buffer (mM)	Suspension buffer (mM)
NaCl	142.0	66.8	142.0	68.5
KCl	6.8	6.8	6.8	5.4
$CaCl_2 . 2H_2O$	-	6.2	1.6	1.6
$MgCl_2 . 6H_2O$	-	-	-	1.4
KH_2PO_4	-	-	-	1.1
Na_2SO_4	-	-	-	0.7
HEPES	10.0	100.0	10.0	30.0
TES	-	-	-	28.9
Tricine	-	-	-	36.3
NaOH	5.5	66.0	5.5	52.5
bovine serum albumin	0.3	0.3	0.3	0.3
glucose	-	-	-	10.0
collagenase, Type IV (g/100ml)	-	0.05	-	-
pH	7.4	7.6	7.4	7.6

* As outlined in the methods of Seglen (1976) the two strongly buffered solutions (collagenase and suspension) are designed to withstand continuous acidification by the hepatocytes, and they therefore have a high initial pH. Abbreviations used: HEPES, N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid; TES, N-Tris (hydroxymethyl)-2-aminoethane sulfonic acid; Tricine, Tris(hydroxymethyl) methyl-glycine.

Type IV collagenase was used as this type is recommended by Sigma Chemical Company Inc. for the isolation of rat hepatocytes using the methods of Seglen (1976). Between production lots of this type of collagenase I found that there was substantial variation in the effectiveness of the collagenase in isolating viable hepatocytes. Therefore before conducting the experiments with the animals in DIO study 2, I tested collagenase samples from three different production lots, identified the lot which I found to be most effective, and ordered enough collagenase from that same lot for all of the liver perfusions to be conducted in DIO study 2.

Following the collagenase perfusion the liver was removed and placed in approximately 80ml of the suspension buffer. Cells were liberated from the connective tissue by cutting with scissors (avoiding the poorly perfused areas) and gently shaking the cells loose from the tissue into the suspension buffer. The cell suspension was then filtered through a course Nitex nylon filter (250μm) and incubated and gently shaken at 37°C in a 300ml plastic Erlenmeyer flask for 30 min. The suspension was then filtered through a double layer of nylon filters (250μm and 100μm), and the cells were carefully washed twice using centrifugation (10*g*, 200rpm, 5 min.)/resuspension cycles. After the final wash the cells were resuspended in the suspension medium and the cell concentration was determined (see Hepatocyte counting section, below).

Hepatocyte K^+ influx determinations: For K^+ influx measurements aliquots of 0.5-1.0 x 10^6 cells/ml were suspended in the presence of ^{86}Rb (371 kBq/ml or

10 μCi/ml) with or without ouabain (2.5 mM), or with ouabain (2.5mM) and bumetanide (0.2 mM). K^+ influx was stopped after exactly 25 min. by the dilution of cell suspension aliquots in ice-cold isotonic choline chloride solution, and the immediate centrifugation (Eppendorf 3200 microcentrifuge) through a corn oil:dibutyl phthalate mixture (3:10; v/v). The radioactivity of the dissolved cell pellet was then measured using liquid scintillation counting. The K^+ influx data are expressed as nmol K^+/ 10^6 cells/ min. The K^+ influx pathways were identified as described for erythrocytes and thymocytes; the pathways are defined and depicted in Fig. 3.1. Figure 4.0.3 describes the results from rat hepatocyte K^+ influx time course experiments and show the time during which influx is linear.

Hepatocyte counting: The cell concentration of each hepatocyte suspension was determined using a hemocytometer (Improved Neubauer) as described by Freshney (1983). This method involves placing a small volume of cell suspension in the optically flat chamber of the hemocytometer and viewing under a microscope (Zeiss). The number of cells within a defined area (corresponding to 10^{-4} ml) is counted and the cell concentration is derived from this count.

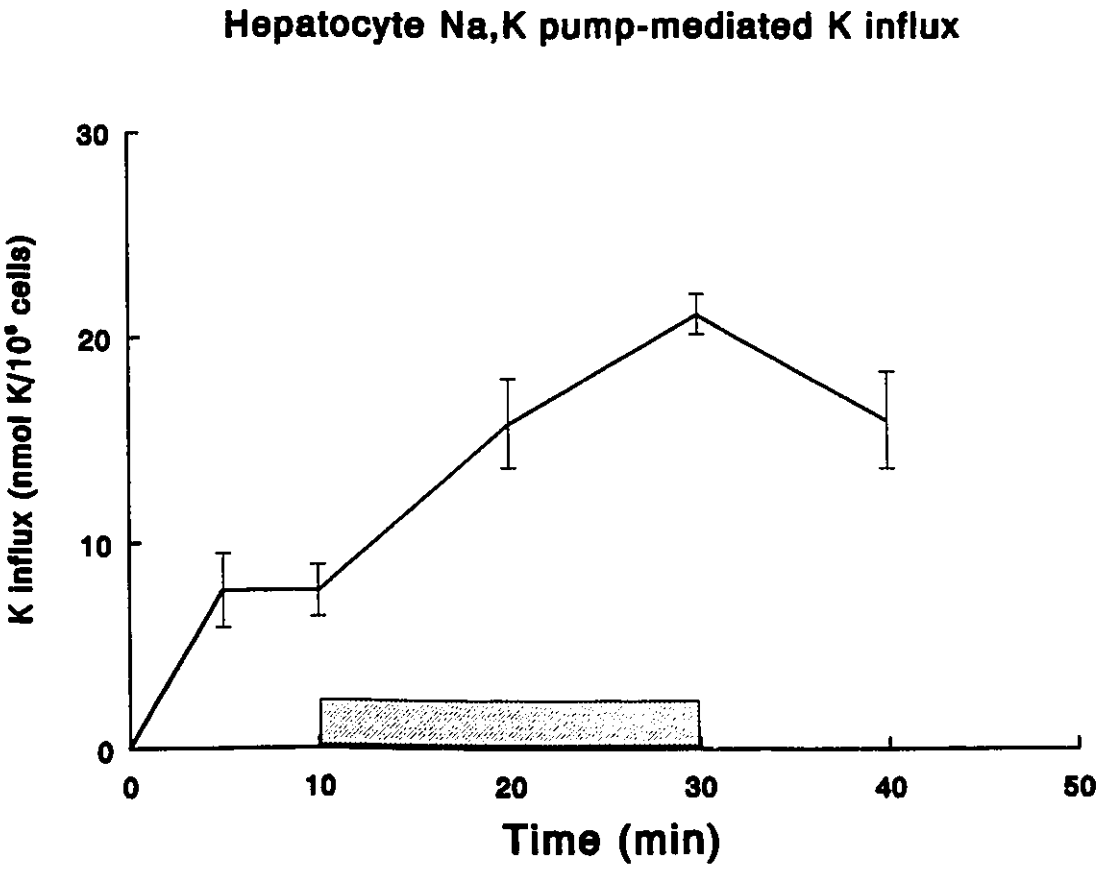

Figure 4.0.3: Time course of rat hepatocyte Na^+,K^+ pump-mediated K^+ influx. Each of the data points is a mean (±SEM) of the results from three control SD rats. The ouabain-sensitive K^+ influx rate between 10 and 30 minutes (during which time influx is linear) is 0.7 nmol/10^6 cells/min (SEM, ±0.03). The shaded area corresponds to the time over which influx was linear; influx assays were thus conducted only during this time.

Cell viability:

Trypan blue exclusion tests were used to assess the viability of thymocytes and hepatocytes. This test relies on the controlled permeability of the plasma membrane of living cells and the methods as described by Freshney (1983) were used. The viability of the thymocyte and hepatocyte suspensions were usually approximately 98-99% and 90-95%, respectively.

Effectiveness of cell isolation: In order to assess the effectiveness of the techniques used to isolate cells from their isotope-containing flux media following the periods over which fluxes were measured, the following techniques were used. The results of these experiments are shown in Figure 4.0.4.

Hepatocyte and thymocyte determinations: The methods of Wohlhueter and Plagemann (1989) were used to assess the extracellular aqueous volume that is typically carried through the oil phase during centrifugation when thymocyte or hepatocyte ion fluxes are stopped. These methods rely on the metabolically inert and impermeable characteristics of inulin and the high cellular permeability of water in cell suspensions.

Cells were isolated as standardly done during flux experiments. The concentrations of cells used were similar to those used in the K^+ influx experiments, and were approximately 1.0×10^6 hepatocytes/ml and 20×10^6 thymocytes/ml. These cell suspensions were incubated for one minute at room temperature in solutions which had the same composition as the media which were used for ion fluxes, except

that they also contained approximately 5 x 10^6 dpm/ml of [carboxyl-^{14}C]carboxylinulin (NEN) and 1 x 10^6 dpm/ml of ^3H_2O (NEN). Then, similar to the flux experimental protocols, each 0.25 ml cell suspension aliquot was diluted into 0.75mL isotonic choline chloride solution (0°C) which was layered over 0.30 mL of a corn oil-dibutyl phthalate mixture (3:10; v/v) and then centrifuged at 15,000*g* for 20-30 seconds. The radioactivity of the cell pellets and of a series of standards and blanks were measured in a Beckman LS 6800 liquid scintillation counter using an appropriate dual-isotope program. Total pellet volume was estimated from the ^3H_2O counts; the extracellular volume of the pellet was estimated from the [carboxyl-^{14}C]carboxylinulin counts, and the intracellular volume was estimated as the difference between the total pellet volume and the extracellular volume.

Erythrocyte determinations: The methods of Wohlhueter and Plagemann (1989) were adapted to assess the extracellular aqueous volume that typically remains after the ion fluxes are stopped and the cells are washed free from the isotope-containing suspension medium (see erythrocyte K^+ influx techniques section). The concentration of cells used in these determinations, 4% hematocrit, is similar to that used in the ion flux experiments. Each erythrocyte suspension was incubated for 10 minutes (37°C) in a solution having the same composition of the medium used during ion fluxes, except that it also contained approximately 5 x 10^6 dpm/ml of [carboxyl-^{14}C]carboxylinulin (NEN) and 1 x 10^6 dpm/ml of ^3H_2O (NEN). The erythrocytes were isolated as standardly done during Na^+ and K^+ flux experiments: each cell suspension

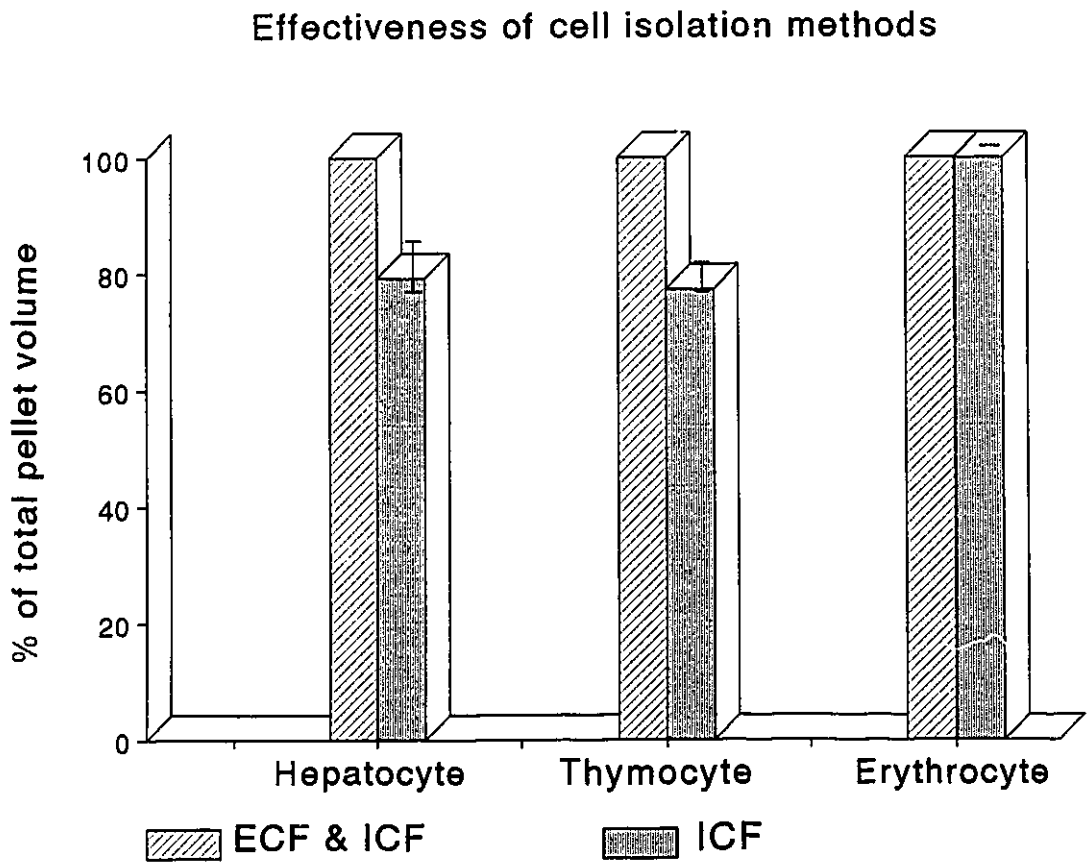

Figure 4.0.4: Effectiveness of cell isolation methods. For hepatocytes and thymocytes: results are from one representative experiment in which each mean (±SEM) is calculated from pentuplicate determinations for one minute incubation periods. The concentrations of cells used are similar to those used in the K^+ influx experiments, and are 1.0 x 10^6 hepatocytes/ml and 19.6 x 10^6 thymocytes/ml. The cells were isolated as standardly done during flux experiments: each 0.25 ml cell suspension aliquot was diluted into 0.75ml isotonic choline chloride solution (0°C) which was layered over 0.30 ml of a corn oil:dibutyl phthalate mixture (3:10; v:v) and then centrifuged at 15,000*g* for 25 seconds. For erythrocytes: results are presented as the mean (±SEM) from one representative experiment in which determinations were carried out in triplicate after a 10 minute incubation period. The concentration of cells used, 3.9% hematocrit, is similar to that used in Na^+ and K^+ flux experiments. The erythrocytes were isolated as standardly done during Na^+ and K^+ flux experiments: each cell suspension was spun at 15,000*g* for 10 seconds; the supernatant was aspirated, and the cell pellet was resuspended and washed three times with approximately 1.0 ml of isotonic $MgCl_2$ solution (0°C) by sequential centrifugation/resuspension cycles. ECF: extracellular fluid; ICF: intracellular fluid.

was spun at 15,000*g* for 10-15 seconds; the supernatant was aspirated, and the cell pellet was resuspended and washed three times with approximately 1.0 mL of isotonic $MgCl_2$ solution (0°C) by sequential centrifugation/resuspension cycles. The total, extracellular and intracellular volumes of the final pellet were determined as described for the thymocytes and hepatocytes.

Plasma and intracellular [Na^+] and [K^+]:

These were determined by flame photometry (Instrument Laboratory, Model 443) using Li^+ as the internal standard. Erythrocyte intracellular and plasma [Na^+] and [K^+] were determined as described in chapter 3. For thymocyte intracellular [Na^+] and [K^+] cells were washed 3 times with isotonic Na^+ and K^+-free media at room temperature. The cells in each sample were then lysed with 5% trichloroacetic acid, lyophilized (VirTis, Freezemobile Model 12) to a constant dry weight, and the contents were resuspended in 15mM Li^+/l for flame photometry. During the analysis of each set of samples several standards were run; the standards were prepared by serial dilution of Instrumentation Laboratory [Na^+] and [K^+] standards. No significant differences were observed between diet groups in either of the following described studies.

Analyses of BAT and its activity:

Immediately after each rat was killed the interscapular BAT was removed and placed in ice-cold isolation medium (0.25 M sucrose, 1 mM N-2-

hydroxyethylpiperazine-N'-ethane sulfonic acid (HEPES), 0.2 mM ethylenediamine tetraacetic acid (EDTA) potassium salt, pH 7.2). The BAT was then cleaned of adhering muscle and white adipose tissue and weighed. It was then homogenized in isolation medium using a motor-driven glass-teflon homogenizer. Samples of homogenate were removed, frozen in liquid nitrogen and stored at -80°C for later assays of protein content, UCP content, cytochrome c oxidase activity and DNA content. The remainder of the homogenate was used for the analysis of mitochondrial GDP binding.

Measurement of mitochondrial GDP binding:

Isolation of BAT mitochondria: Mitochondria were isolated as described by Cannon and Lindberg (1979). Mitochondria, nuclei and cellular debris in the homogenate were pelleted during an initial centrifugation at 8,500*g* (0°C). The pellet was then resuspended in isolation medium and this suspension was centrifuged at 650*g* for 5 minutes (0°C). The resulting supernatant which contained the mitochondria was then filtered through a double layer of cheesecloth. The mitochondria in this suspension were washed twice with isolation medium (8,500*g*, 10 min., 0°C). The final mitochondrial pellet was resuspended in less than 1 ml of isolation medium and kept on ice for less than one hour before the GDP binding assay was conducted. Mitochondrial protein content was determined using 5μl of mitochondrial protein suspension which was placed directly into 0.5 N sodium hydroxide (NaOH), omitting the precipitation step necessary with homogenates, and

the protein content was assessed using the methods described below.

GDP binding assay: The measurement of mitochondrial GDP binding provides an estimate of uncoupling protein activity, and therefore a measure of the tissue's thermogenic activity. The method of Desautels *et al* (1978) was used with the minor modifications, as described here. Two hundred micrograms of freshly prepared mitochondrial protein (in 20 μl) was added to 280μl incubation medium [100 mM sucrose, 20 mM N-Tris(hydroxymethyl)-2-aminoethane sulfonic acid (TES), 10 mM choline chloride, 1mM EDTA salt (to inhibit adenylate kinase activity), 5 μM rotenone, 100 μM potassium atractyloside (to inhibit the adenine nucleotide translocator), 10 μM [^{3}H]GDP (Amersham, 13.1 Ci/mmol) and 0.1 μCi/ml [^{14}C]sucrose (Amersham, 540mCi/mmol), with or without 100 μM ADP for the estimation of nonspecific binding]. After vortex mixing, the mitochondria were incubated for 2 minutes at room temperature. Mitochondria were then separated by centrifugation for 2 minutes in an Eppendorf 3200 microcentrifuge. The supernatant was aspirated and the pellet was dissolved in NCS tissue solubilizer (Amersham) overnight at 55°C. The dissolved pellet was counted in a cocktail containing 50 μl 10% ascorbic acid and 10 ml toluene containing 0.7% 2,5-diphenyloxazole (PPO) in a Beckman LS 6800 liquid scintillation counter. Specific binding was calculated as the difference between binding in the presence and absence of 100 μM ADP, after correction for the amount of incubation medium trapped in the pellet, as estimated from the [^{14}C]sucrose values. Binding was expressed as pmol GDP bound per mg

protein.

Measurement of protein content: The protein contents of BAT homogenate and mitochondrial protein samples were measured by a modified Lowry assay (Schacterle and Pollack, 1973). As compared to the original Lowry method (Lowry, Rosebrough, Farr *et al*, 1951), the modified method involves a shorter overall amount of time and a single more stable alkaline copper reagent. Bovine serum albumin (fraction V) was used as the standard for all assays. Samples were analysed in pentuplicate in a Spectronic 20 spectrophotometer (Bausch and Lomb).

Measurement of DNA content: The cellularity of BAT was estimated through the measurement of BAT homogenate DNA content. A fluorometric DNA assay was used (Downs and Wilfinger, 1983). This method involves a one step DNA extraction procedure and utilizes the DNA fluorochrome, bisbenzimidazole (Hoechst 33258) which is highly sensitive (optimum range is 5-150ng DNA) and specific for DNA. The results are reported as the total DNA content of the interscapular BAT (mg).

Measurement of uncoupling protein content:

Protein purification and antibody production: Uncoupling protein (UCP) for use as standard was derived from cold-acclimated rat brown adipose tissue mitochondria. The UCP was purified according to the method of Lin and Klingenberg (1982) except that the sucrose density gradient centrifigation step was omitted.

The immunization schedule used for rabbits was that of Fernandez, Nicholls

and Rial (1987). One hundred micrograms of similarly purified hamster UCP (from cold-acclimated hamsters) in Freund's complete adjuvant was injected intramuscularly into the hind legs and subcutaneously on the back. In the second week 100 μg UCP in Freund's incomplete adjuvant was subcutaneously injected at several sites on the back. In the next week a booster injection of 100 μg UCP in 0.9% NaCl was given in the ear vein. The following week, blood was obtained by cardiac puncture and it was allowed to clot overnight at 4°C. The serum was then collected through centrifugation, first at 2500*g* for 30 min. and then at 2500*g* for 15 min.

To adsorb trace contaminating antibodies to other mitochondrial proteins in the antiserum 500 μl aliquots of antiserum were incubated at 18°C for 2 hours with 50 μl of sonicated rat liver mitochondria (40-50 mg/ml), centrifuged at 11,000*g* for 2 min. and then frozen at -80°C. The antiserum was diluted in glycerol 1:1 (v:v) shortly before use and stored at -20°C. Immediately before use it again was diluted 1:160 (v:v) in a phosphate-buffered saline solution.

Dr. Gloria Zaror-Behrens conducted the UCP purification and the antibody production procedures for the following described radioimmunoassay.

UCP Radioimmunoassay: The measurement of uncoupling protein (UCP) in BAT homogenates, which provides an estimate of BAT thermogenic capacity, was performed by a solid phase radioimmunoassay (Lean, Branch, James *et al*, 1983). Aliquots of rat BAT homogenate were diluted to 2.0 mg protein/ml with phosphate buffered saline (PBS; 6.5mM, containing NaCl 140mM and KCl 3mM). Then 12.5 μl

of diluted homogenate was added to 32.5 μl of PBS and 5.0 μl of 5% Triton X-100; this was mixed well and incubated at room temperature for 30 min. Samples were then diluted 5 times with PBS and centrifuged for 2 min. in an Eppendorf 3200 centrifuge. The infranatant was removed and stored at -20°C for less than 3 days. Immediately before the assay the samples were diluted five times with PBS in order to decrease the Triton concentration to 0.02% and achieve a final protein concentration of approximately 5 μg/ml.

The flexible 96-well microtitre plates (Falcon 3912) were washed and rinsed thoroughly with Pierce RBS 35 detergent and distilled water in order to remove any residues from the manufacturing process and to enhance protein binding to the plates. The plates were then coated with 10 μg rat UCP/ml (50 μl/well). Those wells to be used in the measurement of nonspecific binding contained 50 μl 0.02% Triton in PBS. The plates were then incubated for 2 hours at 37°C, and then washed twice with PBS + 1% bovine serum albumin (BSA) and 0.1% sodium azide (PBS-BSA). The last wash was incubated for 10 min. at 37°C in order to block the sites not bound with UCP. After blocking, the plates were washed three times and then well drained.

The samples were added to the wells to produce a final homogenate protein concentration of 0.2 μg/well. Rat UCP standards were diluted in PBS to produce a range of concentrations (2-64 ng UCP). In the assay rows of wells were included for total and nonspecific binding and for standard and sample nonspecific binding. After the samples and standards were added to the wells, antiserum was added to

each well (10 μl/well; final dilution in PBS, 1:1600). The plates were then covered with parafilm and incubated overnight at 4°C.

On the following day the plates were washed 5 times with PBS-BSA and well drained. Then 60,000 to 70,000 cpm/well of ^{125}I protein A (ICN, 30 μCi/ml) in PBS (50 μl/well) was added. The plates were then covered and incubated at room temperature for 1.5 hours. Following incubation the plates were washed 5 times with PBS-BSA, drained, and the wells were cut out and counted in a Beckman 5500 gamma counter. The results were corrected for nonspecific binding and then calculated from the linear regression line of logit-ln UCP. The data are expressed as μg UCP/mg protein.

Measurement of cytochrome c oxidase activity: To estimate total BAT mitochondrial content and oxidation capacity, cytochrome c oxidase activity in homogenates was assessed using the method of Yonetani and Ray (1965). This method involves a spectrophotometric determination of the oxidation of reduced cytochrome c by the cytochrome c oxidase enzyme in the sample being analysed.

Aliquots of BAT homogenate were treated with Lubrol WX 0.3% (1 μl Lubrol/mg protein) and diluted in 100 mM phosphate buffer (pH 7.0) to achieve a final protein concentration of 0.65 mg/ml. The solution containing the cytochrome c substrate (ferrocytochrome c) contained 1 mg% ascorbic acid so as to maintain the cytochrome c (Sigma, horse heart type III) in the reduced form before the assay. For each determination an aliquot of the diluted BAT homogenate (10 μg protein) was

added to the ferrocytochrome c solution and the absorbance was measured immediately for 2 minutes at 550nm in a Beckman DU-50 spectrophotometer using a kinetics soft pac module (program kindata). The plot of initial velocity against protein concentration was shown to be linear up to 15 μg protein. To determine the Vmax for each sample, measurements were done in duplicate at 3 different cytochrome c concentrations (26.6, 33.0 and 39.6 μM). The cytochrome c oxidase activity results are expressed as μmol/min.

Measurement of plasma thyroid hormones:

Plasma samples which were stored at -70°C and which had been thawed no more than twice were used in the thyroid hormone assays. Commercially available radioimmunoassay kits for T_3 and T_4 were used (Amerlex-M, Amersham), and the instructions provided by the manufacturer were followed. These kits are liquid phase systems and the antibodies are bound to magnetisable polymer particles.

The validity of using these kits for rat plasma was verified through the simultaneous use of rat thyroid hormone standards and the manufacturer-supplied human thyroid hormone standards. The standards for rats were prepared from thyroid hormone-stripped plasma. To prepare the hormone-stripped plasma approximately 50,000 cpm [^{125}I] T_4/ml and 23 mg Norit A charcoal (Sigma) per ml were added to the rat plasma. This mixture was stirred overnight at 4°C. The charcoal and plasma were separated through centrifugation (100,000*g* or 35,000 rpm using the Beckman Ti 55 rotor in the Beckman L8-55M ultracentrifuge) for 30 min.

at 4°C. The supernatant was transferred to a clean tube and the same amount of charcoal was added. The mixture was stirred overnight (4°C) and the plasma and charcoal were again separated through centrifugation. This process was repeated until the plasma was essentially thyroid hormone-free (*ie.* 1-5% of the original counts remaining).

This thyroid hormone-free plasma was then used to prepare standards for the RIA. Known amounts of T_3 and T_4 were added to the plasma and their concentrations were verified spectrophotometrically (Beckman DU50). The standards were frozen once at -70°C before use in the RIA kits.

The plasma concentrations of T_3 and T_4 are reported as ng/dl and μg/dl, respectively.

Data presentation and analysis

Unless otherwise stated, each of the results is presented as a mean (±SEM) for the numbers of animals as indicated. The statistical analyses of the data from DIO study 1 were carried out using True Epistat software on a personal computer. The statistical analyses of the data from DIO study 2 were carried out using True Epistat software on a personal computer and the Statistical Analysis Systems (SAS) software on the University of Ottawa main frame computer. Statistical tests included t tests, linear regression, Pearson correlation coefficients and ANOVA (completely randomized and two way) which in each case was followed by post hoc tests including

Tukey's and Newman-Keuls' tests. The specific statistical tests employed are identified in the Results sections. An α value of 0.05 was taken as the critical level of significance for all tests. It should be noted that in DIO study 2 the statistical comparisons which are presented in the tables and graphs are limited to the more relevant comparisons between *ad libitum* fed groups at each time point (ANOVA) and between *ad libitum* fed groups and their corresponding DR group (Student's t test). This was done because of the very large number of possible comparisons.

DIO Study 1: Effects of a high saturated fat diet versus a moderately high polyunsaturated fat diet on the induction of obesity and on mechanisms of altered energy expenditure.

Objective: The objective of this study was to elucidate the effects of a high saturated fat diet and a diet which was moderately high in polyunsaturated fat, as compared to a control diet, on the development of obesity and on the two mechanisms of altered energy expenditure, Na^+,K^+ pump and BAT activities. In this first study the effects of undernutrition through food restriction were not assessed.

Experimental design: In total 72 male weanling rats were received for the following described study. On account of the time involved in the analyses of Na^+,K^+

transport and BAT at the end point of the study (only the tissues from 3 rats could be analysed per day), the entire study was carried out using 2 time-staggerred groupings of 36 rats. The second group of rats was received and studied exactly 4 weeks following the first group. This was done in order to minimize the variation of results within diet groups that may have occurred due to the possible effects of age and the differing lengths of dietary treatments. The protocols used for both groupings were exactly the same. As no differences were found between results from the two groupings the results from the two groups were pooled for data analyses.

After a two week baseline period during which the weanling rats were fed chow, samples were collected for baseline measurements. For baseline erythrocyte Na^+,K^+ transport analyses blood samples were drawn by cardiac puncture from 66 rats under isofluorane anesthesia. For baseline thymocyte Na^+,K^+ transport analyses 6 rats were killed by decapitation. Blood was also obtained from these rats. 66 rats were then fed *ad libitum* one of three defined diets for nine weeks. The defined diets were begun 48 h after the cardiac puncture procedures; this corresponded to approximately 44 days of age. Following the nine week feeding period the rats were killed by decapitation, the trunk blood was collected and the interscapular BAT and the thymus were removed. The rats were not fasted before killing. Dietary compositions are outlined in Table 4.1.1.

Table 4.1.1: The composition of diets used in DIO study 1*

COMPONENT	HSF	MPF	C
casein (% wt)	27.0	22.0	19.5
corn oil	7.0	7.0	5.0
safflower oil	-	9.0	-
lard	31.0	-	-
sucrose	20.0	16.3	18.0
corn starch	3.1	35.5	47.5
Alphacel™	4.5	4.5	5.0
AIN vitamin mix	1.6	1.2	1.0
AIN mineral mix	5.2	3.9	3.5
D, L-methionine	0.3	0.3	0.3
choline bitartrate	0.2	0.2	0.2
D, L-α-tocopherol	0.1	0.1	-
Energy (kJ/g)	22.7	18.4	16.1
% protein (% energy)	20	20	20
% carbohydrate	17	47	68
% fat	63	33	12

* The vitamin and mineral contents of the HSF and MPF diets were adjusted to compensate for their high energy densities.

Results

Changes in total body weight: The body weight data which were collected during the nine week feeding study are shown in Figure 4.1.1. Following approximately three weeks of dietary treatments (*ie.* 67 days of age) the average body weight of the rats fed the HSF diet was significantly greater than those for rats fed the MPF or C diets ($P < 0.05$; ANOVA with a Newman-Keuls' post hoc test).

Energy intake: The daily energy intakes were assessed for each diet group following one, three and nine weeks of dietary treatments. The results of the assessments are presented in Figure 4.1.2. The rats which were fed the HSF diet consistently consumed more energy per day than the rats in either the MPF or C diet groups ($P < 0.05$; ANOVA with a Newman-Keuls' post hoc test).

Metabolic efficiency: Estimates of metabolic efficiency (mg body weight gained per day/ daily energy intake (kJ)) were made following one, three, and nine weeks of dietary treatments. The results are shown in Figure 4.1.3. Following both three and nine weeks of dietary treatments the metabolic efficiency of the rats fed the HSF diet was significantly greater than that of either the MPF or C diet fed rats ($P < 0.05$; ANOVA with a Newman-Keuls' post hoc test). At no point were there any differences in the metabolic efficiency measurements between the groups fed the MPF and C diets.

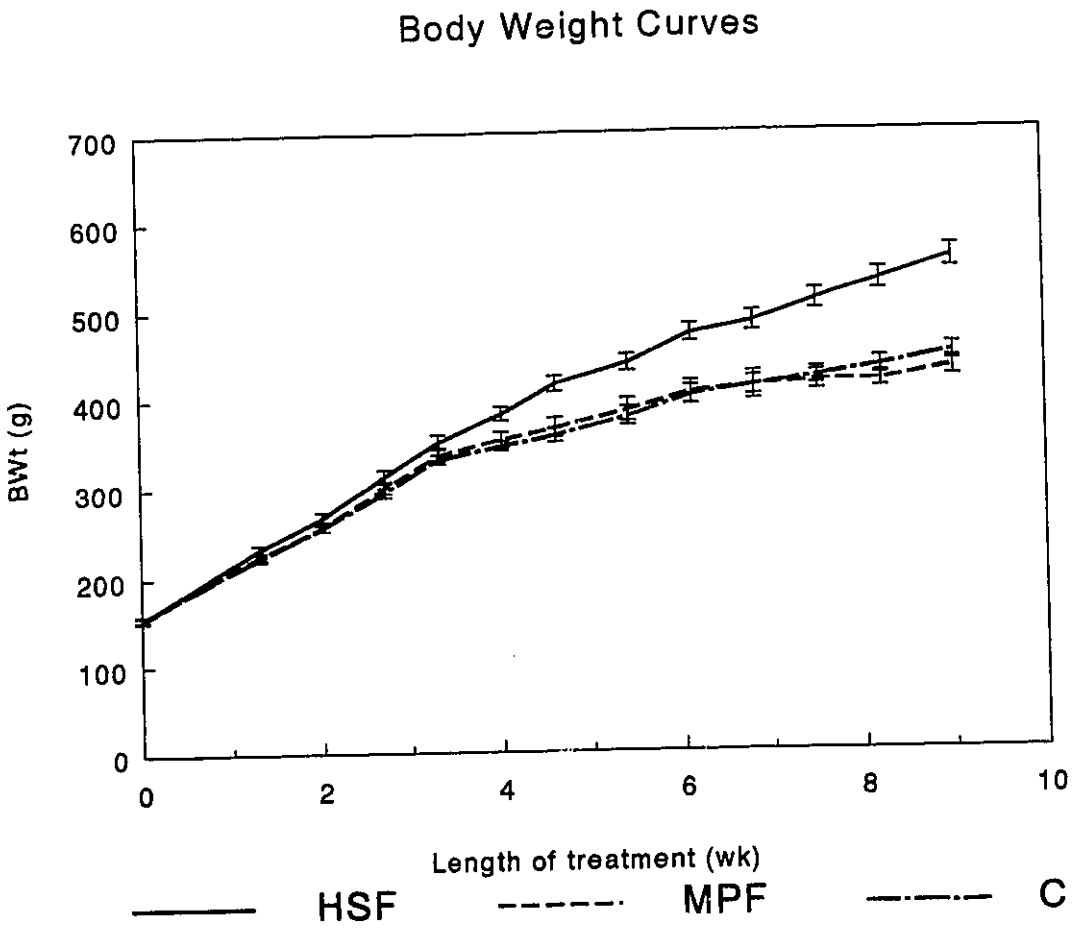

Figure 4.1.1: Body weight changes in high saturated fat diet-fed (HSF), moderately high polyunsaturated fat diet-fed (MPF) rats and control diet-fed (C) rats. Each data point is a mean (±SEM) for approximately 22, 19 and 18 rats in the HSF, MPF and C groups, respectively. Following three weeks of dietary treatments (*ie.* 67 days of age) the mean body weight of rats in the HSF group was significantly greater than the mean body weights of rats in the MPF or C groups ($P < 0.05$; ANOVA, followed by a Newman-Keuls' post hoc test).

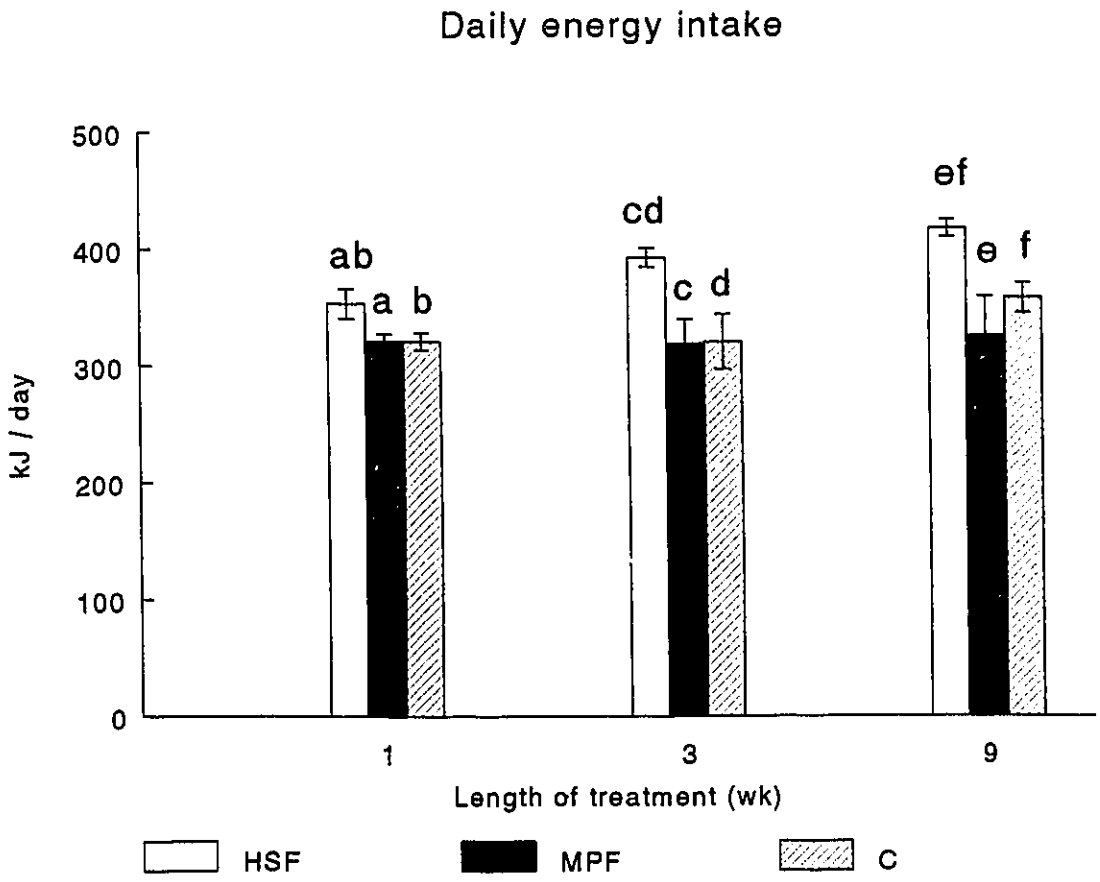

Figure 4.1.2: Daily energy intakes (kJ/d) of HSF, MPF and C rats after one, three and nine weeks of dietary treatments. Average energy intakes were determined daily during five day food intake studies. After accounting for spillage the net weight of diet eaten was used along with Atwater coefficients of 17, 17, and 38 kJ/g dietary protein, carbohydrate and fat, respectively, to calculate the corresponding mean daily energy intake (±SEM) for each diet group. The numbers of rats in each group are: HSF, 22; MPF, 19; C, 18. Similar letters denote significant differences at $P < 0.05$ between means as determined by ANOVA, followed by Newman-Keuls' tests.

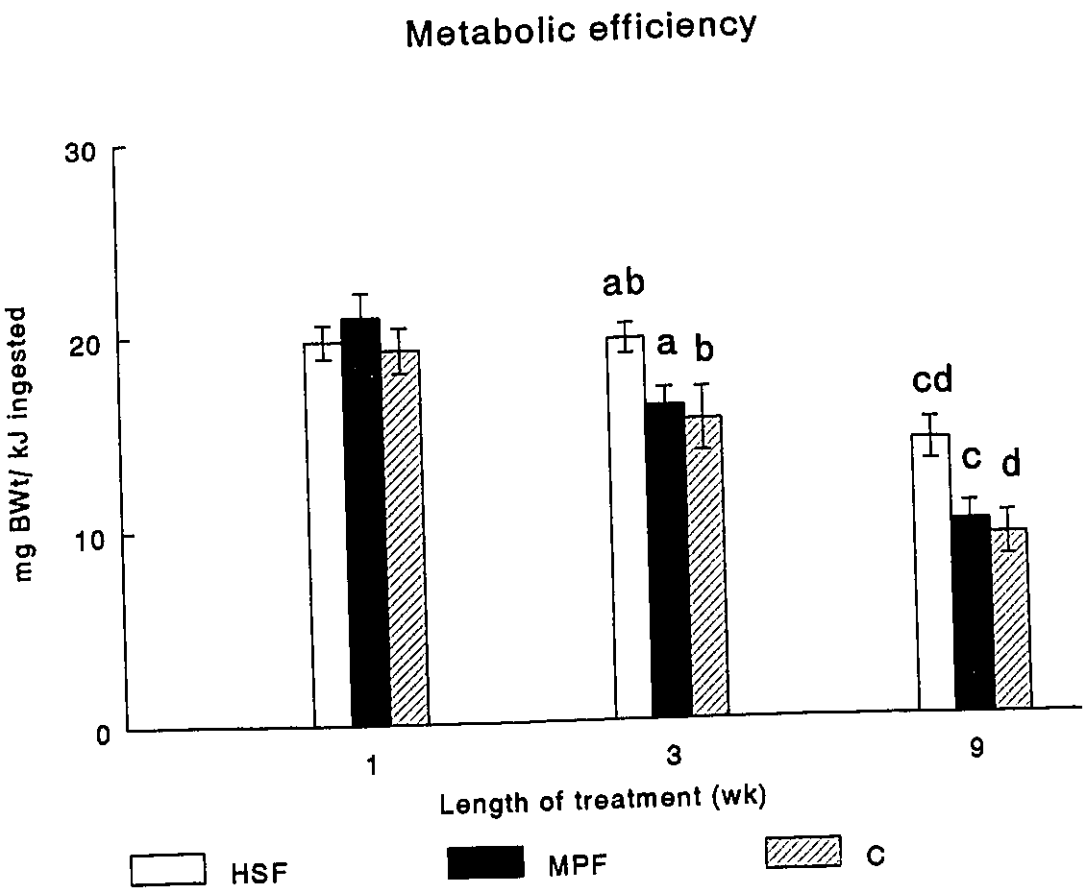

Figure 4.1.3: Metabolic efficiency (mg BWt/ kJ ingested) of HSF, MPF and C rats after one, three and nine weeks of dietary treatments. Average energy intakes and body weights were determined daily over five day periods. To calculate the daily metabolic efficiency the daily body weight gain (mg) was divided by the daily energy intake (kJ). The numbers of rats in each group are: HSF, 22; MPF, 19; C, 18. Similar letters denote significant differences (at $P < 0.05$) between means as determined by ANOVA, followed by Newman-Keuls' tests.

Body Composition: The rats fed the HSF diet became significantly fatter than the rats fed either the MPF or the C diets. The results of the analysis of body composition using TOBEC techniques are presented in Figure 4.1.4. When these techniques were used following three weeks of dietary treatments no significant differences in total body fat mass or lean body weight were observed ($P > 0.05$; ANOVA with a Newman-Keuls' post hoc test; data not shown). However, following eight weeks of dietary treatments the total body fat mass was approximately 79% and 60% greater in the HSF group as compared to the MPF and C groups, respectively (both, $P < 0.01$; ANOVA with Newman-Keuls' post hoc tests). There were no significant differences in BWt or total body fat mass between the MPF and C groups. No differences in lean body mass were observed between any of the diet groups.

The weights of well defined adipose depots such as perirenal or epididymal fat pads are sometimes used as indices of adiposity in laboratory animals. Because TOBEC methodology in studies of obese and undernourished animals has not as yet been validated, I assessed the correlation of perirenal and epididymal fat pads weights to the total body fat weight results. The fat pad weights were assessed immediately after the rats were killed, and the total body fat weight estimates were determined one week earlier (*ie.* after 8 wk. dietary treatments). Figures 4.1.5 to 4.1.7 show the results of the correlation analyses; Figure 4.1.7 shows the correlation of the sum of the fat pad weights with total body fat weight. The Pearson correlation coefficients were calculated including the data from all diet groups. In each case the correlation is weak; the strongest correlation ($r = 0.524$) was found between

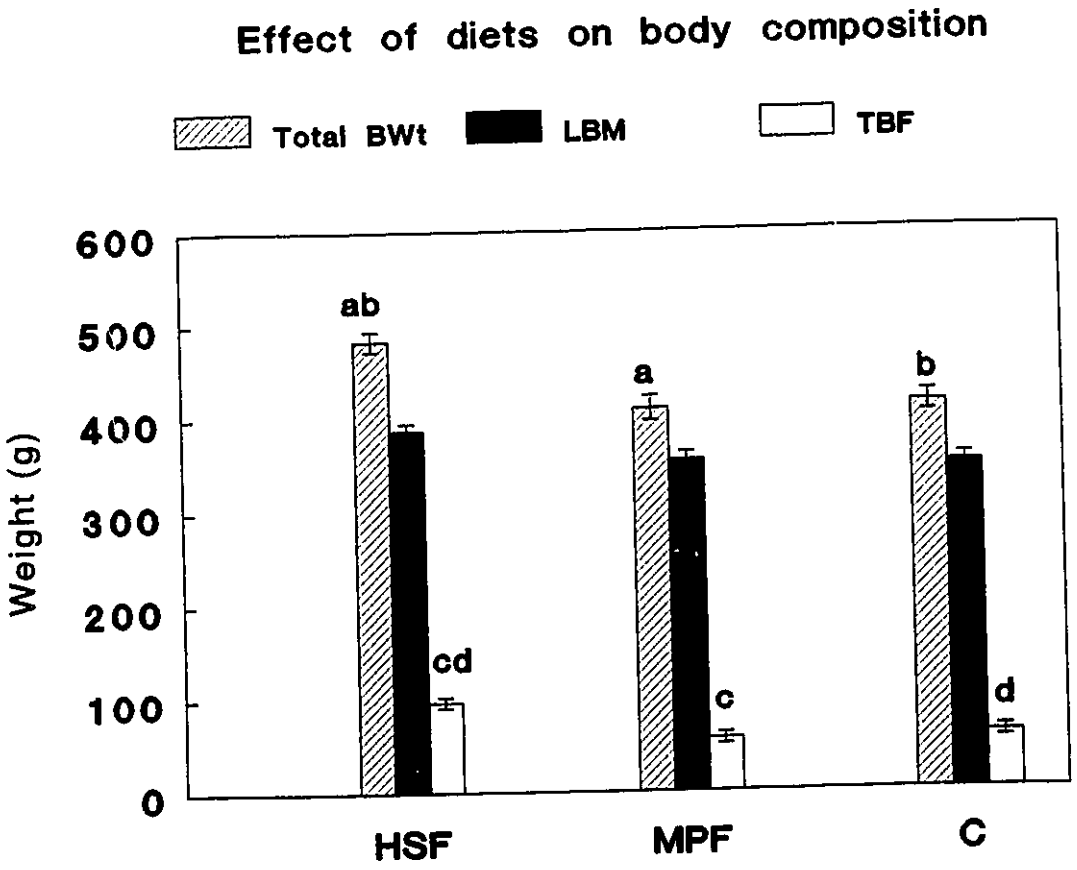

Figure 4.1.4: Effect of eight weeks of dietary treatments on total body weight (BWt), lean body mass (LBM) and total body fat weight (TBF) as determined by TOBEC. The numbers of rats in each group are: HSF, 22; MPF, 20; C, 19. Similar letters denote significant differences (at $P < 0.01$) between means as determined by ANOVA, followed by Newman-Keuls' tests.

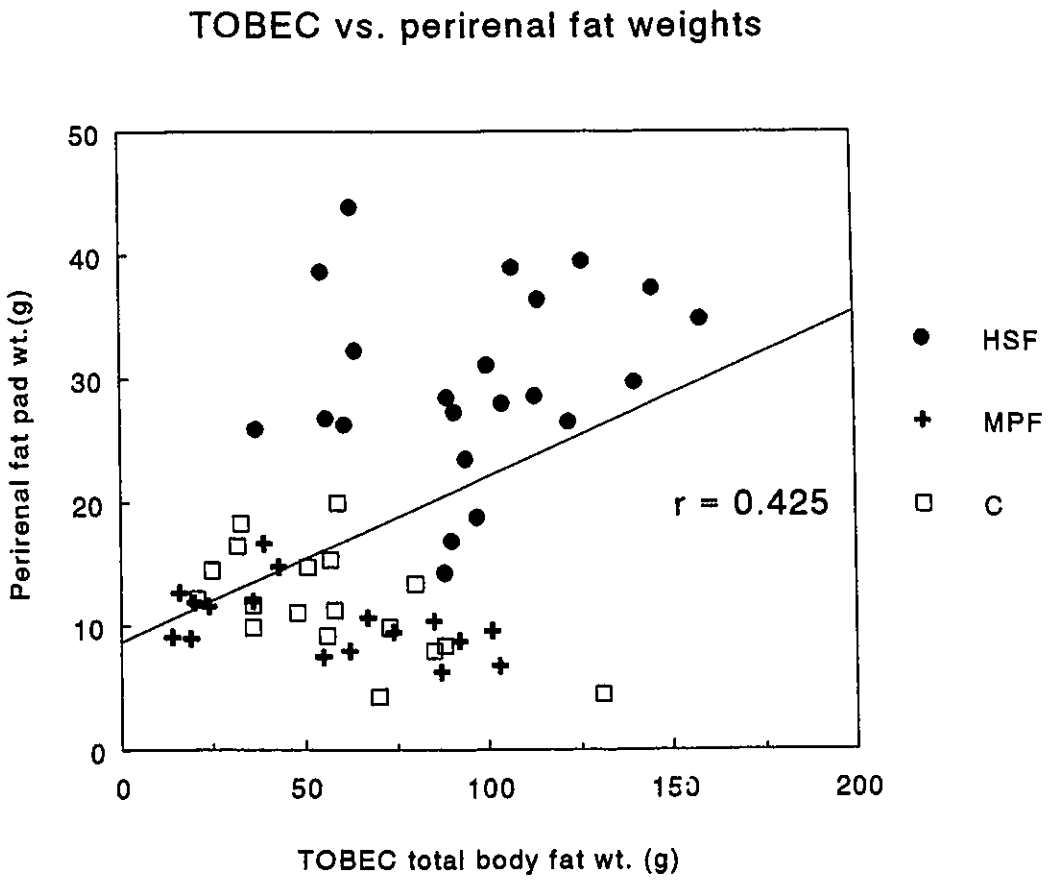

Figure 4.1.5: Correlation of TOBEC total body fat weight and perirenal fat pad weight results. Each data point represents results for one rat. Results from each diet group are indicated as shown in the legends. The numbers of results from each group are: HSF, 22; MPF, 17; C, 18. The TOBEC total body fat weights were determined after eight weeks of dietary treatments and perirenal fat pad weights after nine weeks of dietary treatments were determined immediately after the rats were killed.

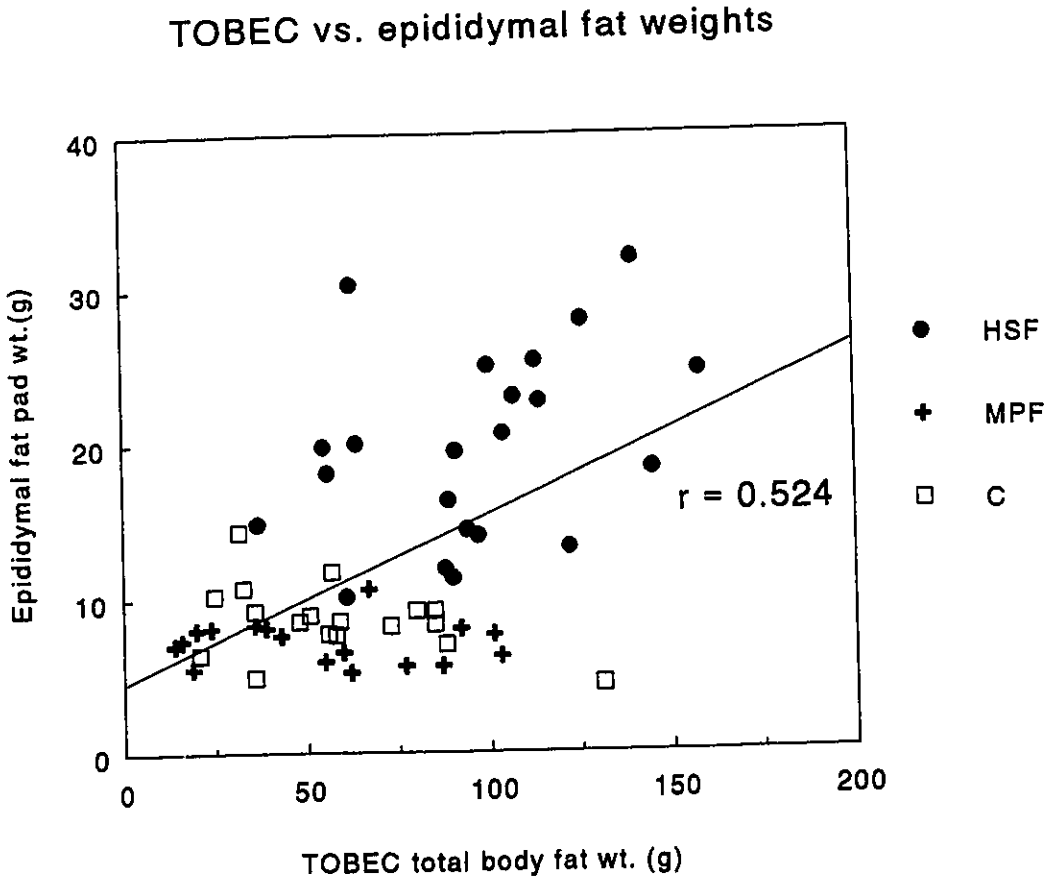

Figure 4.1.6: Correlation of TOBEC total body fat weight and epididymal fat pad weight results. Each data point represents results for one rat. Results from each diet group are indicated as shown in the legend. The numbers of results from each group are: HSF, 22; MPF, 17; C, 18. The TOBEC total body fat weights were determined after eight weeks of dietary treatments and epididymal fat pad weights after nine weeks of dietary treatments were determined immediately after the rats were killed.

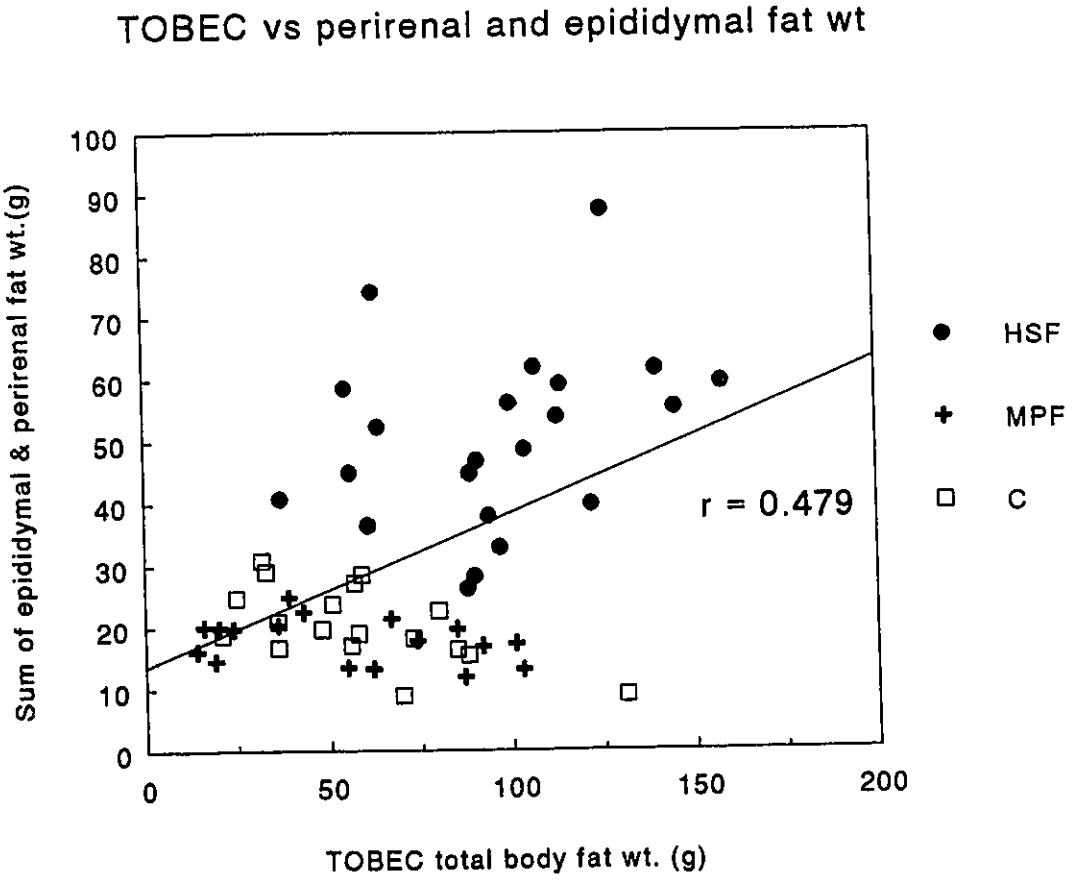

Figure 4.1.7: Correlation of TOBEC total body fat weight and the sum of perirenal and epididymal fat pad weight results. Each data point represents results for one rat. Results from each diet group are indicated as shown in the legend. The numbers results from each group are: HSF, 22; MPF, 17; C, 18. The TOBEC fat weights were determined after eight weeks of dietary treatments and fat pad weights after nine weeks of dietary treatments were determined immediately after the rats were killed.

epididymal fat pad weight and total body fat weight. These results suggested that further studies should be conducted to determine whether TOBEC techniques are valid in the assessment of adiposity in studies of DIO.

Erythrocyte Na^+ and K^+ Transport and plasma [Na^+] and [K^+]: No changes were observed in any of the erythrocyte Na^+,K^+ transport measurements (Figure 4.1.8). Between diet groups there were no significant differences in intracellular or plasma concentrations of Na^+ or K^+. On average (±SEM) they were (mmol/l): intracellular [Na^+],[K^+]: 3.6 (0.2), 98 (2.1); plasma [Na^+],[K^+]: 143 (0.5), 7.1 (0.2).

Thymocyte Na^+ and K^+ Transport: Significantly greater thymocyte Na^+,K^+ pump rates were observed in HSF group as compared to the MPF group; results from group C were intermediate between, and not significantly different from those of the HSF and MPF groups (Figure 4.1.9). There were no significant differences in thymocyte intracellular [Na^+] between diet groups ($P > 0.05$; data not shown). Total and ouabain-sensitive K^+ influx rates were also higher in thymocytes of the younger animals ("baseline") as compared to rates determined following the nine week feeding period. No differences in Na^+,K^+,Cl^- cotransporter-mediated or residual K^+ influx were observed.

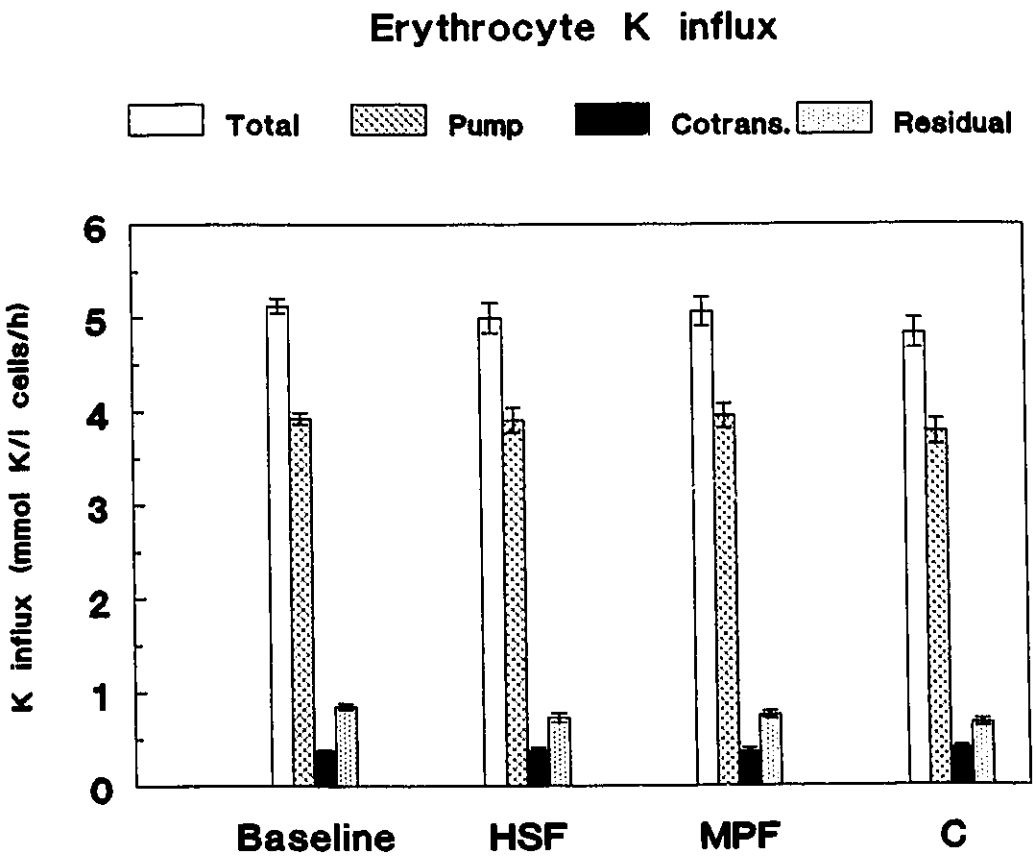

Figure 4.1.8: Erythrocyte K^+ influx at baseline and following nine weeks of dietary treatments. The modes of K^+ influx which are identified in the legend are defined as in Figure 3.1. Each result is reported as a mean (±SEM). The numbers of determinations per group are: Baseline, 72; HSF, 22; MPF, 17; C, 18. Comparisons of the means for each of the studied modes of K^+ influx using ANOVA followed by Newman-Keuls' tests revealed no significant differences ($P > 0.05$) between groups.

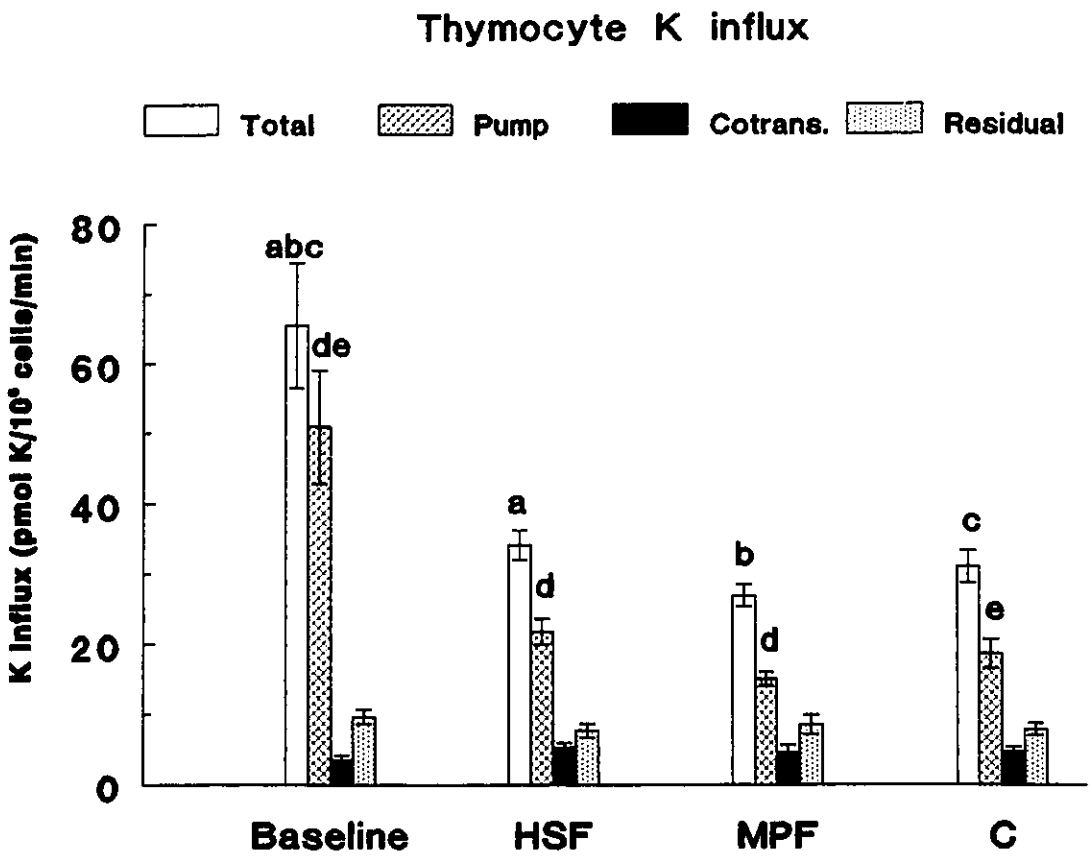

Figure 4.1.9: Thymocyte K^+ influx at baseline and following nine weeks of dietary treatments. The modes of K^+ influx which are identified in the legend are defined as in Figure 3.1. Each result is reported as a mean (±SEM). The numbers of determinations per group are: Baseline, 6; HSF, 21; MPF, 16; C, 18. Similar letters denote significant differences ($P < 0.05$) between group means as determined by ANOVA which was followed by Newman-Keuls' tests.

Analyses of BAT and its activity: The effects of the dietary treatments on various parameters of BAT function are described in Table 4.1.2. The marked increases in both UCP content (total) and concentration (specific) in the HSF group versus the MPF and C groups indicate an augmented BAT thermogenic capacity. There were no differences in the values for cytochrome c oxidase activity between the three groups which suggests that there were no differences in BAT total mitochondrial content. The total interscapular BAT protein content, a measure which provides a general estimate of the growth of the tissue, was significantly greater in the HSF group as compared to either of the MPF or C groups. The results of the DNA assays also show that there were significantly more cells in the BAT of the HSF group as compared to that of the MPF group. However in the interpretation of this data it must be kept in mind that DNA content data provides only a rough index of BAT brown adipocyte cellularity because only about 40% of BAT cells are mature brown adipocytes (Bukowiecki *et al*, 1982).

Table 4.1.2: Effects of nine weeks of dietary treatments on various parameters of BAT function.*

Diet Group	UCP total (mg)	UCP specific (μg/mg protein)	CYTOCHROME C OXIDASE total (μmol/min)	PROTEIN total (mg)	DNA total (μg)
HSF (n=21)	5.4 (0.34)^{ab}	91.5 (5.31)^{ab}	57.6 (10.11)	59.4 (1.30)^{ab}	1857 (39.2)^{b}
MPF (n=16)	2.0 (0.47)^{b}	32.8 (6.91)^{b}	44.1 (4.74)	45.1 (2.16)^{b}	1702 (47.5)^{b}
C (n=17)	1.3 (0.16)^{a}	29.9 (3.59)^{a}	44.5 (6.44)	45.6 (1.58)^{a}	1737 (45.2)

* similar superscript letters denote significant differences at $P < 0.05$ as determined using ANOVA which was followed by Tukey's post hoc tests.

DIO Study 2: Effects of high saturated fat diets and undernutrition on diet-induced obesity and on mechanisms of altered energy expenditure.

Objective: The objective of this study was to examine the effects of two high saturated fat diets, as compared to a control diet, on the development of obesity and on the two mechanisms of altered energy expenditure, Na^+,K^+ pump and BAT activities. In this second study the effects of undernutrition through food restriction were also assessed. Based on results in the literature (McCarter and McGee, 1989) it was thought that following a dietary restriction protocol similar to that of McCarter and McGee the resting metabolic rate would be lowered and that decreases in Na^+,K^+ pump activity and BAT activity might also occur. Because of the poor correlations between fat pad and total body fat weights found in the first study, I also aimed in this second study to examine the validity of TOBEC methods for the determination of total body fat content in obese, control and undernourished rats.

In the first study a marked stimulation of BAT activity was observed in the rats which were fed a high saturated fat diet comprised of lard (pork fat). As discussed earlier the results in the literature have largely supported the idea that polyunsaturated fat is more effective than saturated fat in the induction of BAT activity. Most of these previous studies have included tallow (beef fat) as their source of saturated fat (see Knehans and Romsos, 1984; Mercer and Trayhurn, 1987; Vander Tuig and Romsos, 1984). Despite the fact that the composition of these two fats is very similar (see Appendix 1), I thought that there may be a difference

between lard- and tallow- based diets in the induction of BAT activity. Therefore in this study I included two high saturated fat diets which were identical except that the source of the saturated fat was either lard or tallow.

Experimental design: In total 128 male weanling rats were received for the following described study. An outline of some of the determinations carried out in this study is provided in Table 4.2.1.

After a three week baseline period, at which point the rats were 44 days of age, eight rats were killed for baseline analyses. Then the remaining 120 rats were fed *ad libitum* one of three diets. The diets were: high fat diet one, HF1 (% energy from lard and corn oil: 51 and 12, respectively), high fat diet two, HF2 (% energy from tallow and corn oil: 51 and 12, respectively) and control diet, C (% energy from corn oil: 12). The diets were equivalent in protein content (20% energy). The compositions of the diets including the diet which was used for those rats whose intakes were restricted are shown in Table 4.2.2.

After twelve weeks of dietary treatments eight rats from each of the three diet groups were killed for analyses, and eight rats per group began a dietary restriction regime. During dietary restriction periods the rats were given 50% of the energy intake of the control rats in the form of the dietary restriction (DR) diet. Then following another twelve week period eight rats from each of the three diet groups and eight rats in each of the three DR groups were killed for analyses. Of the remaining rats, half within each of the three diet groups began DR and half continued the diets for the final twelve week period. A clearer idea of the

Table 4.2.1: Outline of some determinations carried out in DIO study 2.

Determinations	Baseline	12 wk.	24 wk.		36 wk.	
			DR	Diet	DR	Diet
Whole rat O_2 consumption	---	---	X	X	---	---
Carcass % fat	X	X	X	X	X	X
BAT GDP binding	X	X	X	X	X	X
BAT UCP content	X	X	X	X	X	X
BAT cytochrome c oxidase activity	X	X	X	X	X	X
BAT DNA content	X	X	X	X	X	X
RBC Na^+,K^+ pump activity	X	X	X	---	---	---
Thymocyte Na^+,K^+ pump activity	X	X	X	X	X	X
Hepatocyte Na^+,K^+ pump activity	---	---	---	X	X	X

Table 4.2.2: **The composition of diets used in DIO study 2.***

COMPONENT	HF1	HF2	C	DR
casein (% wt)	27.0	27.0	19.5	19.5
corn oil	7.0	7.0	5.0	5.0
lard	31.0	-	-	-
tallow	-	31.0	-	-
sucrose	20.0	20.0	18.0	15.5
corn starch	3.1	3.1	47.5	45.5
Alphacel™	4.5	4.5	5.0	5.0
AIN vitamin mix	1.6	1.6	1.0	2.0
AIN mineral mix	5.2	5.2	3.5	7.0
D, L-methionine	0.3	0.3	0.3	0.3
choline bitartrate	0.2	0.2	0.2	0.2
D, L-α-tocopherol	0.1	0.1	-	-
Energy (kJ/g)	22.7	22.7	16.1	16.1
% protein (% energy)	20	20	20	21
% carbohydrate	17	17	68	67
% fat	63	63	12	12

* In HF1 and HF2 diets the percentages of energy provided by lard or tallow and corn oil are 51% and 12%, respectively. The vitamin and mineral contents of the HSF diets were adjusted to compensate for their high energy densities. During dietary restriction rats were fed 50% of the energy intake of C rats. The vitamin and mineral contents of the DR diet were also adjusted to compensate for the decreased overall intake of nutrients during the dietary restriction period.

experimental design is gained from Figure 4.2.1.

The rats which were selected from each of the three diet groups for the dietary restriction protocol were selected as follows. The body weights of rats in the diet groups were sorted in descending order and then divided into quartiles. Then the two heaviest rats from each quartile were selected for the restriction regime. A similar sorting procedure was used to select rats killed for analyses.

Beyond the determinations outlined in Table 4.2.1 the following regular measurements were carried out. Body weights of all rats were assessed weekly. Food intake and metabolic efficiency were determined approximately every four weeks. The total body fat content of live rats was determined every four weeks using TOBEC until the rats were too large for the TOBEC equipment (*ie.* at approximately 600 g). It should be noted that the whole rat oxygen consumption determinations identified in Table 4.2.1. were carried out once after approximately 22 weeks of dietary treatments; these assessments involved both fed and DR rats.

Results

Changes in total body weight: The body weight data which were collected during the 36 week feeding study are shown in Figure 4.2.1. Following approximately three weeks of dietary treatments (*ie.* 69 days of age) the average body weights of the rats fed either HF1 or HF2 diets were significantly greater than those of rats fed the C diet ($P < 0.01$; ANOVA with a Newman-Keuls' test).

Using standard linear regression techniques the weight gain slopes were analysed over three time periods. Over the first time period of 0-8 wk. the weight gain slopes (g/d) of HF1 and HF2 rats were not significantly different from each other but both were significantly greater than the slope for C rats. However, over the time period of 8-22 wk.the weight gain slopes of the HF1 rats was greater than that for HF2 rats; the slopes for both HF1 and HF2 rats were greater than that for C rats. Finally over the time period of 22-36 wk., the slope for the HF2 rats was greater than both the slopes of HF1 and C rats.

When the slopes for weight loss during the two dietary restriction periods were analysed it was found that the HF1 and HF2 rats lost weight (g/d) at a greater rate than the C rats. However, if the percentages of weight lost (% BWt) during restriction period 1 and restriction period 2 (*ie.* the younger rats vs. the older rats) are compared it is apparent that a greater percentage of weight is lost in each of the three diet groups during diet restriction period 1. In diet restriction period 1 the percentage of weight lost (±SEM) in each of the diet groups were: HF1: 39.3 (2.11), HF2: 39.8 (4.05) and C: 33.0 (2.65). In restriction period 2 the percentages of weight lost (±SEM) were: HF1: 28.1 (1.87), HF2: 27.5 (4.11), and C, 21.1 (2.21).

The statistical comparisons between the three diet groups at each time point were carried out using ANOVA and Newman-Keuls' post hoc tests; those between *ad libitum* fed and dietary restriction groups were carried out using unpaired Student's t tests.

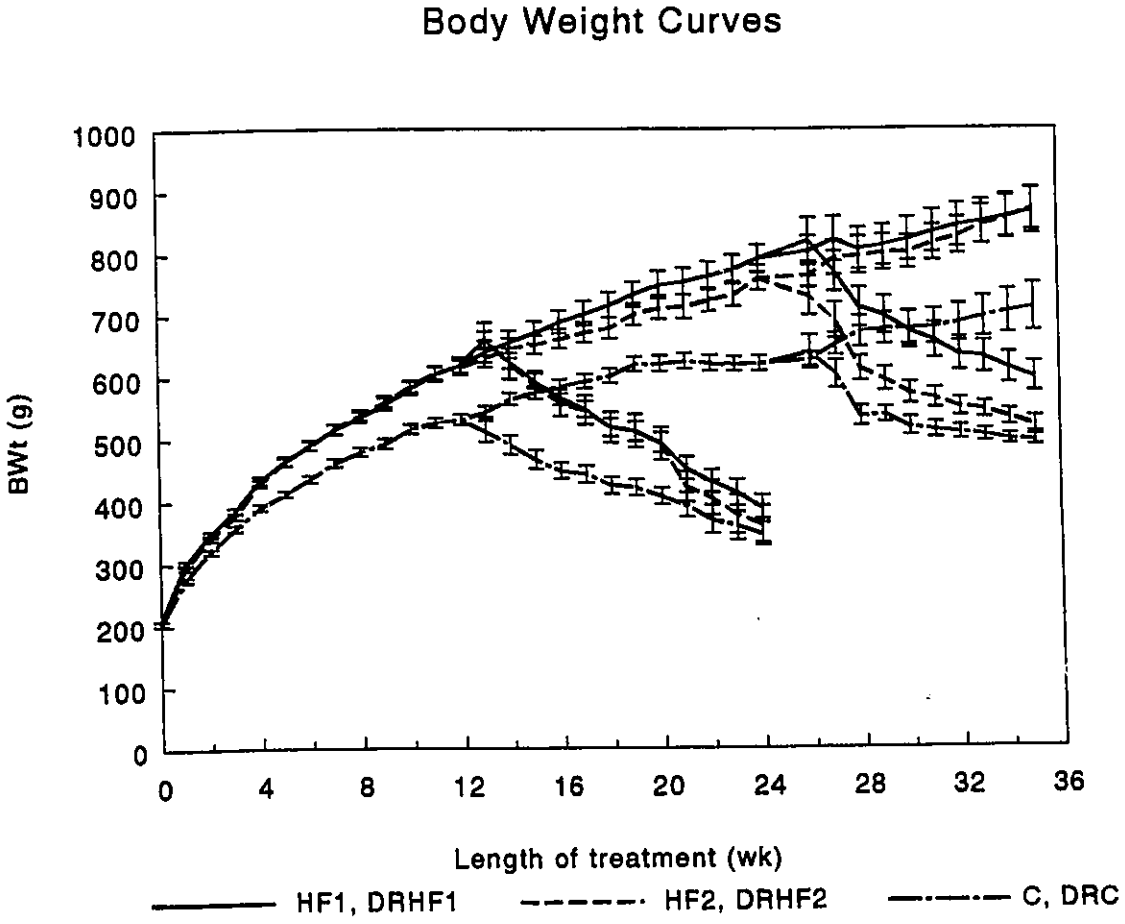

Figure 4.2.1: Body weight changes in high fat diet-fed (HF1 and HF2) and control diet-fed (C) rats. After a three week chow diet baseline period 120 male SD rats were fed a high fat diet, HF1, or HF2, or fed a control diet (C). After 12 weeks eight rats per group began a diet restriction (DR) regime (50% of the *ad libitum* energy intake of C rats) and eight rats per group were killed. After another 12 weeks eight rats per group and the rats in the DR groups were killed. Of the remaining rats half began the DR protocol and half continued eating diets HF1, HF2 and C *ad libitum* for the final 12 wk. period. Each result is presented as a mean (±SEM).

Energy intake: The results of the energy intake determinations are presented in Figure 4.2.2. At all time points, except two, the daily energy intakes of HF1 and HF2 rats were not significantly different from each other, but both were significantly greater than the average intake of C rats ($P < 0.05$; ANOVA with Newman-Keuls' post hoc tests). The two exceptions to this observed hyperphagia in both HF1 and HF2 rats occurred at: 1) one week when the intake of HF1 rats, but not HF2 rats, was greater than the intake of C rats, and 2) at 16 weeks when the intakes of neither HF1 or HF2 was greater than the intake of C rats.

Metabolic efficiency: The estimates of metabolic efficiency (mg body weight gained per day/ daily energy intake (kJ)) are shown in Figure 4.2.3. At all time points, except two, the metabolic efficiencies of HF1 and HF2 rats were greater than the metabolic efficiency of C rats ($P < 0.05$; ANOVA with Newman-Keuls' post hoc tests). The two exceptions to this observation occur: 1) at one week when the efficiency of HF1 rats was greater than that for HF2 rats, and the efficiency of HF2 rats was greater than that for C rats, and 2) at 23 weeks when the efficiency of HF2 rats, but not of HF1 rats, was greater than that of C rats.

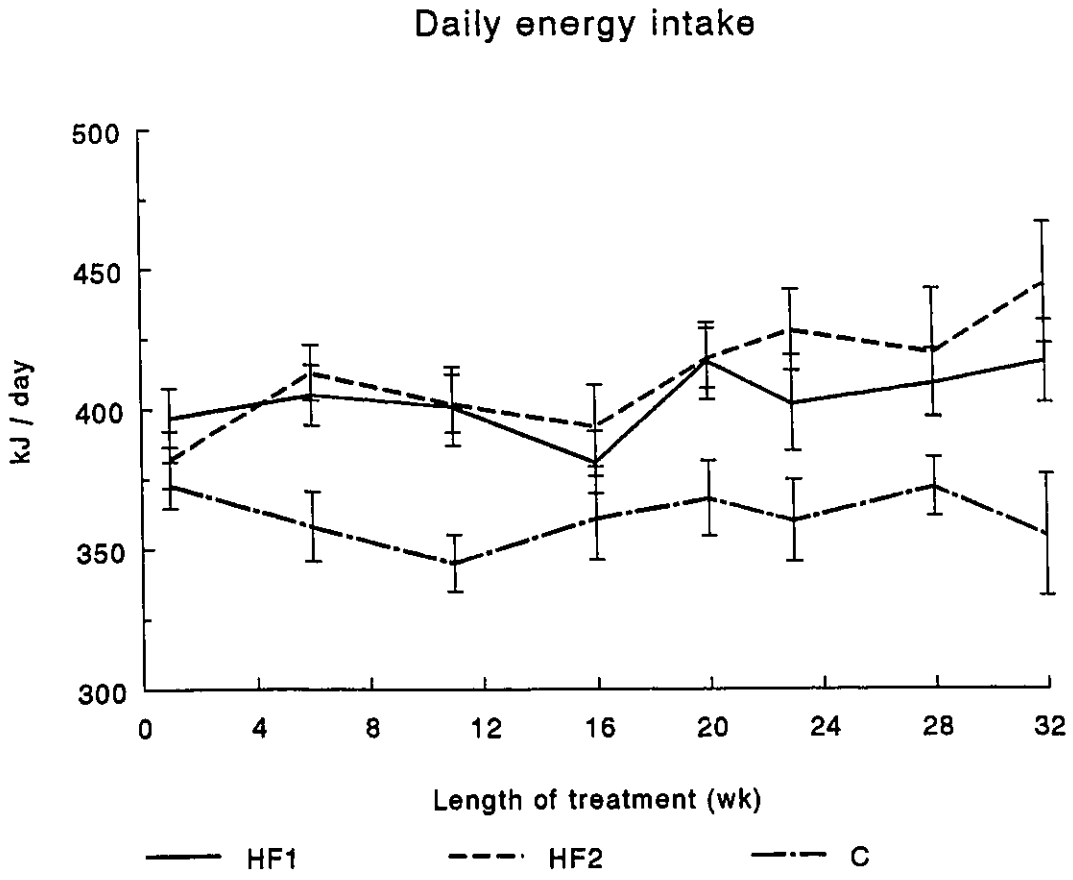

Figure 4.2.2: Daily energy intakes (kJ/d) of HF1, HF2 and C rats. Average intakes were determined during three day food intake studies at approximately four week intervals. After accounting for spillage the net weight of diet eaten was used along with Atwater coefficients to calculate the corresponding mean daily energy intake (±SEM) for each diet group. The average numbers of rats per group at each time point are: 1 wk.: 42; 6 wk.: 42; 11 wk.: 41; 16 wk.: 24; 20 wk.: 24; 23 wk.: 24; 28 wk.: 9; 32 wk.: 9. At all time points except two the daily energy intakes of HF1 and HF2 rats were not significantly different from each other, but both were significantly greater than the daily energy intake of C rats (ANOVA followed by Newman-Keuls' tests; $P < 0.05$). The two exceptions to this hyperphagia in both HF1 and HF2 rats occurred 1) at one week when the intake of HF1 rats, but not HF2 rats, was greater than the intake of C rats, and 2) at 16 weeks when the intakes of neither HF1 or HF2 rats was greater than the intake of C rats.

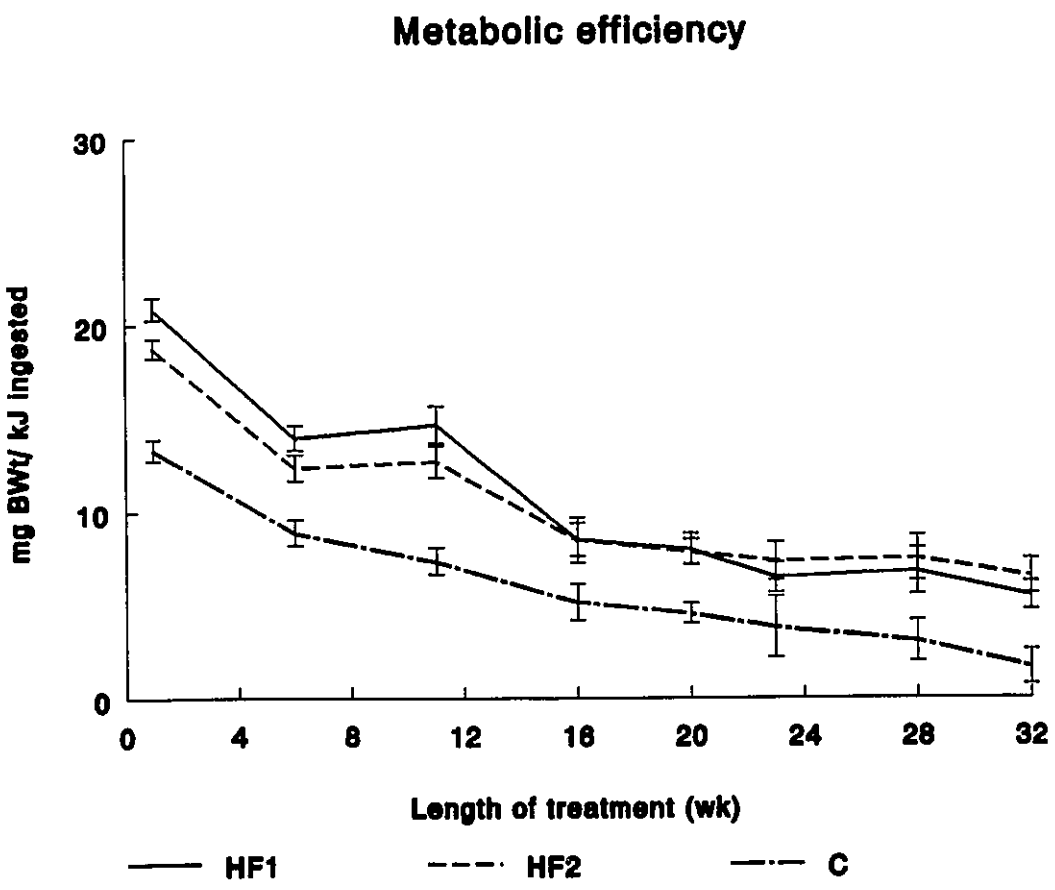

Figure 4.2.3: Metabolic efficiency (mg BWt/ kJ ingested) of HF1, HF2 and C rats. Average energy intakes and body weights were determined daily over three day periods. After accounting for spillage the net weight of diet eaten was used along with Atwater coefficients to calculate the corresponding mean daily energy intakes. To calculate the daily metabolic efficiency the daily body weight gain (mg) was divided by the daily energy intake (kJ). Each result is presented as a mean (±SEM). The average numbers of rats per group at each time point are: 1 wk.: 42; 6 wk.: 42; 11 wk.: 41; 16 wk.: 24; 20 wk.: 24; 23 wk.: 24; 28 wk.: 9; 32 wk.: 9. At all time points except two the metabolic efficiencies of HF1 and HF2 rats were not significantly different from each other, but both were significantly greater than the metabolic efficiencies of C rats (ANOVA followed by Newman-Keuls' tests; $P < 0.05$). The two exceptions to this enhanced efficiency in HF1 and HF2 rats occurred 1) at 1 wk. when the efficiency of HF2 rats was greater than that of C rats, and the efficiency of HF1 rats was greater than that of HF2 rats, and 2) at 23 wk. when the efficiency of HF2 rats, but not HF1 rats, was greater than that of C rats.

Whole rat resting oxygen consumption: Between HF1, HF2 and C groups there were no differences in resting oxygen consumption data (μl/min/cm^2) after approximately 22 weeks of dietary treatments ($P > 0.05$; ANOVA with Newman-Keuls' post hoc tests). These results are shown in Figure 4.2.4. However between the groups of rats which were fed HF1, HF2 and C diets for 12 weeks and subsequently followed the DR protocol for 10 weeks, there were significant decreases in oxygen consumption. That is, there were significant decreases in resting oxygen consumption in all groups during 10 weeks of dietary restriction, regardless of which diet the rats ate previous to the dietary restriction period ($P < 0.05$; Student's t tests). However there were no differences between DRHF1, DRHF2 and DRC groups.

Measurements of adiposity:

1) **Carcass homogenate determinations:** The measurements of total body fat (% BWt) as determined by carcass homogenate lipid extractions are shown in Figure 4.2.5. The results show that after twelve weeks of dietary treatments and at each subsequent analysis throughout the study, rats in groups HF1 and HF2 were significantly fatter than those in group C. As expected, dietary restriction of rats from each of the three diet groups resulted in significant decreases in total body fat. Interestingly, at the 36 week time point, even after dietary restriction, DRHF1

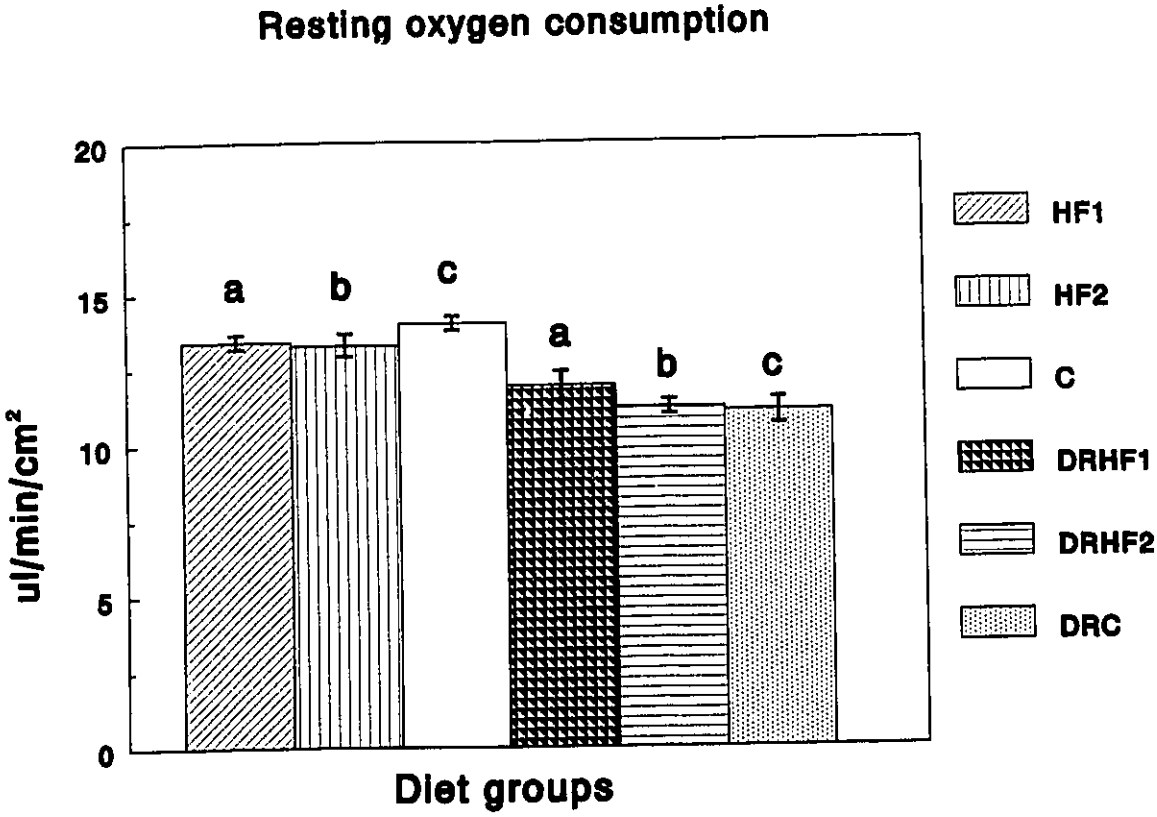

Figure 4.2.4: Whole animal resting oxygen consumption. Measurements were carried out after 22 wk. of dietary treatments; therefore HF1 (n=13), HF2 (n=14) and C (n=14) rats had been fed their respective diets for 22 wk. while DRHF1 (n=8), DRHF2 (n=6) and DRC (n=8) rats had been fed HF1, HF2 or C diets, respectively, for 12 wk. and then fed the restriction diet for 10 wk. Measurements were carried out by placing the unanesthetized rat (not fasted) in a water jacket-enclosed chamber (28°C) and monitoring oxygen consumption using a Beckman Industrial™ Model 755 Oxygen Analyser over approximately 1.5 hr. Resting oxygen consumption was defined as that during periods over which consumption was consistently at a minimal rate. The data are expressed per unit surface area using equations for the surface area of the rat; these equations were $S = 7.47\ W^{0.667}$ for normal rats and $S = 7.64W^{0.667}$ for emaciated rats, where S represents surface area (cm^2) and W represents body weight (g) (Diack, 1930). Each result is presented as a mean (±SEM); similar letters denote significant differences (at $P < 0.05$) between means as determined by ANOVA followed by Newman-Keuls' tests, or by Student's t tests (for comparisons of *ad libitum* fed vs. post DR).

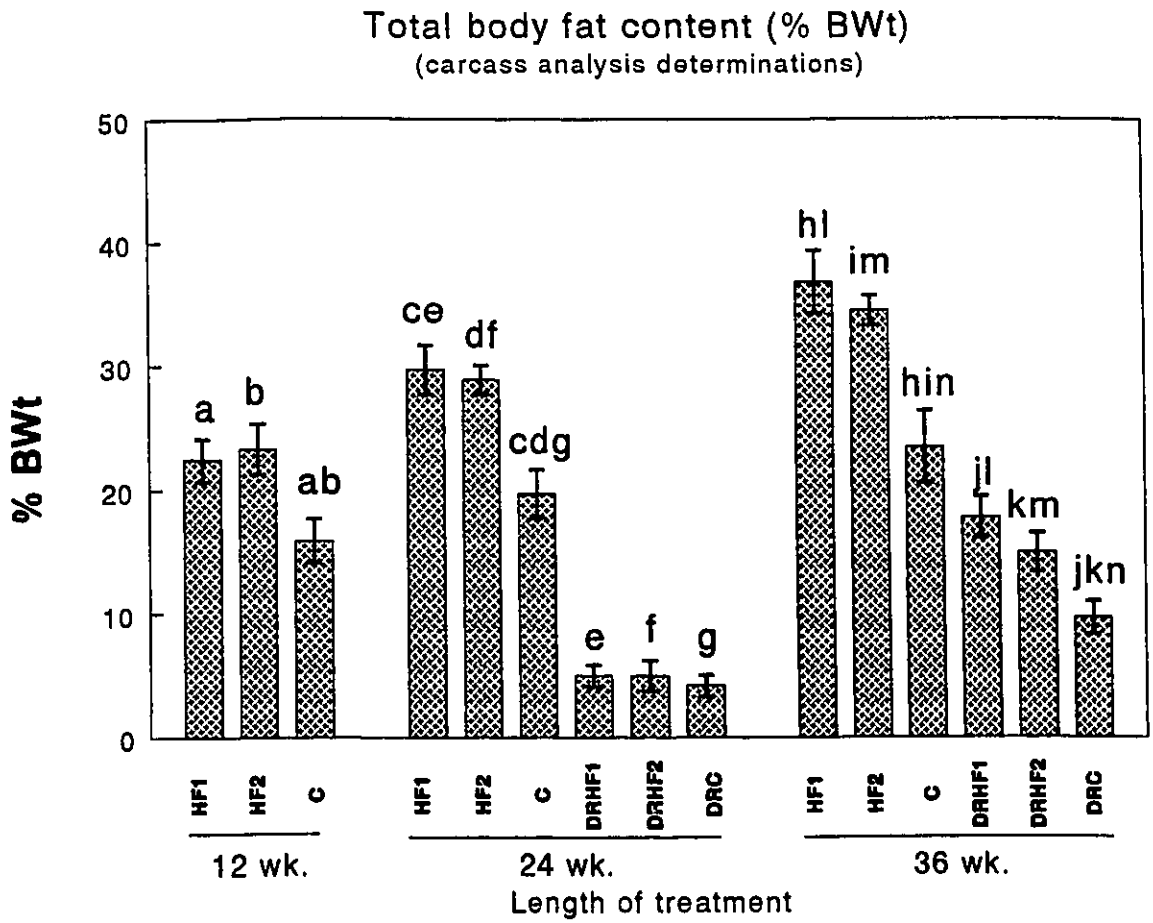

Figure 4.2.5: Total body fat content as determined by carcass lipid extraction. Carcass homogenates were analyzed in duplicate for total lipid content using petroleum ether in a Soxlet extraction system. The number of rats per group in the order (left to right) at each time point are: 12 wk.; 8, 8, 8; 24 wk.; 8, 8, 8, 8, 6, 8; 36 wk.; 7, 8, 8, 7, 5, 5. Each result is presented as a mean (±SEM); similar letters denote significant differences (at $P < 0.05$) between diet groups at each time point as determined by ANOVA followed by Newman-Keuls' tests, or by Student's t tests (for comparisons of *ad libitum* fed vs. post DR at each time point).

and DRHF2 rats remain significantly fatter than DRC. No differences in adiposity were observed at the 24 week time point between DRHF1, DRHF2 and DRC groups. These results are consistent with the idea that long term obesity is more resistant to reversal than is shorter term obesity. The tests of statistical significance were carried out using ANOVA which was followed by Newman-Keuls' post hoc tests, or using unpaired Student's t tests (for comparisons of *ad libitum* fed vs. post DR data at each time point).

2) **TOBEC determinations:** The results of the TOBEC determinations of total body fat content are shown in Figure 4.2.6. The results overall do not follow the pattern of the results obtained through carcass homogenate analysis. After 12 weeks of dietary treatments these data suggest that only those rats in the HF2 group are fatter than the rats in the C group. After 16 weeks the data suggest that rats in the HF2 group are no longer fatter than the rats in the C group and that there are no differences in total body fat content between groups. After 20 weeks the data again suggest that only those rats in the HF2 group are fatter than the rats in the C group. Neither two or eight weeks of dietary restriction had a significant effect on the TOBEC determinations of total body fat content. The tests of statistical significance were carried out using ANOVA which was followed by Newman-Keuls' post hoc tests, or using unpaired Student's t tests (for comparisons of *ad libitum* fed vs. post DR data at each time point).

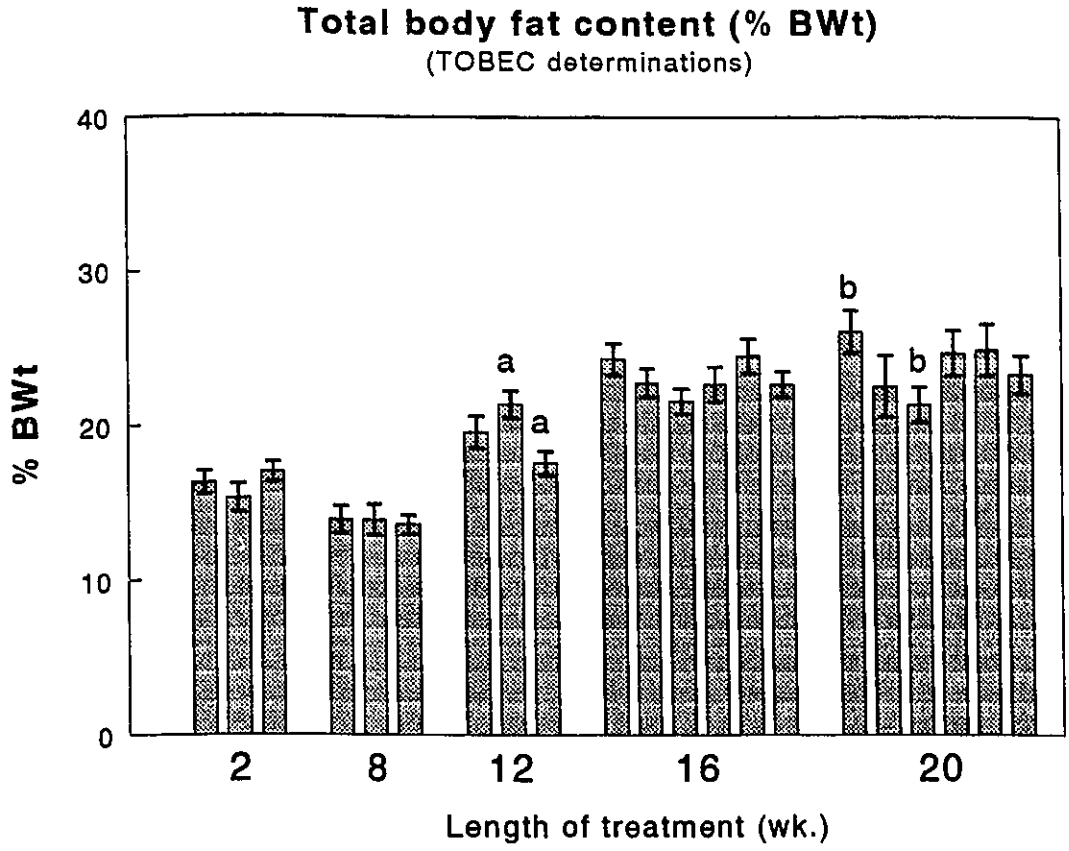

Figure 4.2.6: Total body fat content as determined by TOBEC. Within each cluster of results in the bar graph the bars, left to right, represent HF1, HF2, C (DRHF1, DRHF2 and DRC) groups. The numbers of rats per group in this same respective order are: 2 wk.: 41, 42, 43; 8 wk.: 41, 42, 43; 12 wk.: 29, 31, 40; 16 wk.: 9, 12, 21, 8, 8, 8; 20 wk.: 5, 5, 18, 8, 7, 8. Each result is presented as a mean (±SEM); similar letters denote significant differences (at $P < 0.05$) between diet groups at each time point as determined by ANOVA followed by Newman-Keuls' tests, or by Student's t tests (for comparisons of *ad libitum* fed vs. post DR data at each time point).

3) **Correlation of TOBEC with carcass homogenate determinations of total body fat content:** The results of this comparison are shown in Figure 4.2.7. The correlation between the results of the two methods is fairly weak and it appears that the TOBEC method under-estimates total body fat content by approximately 20 to 30 g. However it is also possible that in removing the skin and fur during the homogenization procedures some of the body fat was lost; this would result in an inaccurate (low) estimation of carcass fat content.

4) **Correlation of fat pad weights with carcass homogenate determinations of total body fat content:** After 12 weeks and 24 weeks of dietary treatment, and after 24 weeks of dietary treatment which included 12 weeks of dietary restriction, the results of the sum of the epididymal and perirenal fat pad weights were correlated with the results of carcass fat content. The results are shown in Figures 4.2.8 to 4.2.10. As compared to the correlation between the sum of the fat pad weights with TOBEC-determined total body fat weight in DIO study 1, these correlations are markedly stronger.

5) **Effect of rat positioning on TOBEC total body fat determinations:** As a result of the weak correlations which were found between TOBEC determinations of total body fat weight and other indices of adiposity further analyses of TOBEC measurements were conducted. The results of these analyses are shown in Figures 4.2.11 and 4.2.12. All tests of statistical significance were carried out using ANOVA which was followed by Newman-Keuls' post hoc tests.

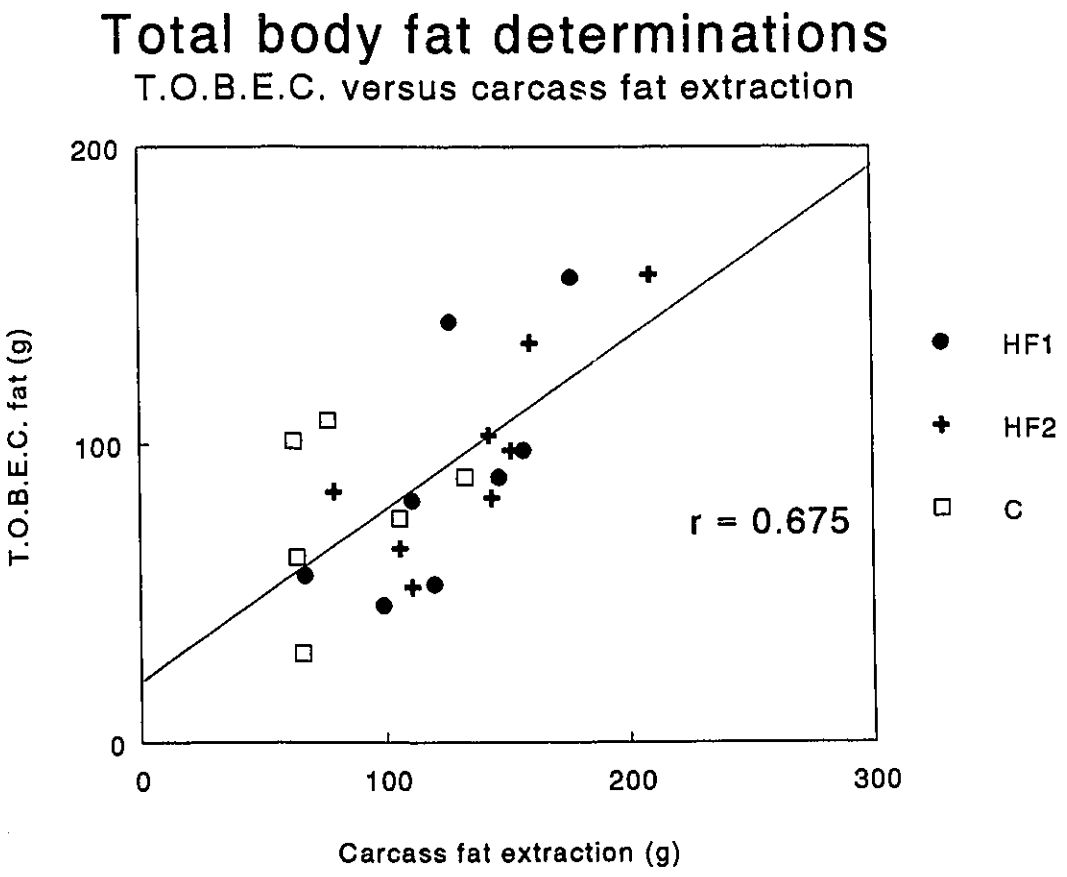

Figure 4.2.7: Correlation of total body fat weights as determined by TOBEC and carcass lipid extraction. TOBEC measurements were carried out after 11 wk of dietary treatments; these results are correlated to the total body fat weight of carcasses of rats which were killed after 12 wk of dietary treatment. Each data point represents results for one rat. Results from each diet group are indicated as shown in the legend. The numbers of results from each group are: HF1, 8; HF2, 8; C, 6.

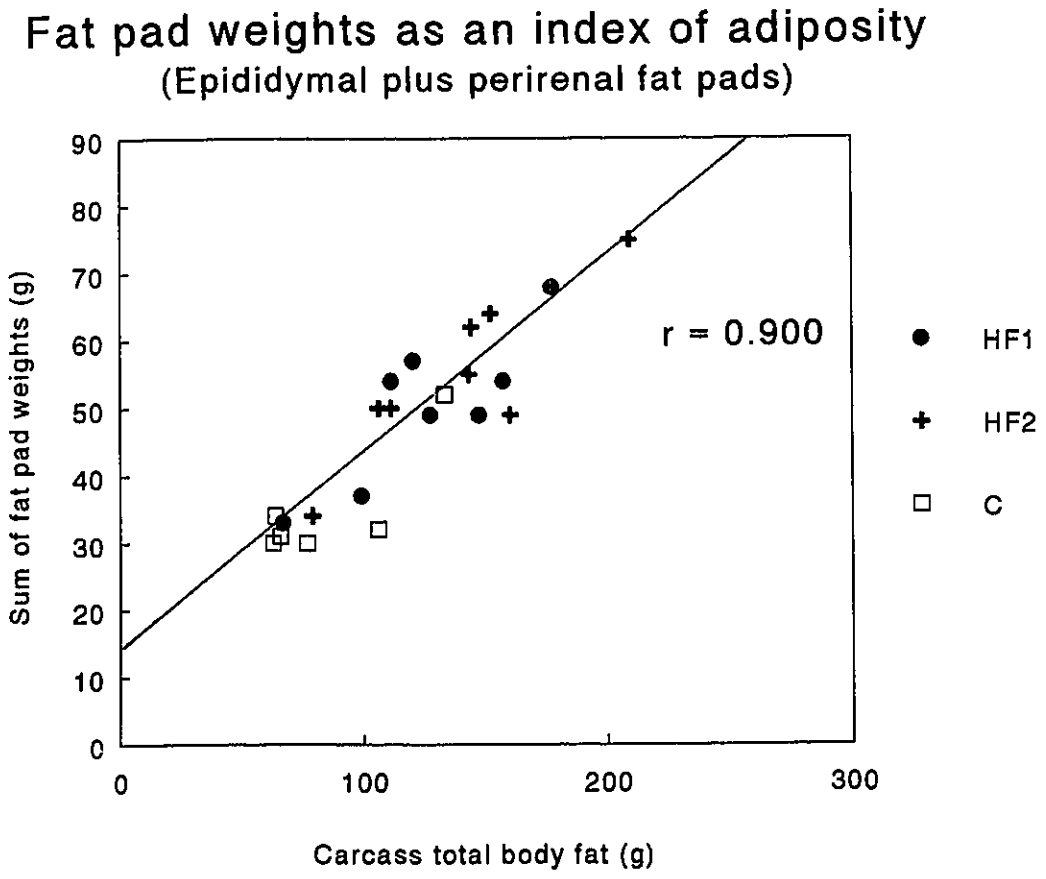

Figure 4.2.8: Correlation of the sum of epididymal and perirenal fat pad weights and total body fat weights as determined by carcass lipid extraction after 12 wk. of dietary treatments. Each data point represents results for one rat. Results from each diet group are indicated as shown in the legend. The numbers of results from each group are: HF1, 8; HF2, 8; C, 6.

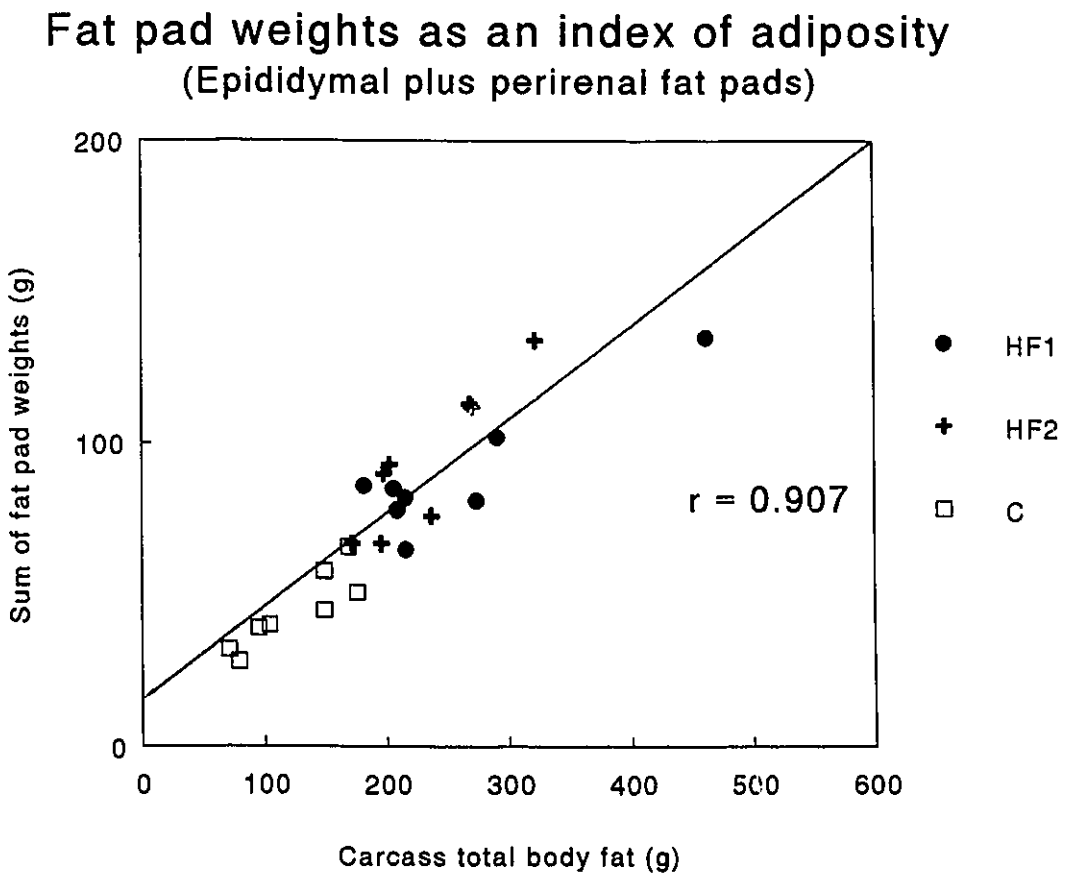

Figure 4.2.9: Correlation of the sum of epididymal and perirenal fat pad weights and total body fat weights as determined by carcass lipid extraction after 24 wk. of dietary treatments. Each data point represents results for one rat. Results from each diet group are indicated as shown in the legend. The numbers of results from each group are: HF1, 8; HF2, 8; C, 8.

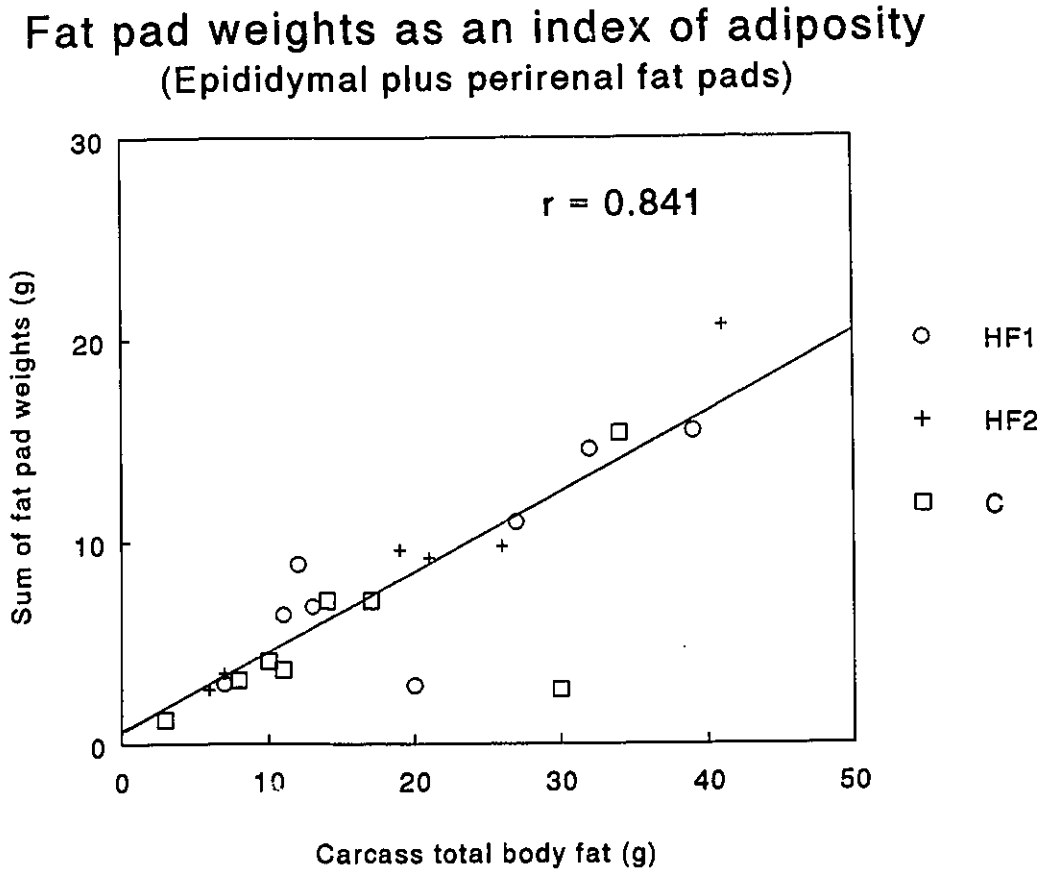

Figure 4.2.10: Correlation of the sum of epididymal and perirenal fat pad weights and total body fat weights as determined by carcass lipid extraction after 12 wk. of *ad libitum* dietary treatments and 12 wk. of the dietary restriction regime. Each data point represents results for one rat. Results from each diet group are indicated as shown in the legend. The numbers of results from each group are: DRHF1, 8; DRHF2, 6; DRC, 8.

Figure 4.2.11 shows that in most cases placing the rat in the supine position as compared to the recommended prone position on the plexiglass carrier platform within the cylinder has a significant effect on the total body fat results. The size of the carrier platform used which affects the vertical position of the rat within the cylinder seems to have less of an effect on the total body fat results, although statistically significant differences were observed.

Figure 4.2.12 shows that the effect of changing the position of rat within the cylinder upon the total body fat result is not consistent between the results for two rats having different body weights. Again these results demonstrate that the supine and prone positioning within the cylinder is critical for reproducible results from each rat. However, the results also show that for different rats, similar changes in positioning within the cylinder sometimes have different effects on the total body fat results. For example, when each rat's position was changed from the supine to the prone position, and the same carrier was used in each case, the total body fat result for the heavier rat decreased significantly while the result for the lighter rat increased significantly.

While it was not systematically tested, the position of the rat lengthwise within the cylinder also appeared to have large effects upon the results. It should be noted that careful attention was paid to the consistent positioning of each rat within the cylinder throughout the studies involving this equipment.

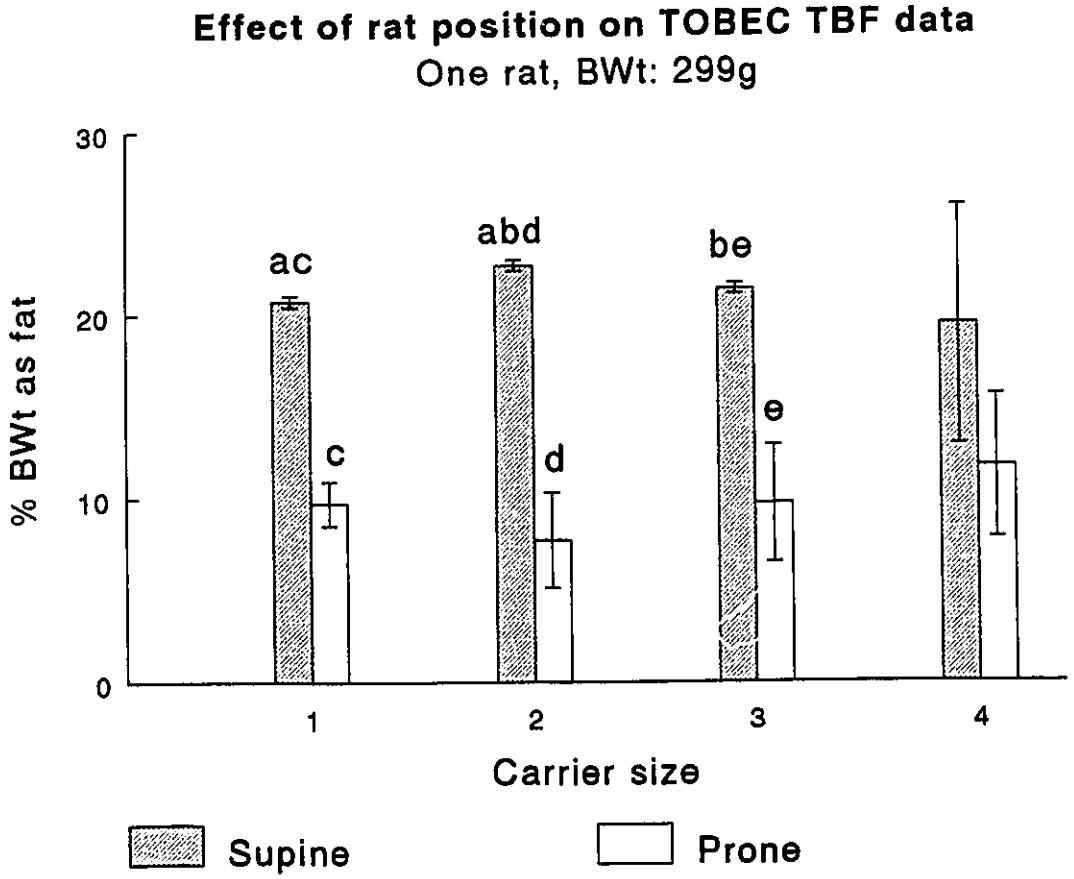

Fig 4.2.11: Effect of rat positioning on TOBEC determinations of total body fat weights: variation in vertical positioning. Results (mean ± SEM) are reported for one control rat. The rat was placed in the cylinder in either a supine (S) or in a prone (P) position, and the vertical position of the rat was varied by increasing the size (widths 1 to 4) of the plexiglass carrier band under the rat within the cylinder. Thus through the use of wider plexiglass carrier bands in the cylinder the position of the rat in the cylinder is moved upwards. Statistical analyses involved ANOVA which was followed by Newman-Keuls' post hoc tests; similar letters denote significant differences ($P < 0.05$).

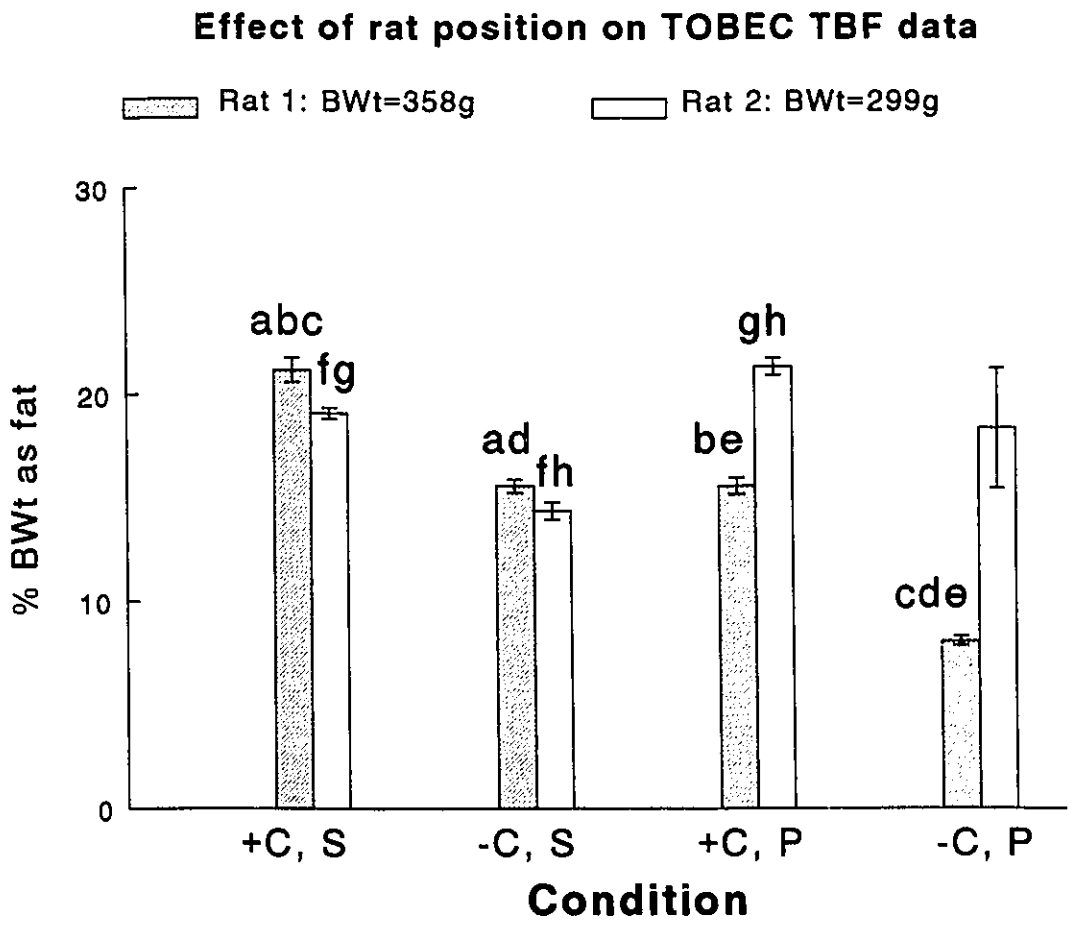

Fig 4.2.12: Effect of rat positioning on TOBEC determinations of total body fat weights: variation in body weight and vertical positioning. Results (mean ± SEM) are reported for two control rats. The positioning of each rat in the cylinder is shown on the x axis. +C and -C represent the presence and absence, respectively, of the plexiglass carrier band under the rat within the cylinder; S and P represent supine and prone positioning, respectively, of the rat within the cylinder. Statistical analyses involved ANOVA which was followed by Newman-Keuls' post hoc tests; similar letters denote significant differences ($P < 0.05$).

Erythrocyte Na^+ and K^+ Transport: No changes were observed in erythrocyte Na^+,K^+ pump-mediated K^+ influx between diet groups at the 12 week or the 24 week time points (Table 4.2.3; Figure 4.2.13). However, following 12 weeks of dietary restriction Na^+,K^+ pump activity decreased in the rats which were fed HF2 or C diets prior to DR (*ie.* HF2 *vs.* DRHF2 and C *vs.* DRC).

No significant differences in total K^+ influx or Na^+,K^+,Cl^- cotransporter-mediated K^+ influx were observed between diet groups at any time point or between pre and post dietary restriction values (*eg.* C *vs.* DRC). Residual K^+ influx increased with DR in each of the three diet groups. These data are summarized in Table 4.2.3.

Statistical tests of significance involved unpaired Student's t tests between *ad libitum* fed and post dietary restriction data; ANOVA followed by Newman-Keuls' post hoc tests were used for the comparisons between the three diet groups.

Thymocyte Na^+ and K^+ Transport: After 12 weeks of dietary treatments thymocyte Na^+,K^+ pump activity was significantly greater in the group which was fed the HF1 diet as compared to the activities determined in rats which were fed either the HF2 or C diets (Table 4.2.4; Figure 4.2.14). However, after 24 weeks Na^+,K^+ pump activity was significantly greater in the group which was fed the HF2 diet as compared to the data from either the HF1 or C groups. At 24 weeks it was evident that 12 weeks of DR had no effect on Na^+,K^+ pump activity in any of the groups. At 36 weeks there were no longer any differences in activity between HF1, HF2 and C

Table 4.2.3: Total, Na^+,K^+ pump-mediated, Na^+,K^+,Cl^- cotransporter-mediated and residual K^+ influx in erythrocytes.*

Diet Group	Time (wk)	Total (mmol/l cells/h)	Na^+,K^+ pump (mmol/l cells/h)	Na^+,K^+,Cl^- cotransporter (mmol/l cells/h)	Residual (mmol/l cells/h)
Baseline n=8	0	4.78 (0.130)	3.71 (0.161)	0.46 (0.070)	0.65(0.122)
HF1 n=8	12	4.60 (0.252)	3.57 (0.215)	0.52 (0.057)	0.50(0.145)[a]
HF2 n=8	12	5.41 (0.431)	4.02 (0.282)[a]	0.87 (0.244)	0.53(0.053)[b]
C n=8	12	4.94 (0.262)	3.89 (0.172)[b]	0.52 (0.073)	0.53(0.040)[c]
DRHF1 n=8	24	4.83 (0.178)	3.29 (0.294)	0.62 (0.118)	0.89(0.051)[a]
DRHF2 n=8	24	4.70 (0.197)	2.95 (0.248)[a]	0.83 (0.170)	0.92(0.039)[b]
DRC n=8	24	4.39 (0.187)	2.81 (0.186)[b]	0.72 (0.169)	0.86(0.099)[c]

* Each of the data is expressed as a mean (±SEM). Significant differences between means at within columns (each time point) are indicated by similar superscript letters.

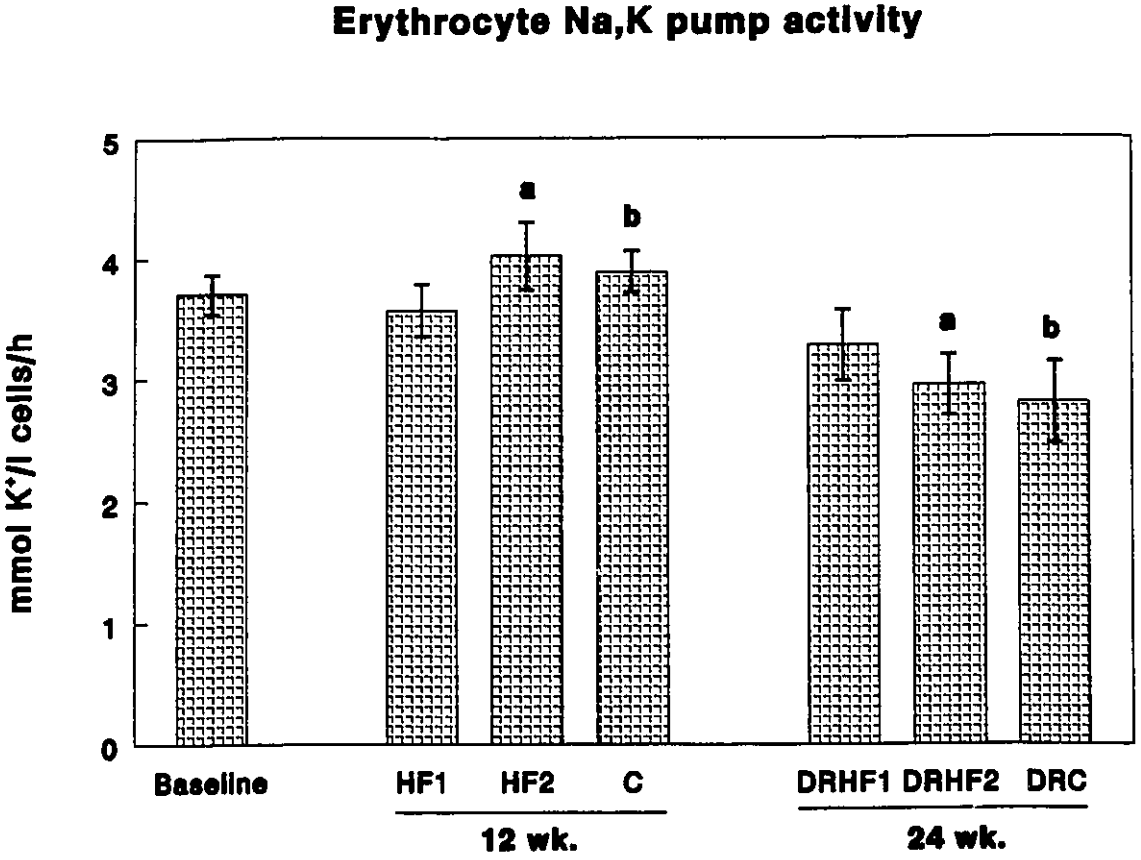

Figure 4.2.13: Erythrocyte Na^+,K^+ pump activity at baseline and after 12 wk. dietary treatments and after 12 wk. of dietary treatments which was followed by 12 wk. of the dietary restriction regime. The numbers of determinations per group are: Baseline, 8; HF1, 8; HF2, 8; C, 8; DRHF1, 8; DRHF2, 8; DRC, 8. Each result is presented as a mean (±SEM); similar letters denote significant differences (at $P < 0.05$) between means at each time point. Statistical tests of significance involved: ANOVA with Newman-Keuls' post hoc tests for comparisons between the three diet groups and unpaired Student's t tests for comparisons of *ad libitum* fed vs. post dietary restriction data at each time point.

groups. The only significant difference in thymocyte Na^+,K^+ pump activities at the 36 week time point was the lower activity in the DRHF1 group as compared to the activity in the HF1 group.

In regards to the other pathways of thymocyte K^+ influx which were studied, few effects of diet or dietary restriction were observed (Table 4.2.4). At the 24 week time point it was evident that 12 weeks of dietary restriction resulted in significant decreases in total K^+ influx in the HF1 - DRHF1 and HF2 - DRHF2 groups. Thymocyte Na^+,K^+,Cl^- cotransporter-mediated K^+ influx after 12 weeks of dietary treatments was greater in the control group than in the group fed the HF1 diet. The negative values for Na^+,K^+,Cl^- cotransporter-mediated K^+ influx indicate that the bumetanide, instead of inhibiting K^+ influx (the proportion being identified as "bumetanide-sensitive" or "$Na^+K^+Cl^-$ cotransporter-mediated" K^+ influx), the bumetanide actually stimulated overall K^+ influx. The reasons for this stimulation of K^+ influx which is sometimes observed (especially, it appears, in cells of older rats) are unknown. No changes in residual K^+ influx were observed.

Statistical tests of significance involved unpaired Student's t tests for the comparison of *ad libitum* fed and post dietary restriction data; ANOVA which was followed by Newman-Keuls' post hoc tests were used for the comparisons between the three diet groups.

Table 4.2.4: Total, Na^+,K^+ pump-mediated, Na^+,K^+,Cl^- cotransporter-mediated and residual K^+ influx in thymocytes.*

Diet Group	Time (wk)	Total (pmol/10^6 cells/min)	Na^+,K^+ pump (pmol/10^6 cells/min)	Na^+,K^+,Cl^- cotransporter (pmol/10^6 cells/min)	Residual (pmol/10^6 cells/min)
Baseline n=8	0	46.99(3.950)	26.57(3.560)	8.105(3.069)	14.25(1.079)
HF1 n=8	12	28.01(3.359)	19.51(2.163)^{ab}	1.63(0.392)^{a}	6.88(1.200)
HF2 n=8	12	25.21(2.298)	14.99(0.947)^{a}	4.18(1.583)	6.15(0.892)
C n=8	12	27.84(2.523)	13.80(0.050)^{b}	4.13(0.556)^{a}	9.70(1.756)
HF1 n=8	24	38.69(3.786)^{a}	16.24(1.846)^{c}	1.81(2.267)	20.74(3.804)
HF2 n=8	24	47.48(5.565)^{b}	24.00(3.102)^{cd}	4.57(1.615)	19.07(3.608)
C n=8	24	35.11(3.182)	16.40(1.736)^{d}	1.73(1.852)	17.01(2.733)
DRHF1 n=8	24	24.36(3.074)^{a}	12.84(3.471)	1.83(5.202)	10.11(1.953)
DRHF2 n=6	24	30.04(4.753)^{b}	17.18(3.228)	3.38(3.646)	9.96(1.887)
DRC n=7	24	26.59(2.361)	15.48(2.144)	-1.20(1.067)	12.64(2.519)
HF1 n=8	36	37.43(2.001)	20.06(2.151)^{e}	-3.38(3.329)	20.72(4.170)
HF2 n=8	36	41.02(5.246)	15.31(3.549)	-0.56(3.304)	26.28(5.373)
C n=9	36	39.13(3.579)	14.32(3.465)	0.31(2.274)	24.56(5.051)
DRHF1 n=7	36	31.96(2.242)	12.27(2.124)^{e}	-0.10(2.474)	19.78(4.145)
DRHF2 n=5	36	34.31(5.554)	11.84(2.877)	4.64(2.502)	17.83(5.048)
DRC n=5	36	28.30(5.522)	9.90(2.677)	-4.93(5.645)	23.33(9.645)

* Each of the data is expressed as a mean (±SEM). The negative values for Na^+,K^+,Cl^- cotransporter-mediated K^+ influx indicate that the addition of bumetanide resulted in a stimulation of K^+ influx, not an inhibition of K^+ influx as is usually observed. Statistical differences between means within columns (at each time point) are indicated by similar superscript letters.

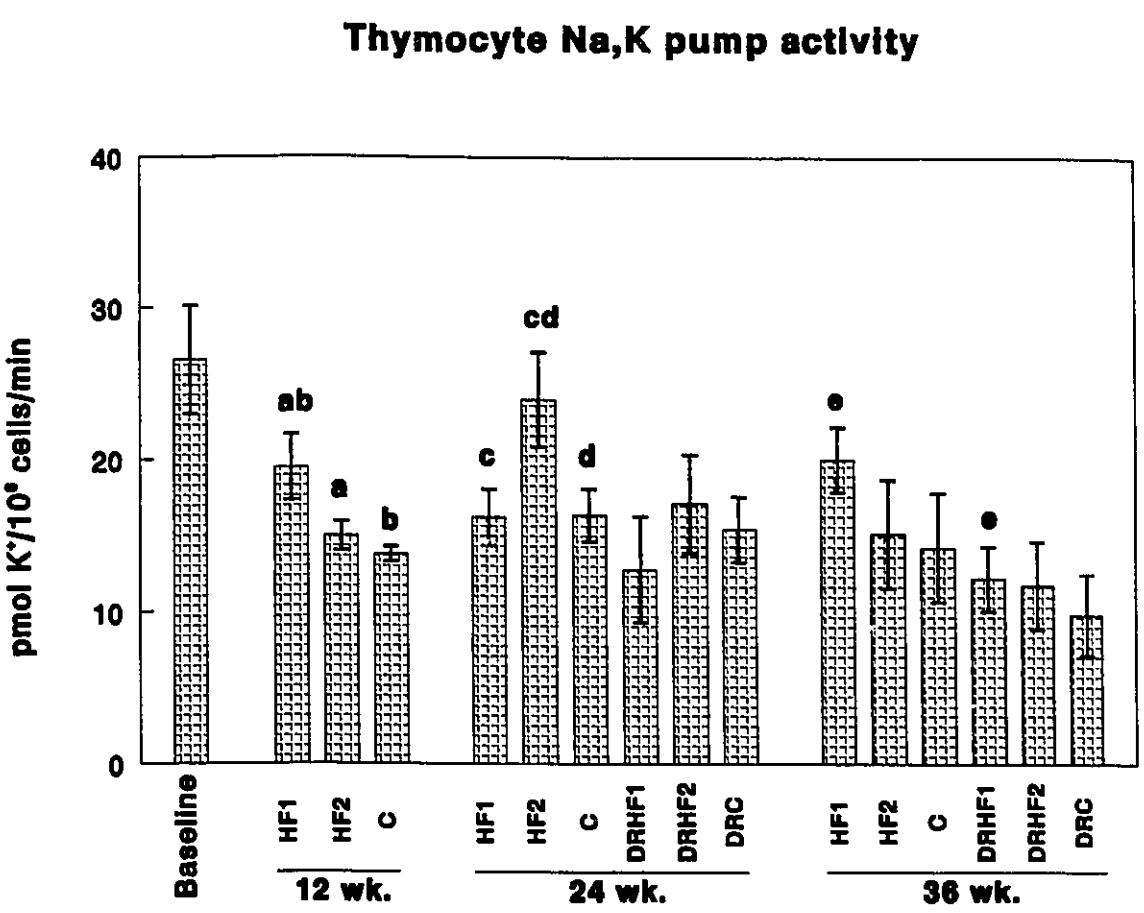

Figure 4.2.14: Thymocyte Na^+,K^+ pump activity at baseline and after 12, 24 and 36 wk. of dietary treatments. The effects of 12 wk. of the dietary restriction regime are shown at 24 and 36 wk. time points. The numbers of determinations per group are: Baseline: 8; 12 wk.: HF1, 8; HF2, 8; C, 8; 24 wk.: HF1, 8; HF2, 8; C, 8; DRHF1, 8; DRHF2, 6; DRC, 7; 36 wk.: HF1, 8; HF2, 8; C, 9; DRHF1, 7; DRHF2, 5; DRC, 5. Each result is presented as a mean (±SEM); similar letters denote significant differences (at $P < 0.05$) between means at each time point. Statistical tests of significance involved: ANOVA with Newman-Keuls' post hoc tests for comparisons between the three diet groups and unpaired Student's t tests for comparisons of *ad libitum* fed vs. post dietary restriction data at each time point.

Hepatocyte Na^+ and K^+ Transport: After 24 weeks of dietary treatments there were no statistically significant differences in hepatocyte Na^+,K^+ pump activities between the three diet groups (Table 4.2.5; Figure 4.2.15). However, if the overall pattern of the hepatocyte Na^+,K^+ pump data is compared to that for thymocyte Na^+,K^+ pump data (Figure 4.2.14) some similarities are observed. Statistical analyses of the Pearson correlation coefficients for the hepatocyte and thymocyte Na^+,K^+ pump data showed that hepatocyte and thymocyte Na^+,K^+ pump data are significantly correlated in diet groups HF1 and HF2 at the 24 week time point. The only significant difference in hepatocyte Na^+,K^+ pump activities at the 36 week time point was the greater activity in the HF2 group as compared to the activity in the DRHF2 group.

Total K^+ influx at 36 weeks was greater in the HF1 group than in the HF2 group (Table 4.2.5). At 36 weeks it was also evident that with 12 weeks of dietary restriction, total K^+ influx decreased in all groups (*ie.* HF1 - DRHF1, HF2 - DRHF2 and C - DRC). Hepatocyte Na^+,K^+,Cl^- cotransporter-mediated K^+ influx after 36 weeks of dietary treatments was greater in the control group than in the groups fed either the HF1 or the HF2 diets. Again, the addition of bumetanide sometimes resulted in an overall stimulation of K^+ influx. Residual K^+ influx determinations at 36 weeks showed greater influx in hepatocytes of HF1 diet-fed than of HF2 diet-fed groups.

Statistical tests of significance involved ANOVA with Newman-Keuls' post hoc tests for the comparisons between diet groups and unpaired Student's t tests for the

Table 4.2.5: Total, Na^+,K^+ pump-mediated, Na^+,K^+,Cl^- cotransporter-mediated and residual K^+ influx in hepatocytes.*

Diet Group	Time (wk)	Total (nmol/10^6 cells/min)	Na^+,K^+ pump (nmol/10^6 cells/min)	Na^+,K^+,Cl^- cotransporter (nmol/10^6 cells/min)	Residual (nmol/10^6 cells/min)
HF1 n=7	24	3.10 (0.465)	0.70 (0.070)	0.47 (0.104)	1.98(0.379)
HF2 n=8	24	3.35 (0.472)	0.92 (0.176)	-0.24 (0.411)	2.68(0.702)
C n=7	24	2.89 (0.309)	0.62 (0.159)	0.46 (0.140)	1.81(0.245)
HF1 n=7	36	1.96 (0.130)^{ad}	0.46 (0.126)	-0.27 (0.116)^{a}	1.85(0.160)^{a}
HF2 n=5	36	1.54 (0.097)^{bd}	0.40 (0.119)^{a}	0.03 (0.049)^{b}	1.14(0.136)^{a}
C n=6	36	2.01 (0.307)^{c}	0.31 (0.180)	0.40 (0.095)^{ab}	1.30(0.387)
DRHF1 n=7	36	1.34 (0.306)^{a}	0.24 (0.185)	0.11 (0.080)	0.99(0.169)
DRHF2 n=5	36	0.99 (0.260)^{b}	0.16 (0.049)^{a}	-0.06 (0.049)	0.89(0.251)
DRC n=5	36	1.18 (0.321)^{c}	0.37 (0.167)	0.02 (0.034)	0.79(0.169)

* Each of the data is expressed as a mean (±SEM). The negative values for Na^+,K^+,Cl^- cotransporter-mediated K^+ influx indicate that the addition of bumetanide resulted in a stimulation of K^+ influx, not an inhibition of K^+ influx as is usually observed. Statistical differences between means within columns (at each time point) are indicated by similar superscript letters.

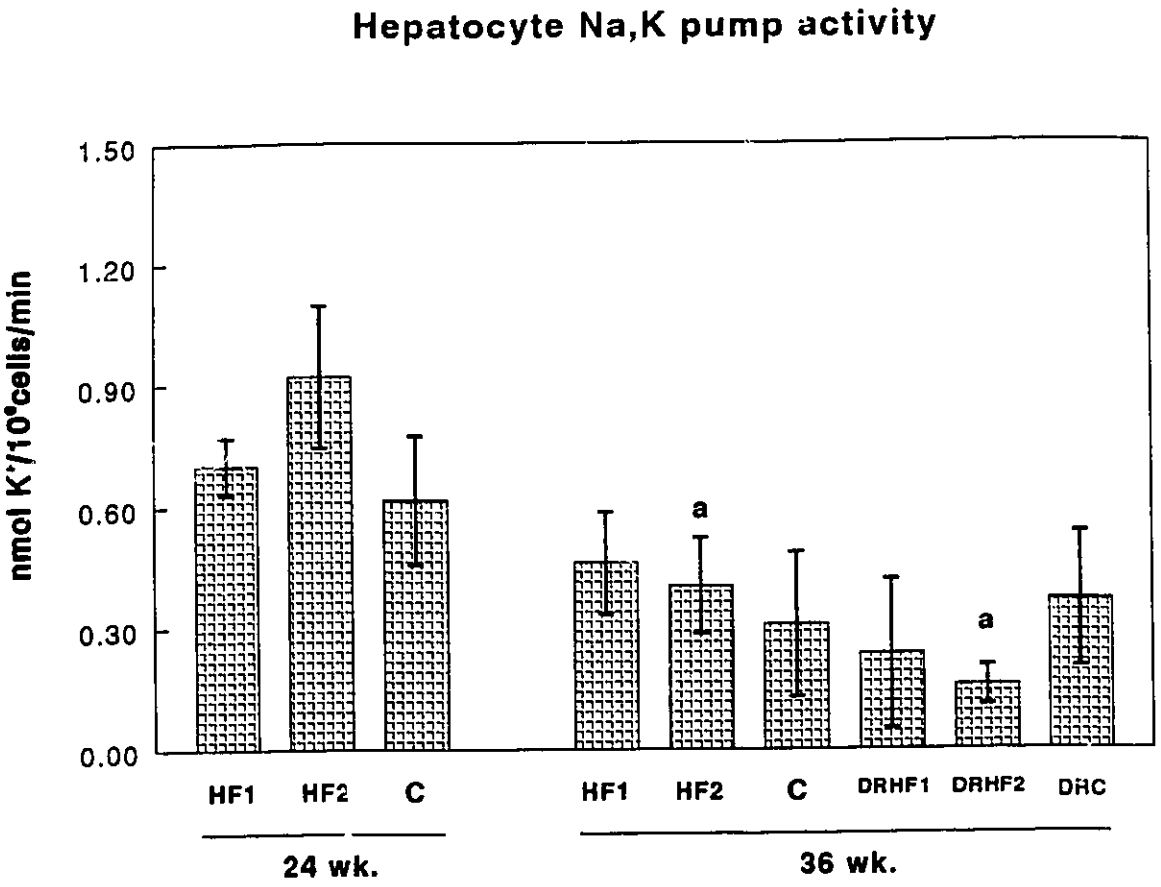

Figure 4.2.15: Hepatocyte Na^+,K^+ pump activity after 24 and 36 wk. of dietary treatments. The effects of 12 wk. of the dietary restriction regime are shown at the 36 wk. time point. The numbers of determinations per group are: 24 wk.: HF1, 7; HF2, 8; C, 7; 36 wk.: HF1, 7; HF2, 5; C, 6; DRHF1, 7; DRHF2, 5; DRC, 5. Each result is presented as a mean (±SEM); similar letters denote significant differences (at $P < 0.05$) between means at each time point. Statistical tests of significance involved: ANOVA with Newman-Keuls' post hoc tests for comparisons between the three diet groups and unpaired Student's t tests for comparisons of *ad libitum* fed vs. post dietary restriction data at each time point.

comparison of *ad libitum* fed versus post dietary restriction data at each time point.

Analyses of BAT and its activity:

In the following analyses of BAT the statistical tests for significant differences between groups of data involved: unpaired Student's t tests for comparisons of *ad libitum* fed and post dietary restriction data; ANOVA (completely randomized) which was followed by Tukey's post hoc tests for the comparisons between the three diet groups at each time point, and two-way ANOVA followed by Tukey's tests to determine the overall effects of dietary treatments upon the outcome variables when variation due to differences in time were controlled for.

1) **Interscapular BAT wet weight:** A two-way ANOVA which was followed by Tukey's post hoc tests revealed that there were significant overall effects of dietary treatments upon BAT wet weight when the variation among data sets due to differences in time was controlled for (data not shown). Weights were significantly greater ($P < 0.05$) in HF1 and HF2 groups as compared to those in C, DRHF1, DRHF2 or DRC groups. HF1 and HF2 weights were not significantly different.

2) **Total protein content:** BAT total protein content results (Figure 4.2.16) demonstrated increases in both HF1 and HF2 groups as compared to C group after 12 weeks of dietary treatments. However after 24 and 36 weeks of dietary treatments no significant differences between the three diet groups were observed. At the 24 week time point it is evident that dietary restriction resulted in decreases in total

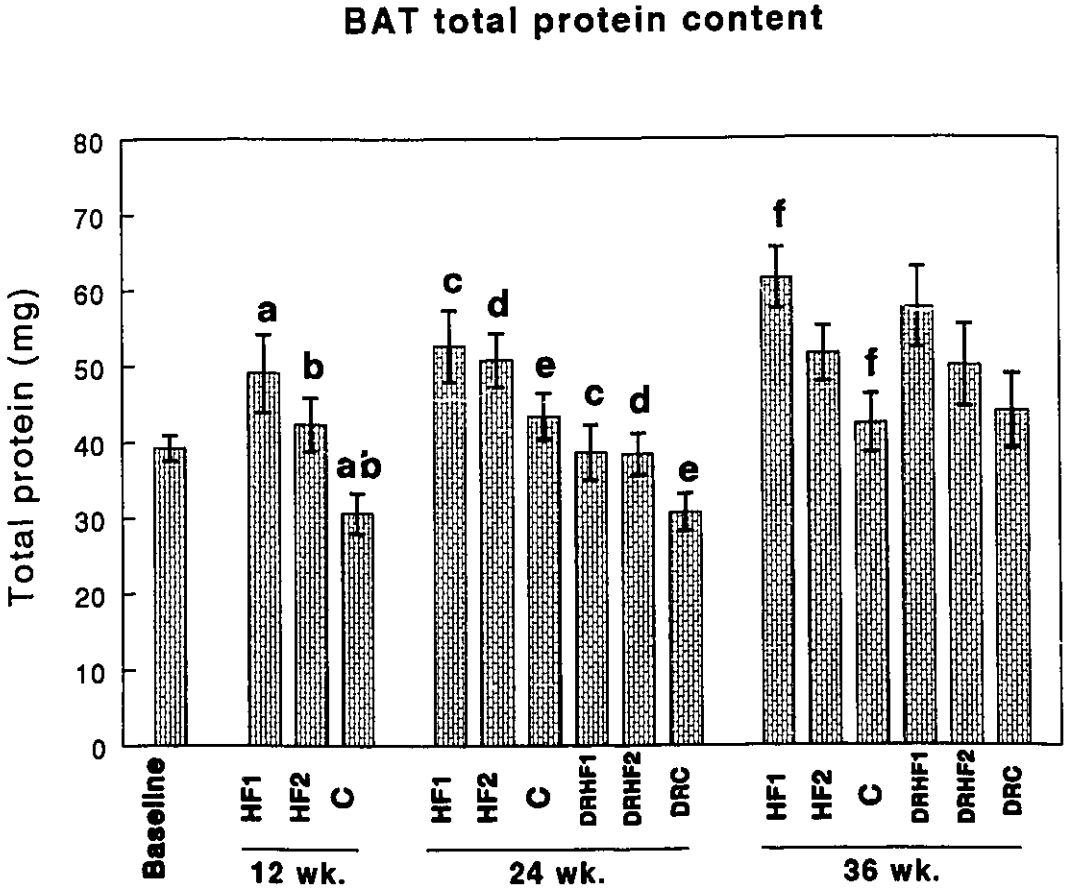

Figure 4.2.16: BAT total protein content at baseline and after 12, 24 and 36 wk. of dietary treatments. The effects of 12 wk. of the dietary restriction regime are shown at the 24 and 36 wk. time points. The numbers of determinations per group are: Baseline: 8; 12 wk.: HF1, 8; HF2, 8; C, 8; 24 wk.: HF1, 8; HF2, 8; C, 8; DRHF1, 8; DRHF2, 6; DRC, 8; 36 wk.: HF1, 8; HF2, 8; C, 9; DRHF1, 7; DRHF2, 5; DRC, 5. Each result is presented as a mean (±SEM); similar letters denote significant differences (at $P < 0.05$). Statistical tests of significance involved: ANOVA with Tukey's post hoc tests for comparisons between the three diet groups and unpaired Student's t tests for comparisons of *ad libitum* fed vs. post dietary restriction data at each time point.

protein content in all three diet groups. At the 36 week time point no effect of dietary restriction upon total protein content was observed.

A two-way ANOVA which was followed by Tukey's post hoc tests revealed that there were significant overall effects of dietary treatments upon BAT total protein content when the variation among data sets due to differences in time was controlled for. Total protein content was significantly greater ($P < 0.05$) in HF1 and HF2 groups as compared to those in C. BAT total protein contents of HF1 and HF2 groups were not significantly different.

3) **GDP binding:** The GDP binding results (Figure 4.2.17) demonstrated increased activity of the uncoupling protein in both HF1 and HF2 groups as compared to C group after 12 and 24 weeks of dietary treatments. At 36 weeks while GDP binding remained greater in HF1 group as compared to C group, there was no longer any difference between results from HF2 group as compared to C group. Following 12 weeks of dietary restriction the increases in GDP binding which were still observed at 24 weeks were eliminated by dietary restriction.

Results from a two-way ANOVA which was followed by Tukey's post hoc tests revealed that there were significant overall effects of dietary treatments upon GDP binding when the variation due to time was controlled for. GDP binding results were significantly greater ($P < 0.05$) in HF1 and HF2 groups as compared to those in C, DRHF1, DRHF2 or DRC groups. Results for groups HF1 and HF2 were not significantly different.

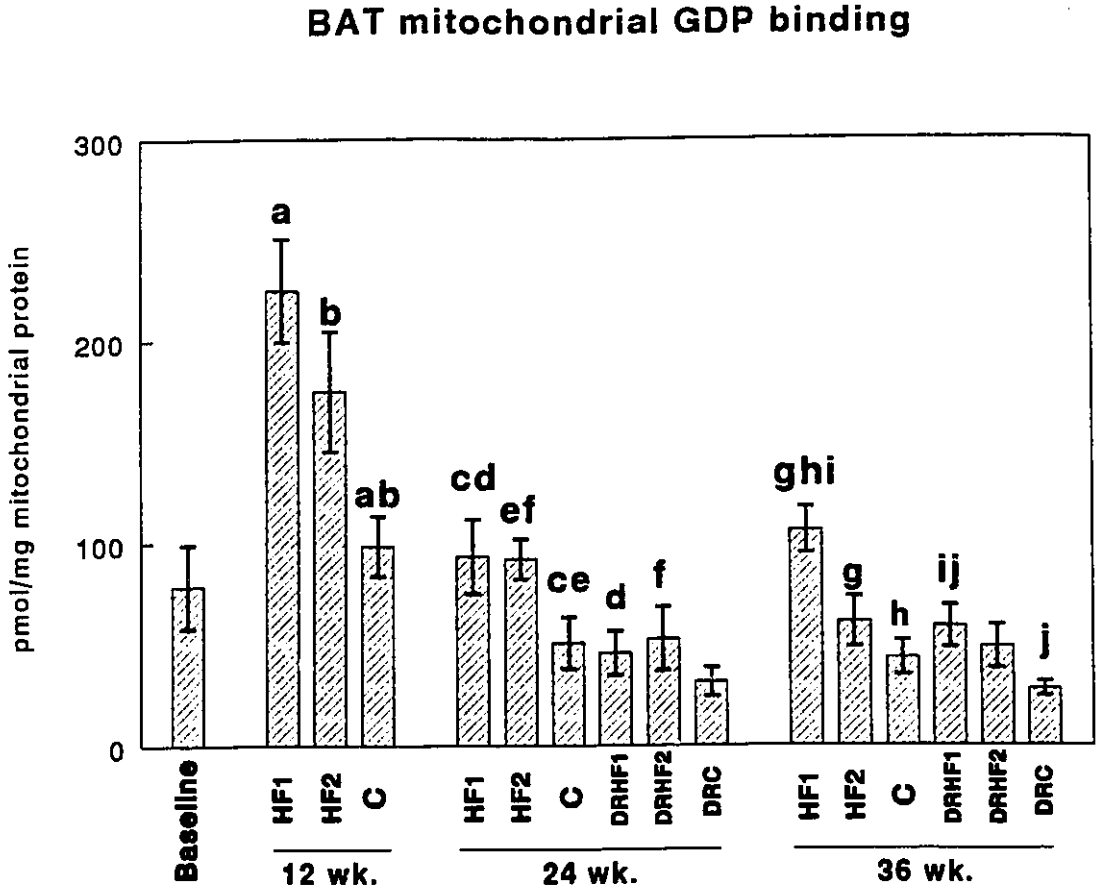

Figure 4.2.17: BAT mitochondrial GDP binding at baseline and after 12, 24 and 36 wk. of dietary treatments. The effects of 12 wk. of the dietary restriction regime are shown at the 24 and 36 wk. time points. The numbers of determinations per group are: Baseline: 8; 12 wk.: HF1, 8; HF2, 8; C, 8; 24 wk.: HF1, 8; HF2, 8; C, 8; DRHF1, 8; DRHF2, 6; DRC, 8; 36 wk.: HF1, 8; HF2, 8; C, 9; DRHF1, 7; DRHF2, 5; DRC, 5. Each result is presented as a mean (±SEM); similar letters denote significant differences (at $P < 0.05$). Statistical tests of significance involved: ANOVA with Tukey's post hoc tests for comparisons between the three diet groups and unpaired Student's t tests for comparisons of *ad libitum* fed vs. post dietary restriction data at each time point.

4) Cytochrome c oxidase activity: The results of the cytochrome c oxidase activities are presented in Figure 4.2.18. These data demonstrate increased mitochondrial contents in HF1 and HF2 groups as compared to C group following 12 weeks of dietary treatments. After 24 and 36 weeks of dietary treatments there were no differences between groups and no effects of dietary restriction.

5) Uncoupling protein content: The uncoupling protein content results (Figure 4.2.19) demonstrate greater BAT thermogenic capacity in groups HF1 and HF2 as compared to C following 12 weeks of dietary treatments. However, after 24 weeks these differences no longer existed. At 36 weeks UCP content in the HF1 groups was greater than either that in the HF2 or the C groups. A decrease in UCP content as a result of dietary restriction was evident only at the 36 week time point (HF1 - DRHF1).

Results from a two-way ANOVA which was followed by Tukey's post hoc tests revealed that there were significant overall effects of dietary treatments upon UCP content when the variation due to time was controlled for. UCP content results were significantly greater ($P < 0.05$) in the HF1 group as compared to those in C, DRHF2 or DRC groups.

6) DNA content: The results of the BAT total DNA content determinations (Figure 4.2.20) demonstrate no hyperplasia of the tissue in any of the diet groups following 12, 24 or 36 weeks of dietary treatments. There were no effects of dietary restriction. However, it must be remembered that DNA content provides only a rough index of

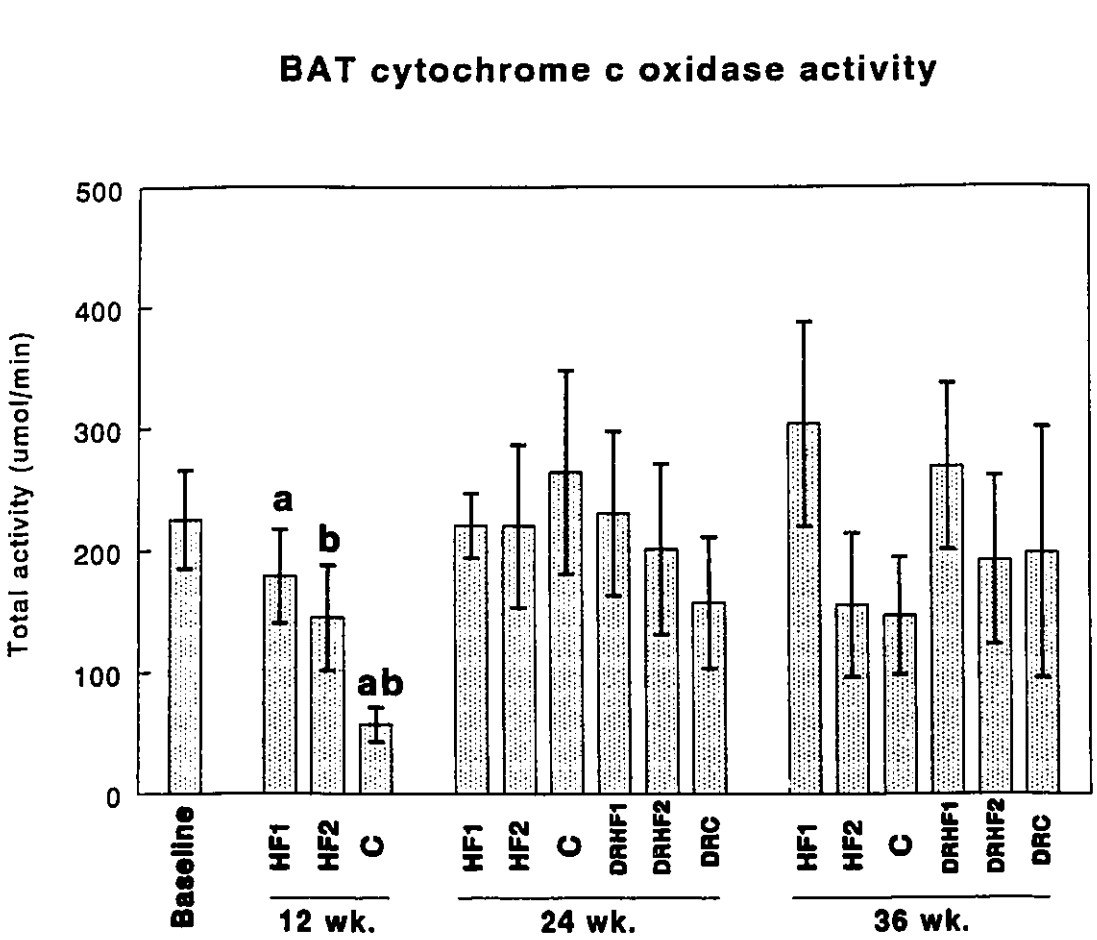

Figure 4.2.18: BAT cytochrome c oxidase activity at baseline and after 12, 24 and 36 wk. of dietary treatments. The effects of 12 wk. of the dietary restriction regime are shown at the 24 and 36 wk. time points. The numbers of determinations per group are: Baseline; 8; 12 wk.; HF1, 8; HF2, 8; C, 8; 24 wk.; HF1, 8; HF2, 8; C, 7; DRHF1, 7; DRHF2, 6; DRC, 8; 36 wk.; HF1, 8; HF2, 7; C, 9; DRHF1, 7; DRHF2, 4; DRC, 3. Each result is presented as a mean (±SEM); similar letters denote significant differences (at $P < 0.05$). Statistical tests of significance involved: ANOVA with Tukey's post hoc tests for comparisons between the three diet groups and unpaired Student's t tests for comparisons of *ad libitum* fed vs. post dietary restriction data at each time point.

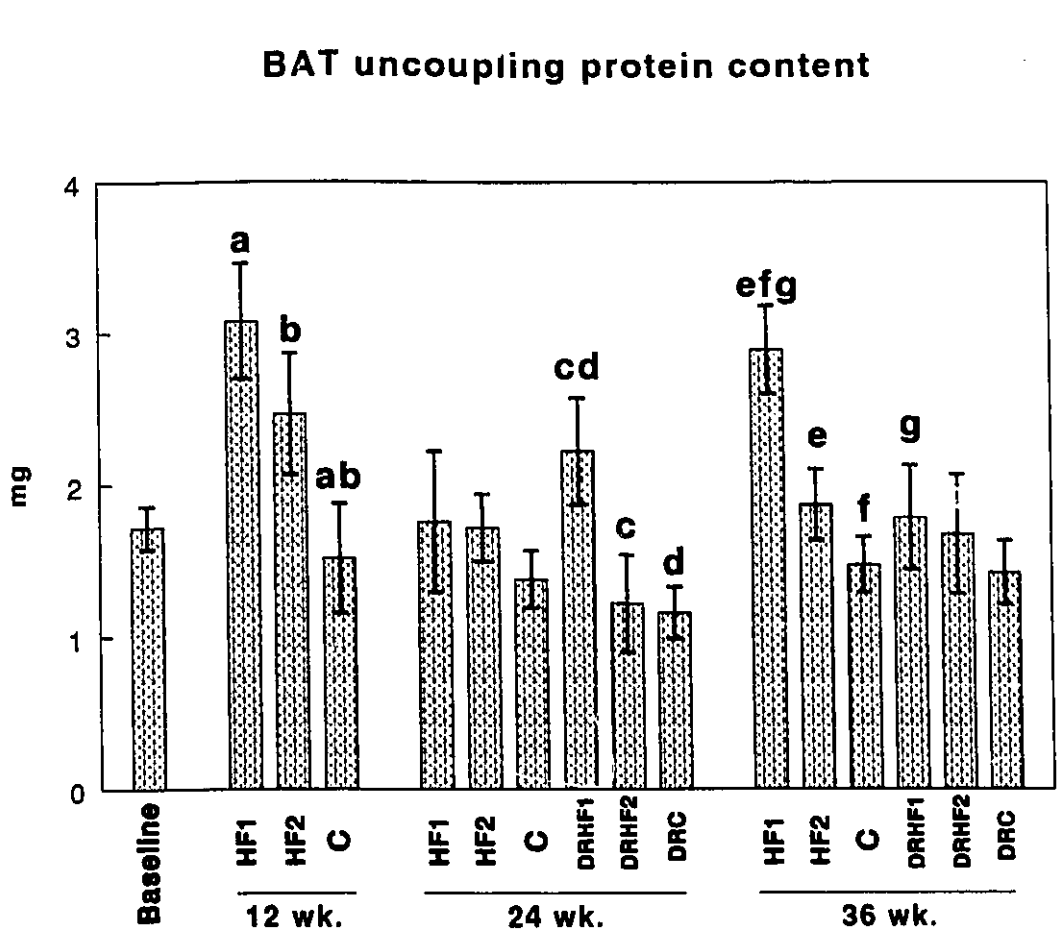

Figure 4.2.19: BAT uncoupling protein content at baseline and after 12, 24 and 36 wk. of dietary treatments. The effects of 12 wk. of the dietary restriction regime are shown at the 24 and 36 wk. time points. The results represent the average total amount of UCP as determined by RIA in the interscapular BAT. The numbers of determinations per group are: Baseline; 8; 12 wk.; HF1, 8; HF2, 8; C, 8; 24 wk.; HF1, 8; HF2, 8; C, 8; DRHF1, 8; DRHF2, 6; DRC, 7; 36 wk.; HF1, 8; HF2, 8; C, 9; DRHF1, 7; DRHF2, 5; DRC, 5. Each result is presented as a mean (±SEM); similar letters denote significant differences (at $P < 0.05$). Statistical tests of significance involved: ANOVA with Tukey's post hoc tests for comparisons between the three diet groups and unpaired Student's t tests for comparisons of *ad libitum* fed vs. post dietary restriction data at each time point.

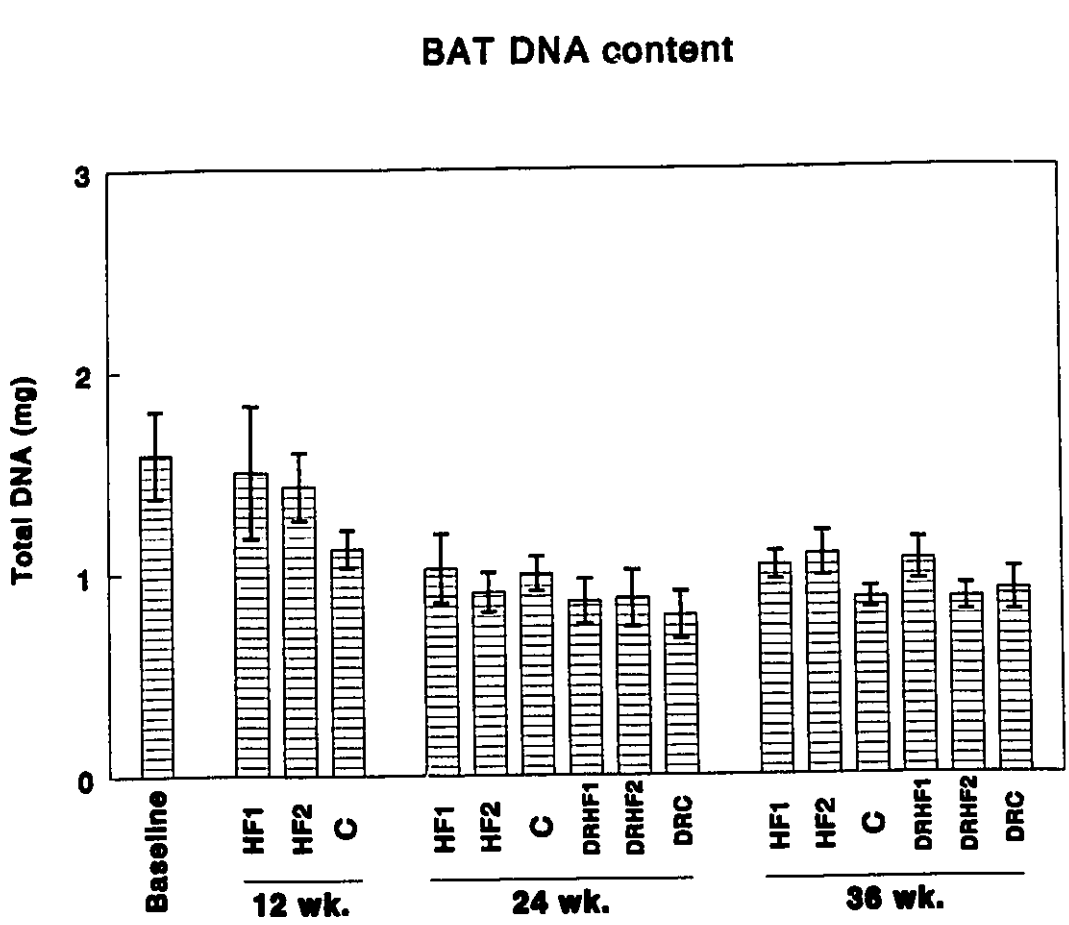

Figure 4.2.20: BAT total DNA content at baseline and after 12, 24 and 36 wk. of dietary treatments. The effects of 12 wk. of the dietary restriction regime are shown at the 24 and 36 wk. time points. The numbers of determinations per group are: Baseline; 8; 12 wk.; HF1, 8; HF2, 8; C, 8; 24 wk.; HF1, 8; HF2, 8; C, 7; DRHF1, 7; DRHF2, 6; DRC, 8; 36 wk.; HF1, 8; HF2, 7; C, 9; DRHF1, 7; DRHF2, 5; DRC, 5. Each result is presented as a mean (±SEM); similar letters denote significant differences (at $P < 0.05$). Statistical tests of significance involved: ANOVA with Tukey's post hoc tests for comparisons between the three diet groups and unpaired Student's t tests for comparisons of *ad libitum* fed vs. post dietary restriction data at each time point.

brown adipocyte cellularity in BAT as only about 40% of BAT cells are mature brown adipocytes (Bukowiecki *et al*, 1982). Moreover if preadipocytes differentiate into brown adipocytes tissue DNA may not change; therefore there could be increased brown adipocytes in the absence of detectable changes in tissue DNA.

Plasma T_3 and T_4 concentrations:

1) **Plasma T_3 concentrations:** The results of the T_3 assays (Figure 4.2.21) demonstrate effects of the two experimental diets and of dietary restriction. At 12 weeks plasma T_3 concentration was greater in HF1 than in either HF2 or C groups. Following 24 weeks of dietary treatments there no longer was any difference between HF1 and HF2 groups, but T_3 concentration was still greater in HF1 group than in C group. At 36 weeks there were no longer any differences between HF1, HF2 and C groups. Effects of dietary restriction were evident at the 24 week and 36 week time points. At 24 weeks dietary restriction resulted in decreases in T_3 concentration for the HF1 - DRHF1 and C - DRC groups. At 36 weeks decreases were evident in all diet groups.

Results from a two-way ANOVA which was followed by Tukey's post hoc tests revealed that there were significant overall effects of dietary treatments upon plasma T_3 concentrations when the variation due to time was controlled for. Plasma T_3 concentrations were significantly greater ($P < 0.05$) in the HF1 group as compared to those in C, DRHF1, DRHF2 or DRC groups. Although not statistically different from those in HF1, T_3 concentrations in the HF2 group were significantly greater ($P < 0.05$) than those in DRHF1, DRHF2 or DRC groups.

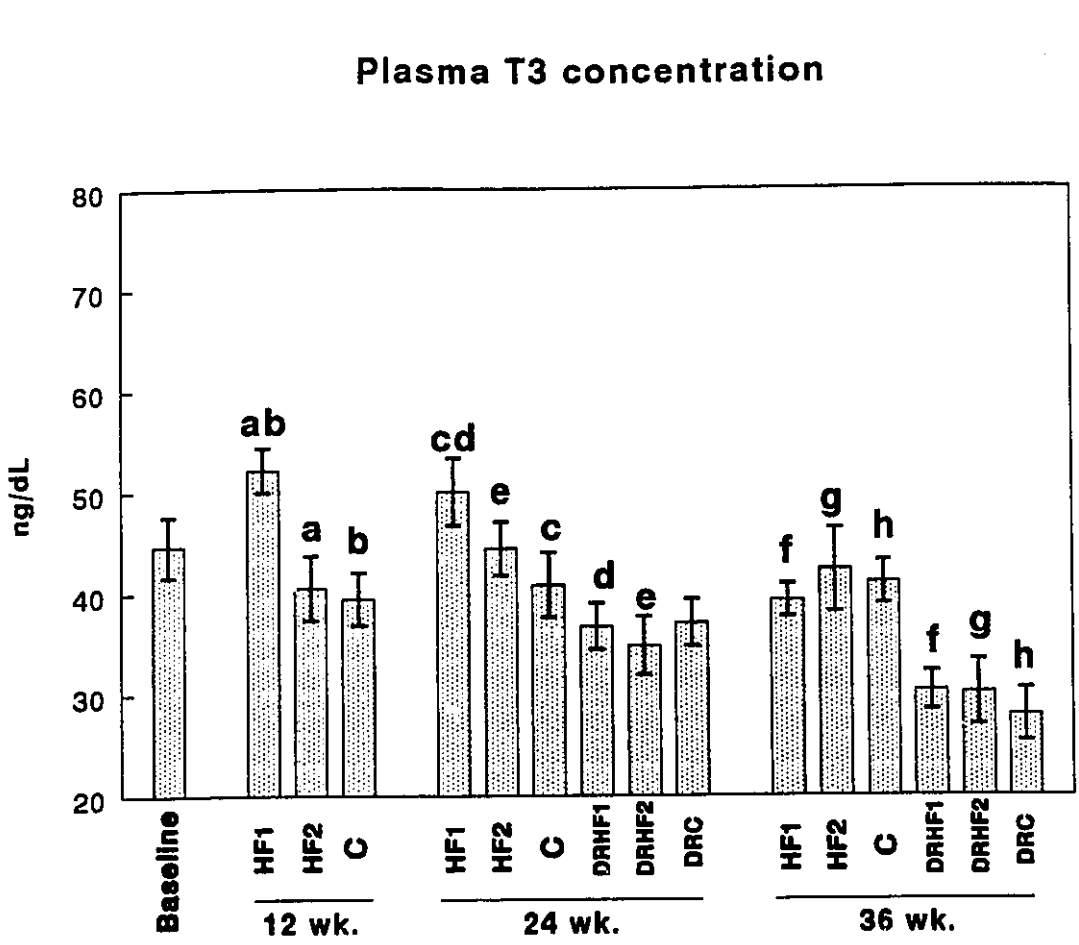

Figure 4.2.21: Plasma T_3 concentrations at baseline and after 12, 24 and 36 wk. of dietary treatments. The effects of 12 wk. of the dietary restriction regime are shown at the 24 and 36 wk. time points. The numbers of determinations per group are: Baseline: 8; 12 wk.: HF1, 8; HF2, 8; C, 8; 24 wk.: HF1, 8; HF2, 8; C, 8; DRHF1, 7; DRHF2, 6; DRC, 8; 36 wk.: HF1, 8; HF2, 8; C, 9; DRHF1, 8; DRHF2, 5; DRC, 5. Each result is presented as a mean (±SEM); similar letters denote significant differences (at $P < 0.05$). Statistical tests of significance involved: ANOVA with Tukey's post hoc tests for comparisons between the three diet groups and unpaired Student's t tests for comparisons of *ad libitum* fed vs. post dietary restriction data at each time point.

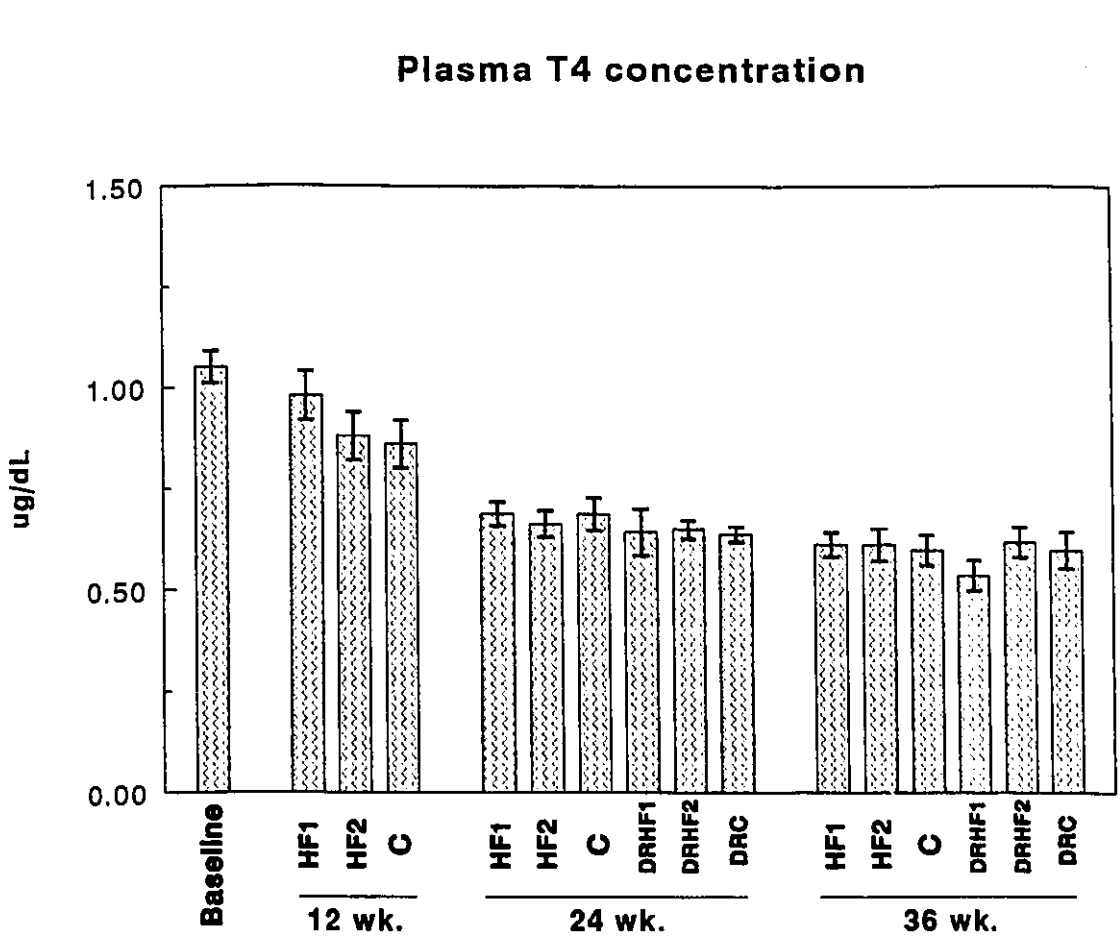

Figure 4.2.22: Plasma T_4 concentrations at baseline and after 12, 24 and 36 wk. of dietary treatments. The effects of 12 wk. of the dietary restriction regime are shown at the 24 and 36 wk. time points. The numbers of determinations per group are: Baseline: 8; 12 wk.: HF1, 8; HF2, 8; C, 8; 24 wk.: HF1, 8; HF2, 8; C, 8; DRHF1, 7; DRHF2, 6; DRC, 8; 36 wk.: HF1, 8; HF2, 8; C, 9; DRHF1, 8; DRHF2, 5; DRC, 5. Each result is presented as a mean (±SEM). A comparison of the means using ANOVA with Tukey's post hoc tests for comparisons between the three diet groups and unpaired Student's t tests for comparisons of *ad libitum* fed vs. post dietary restriction data showed no significant differences at $P < 0.05$.

2) **Plasma T_4 concentrations:** The plasma T_4 concentrations data (Figure 4.2.22) reveal no differences between diet groups and no effects of dietary restriction.

Discussion

1) Metabolic efficiency and the development of diet-induced obesity:

In both of the preceding studies of diet-induced obesity in laboratory rats it was found that the groups of rats which were fed the high saturated fat diets (HSF in DIO study 1 and HF1 and HF2 in DIO study 2) were *more* efficient in converting dietary energy into body weight when compared to control groups. In most instances this enhanced efficiency coincided with hyperphagia. In contrast to other rodent models of obesity where enhanced metabolic efficiency is due to deficient facultative thermogenesis (either in DIT in BAT or in thermoregulatory thermogenesis), the enhanced efficiency here is most likely due to the provision of a high fat diet. Previous studies have indeed shown that high fat diets produce a more efficient conversion of dietary energy into deposited fat than control diets (Wood and Reid, 1975; Mercer and Trayhurn, 1987). Some of the greater efficiency observed for rats fed the HSF diet would be due to the lower metabolic cost of storing dietary lipid in the form of adipose triacylglycerol as compared to the cost of converting dietary protein or carbohydrate into adipose triacylglycerol (Flatt, 1978; Donato and Hegsted, 1985). The estimated cost of storing dietary lipid as adipose tissue is about 1-2% of

the energy content of the food; this is much lower than the estimated cost of storing dietary carbohydrate as glycogen or fat which ranges between 6 and 20%, depending on the involved metabolic route (Himms-Hagen, 1990b).

Other studies have shown that saturated fats are more efficiently stored as adipose tissue than are polyunsaturated fats (Mercer and Trayhurn, 1987; Shimomura, Tamura and Suzuki, 1990). Mercer and Trayhurn (1987) found that both lean and obese mice had higher carcass energy gains when fed a beef tallow diet than when fed a corn oil diet despite isoenergetic intakes of the diets. Their results also showed that the diet rich in polyunsaturated fat appeared to result in the preferential stimulation of BAT thermogenic activity which would deter carcass energy gain. Shimomura *et al* (1990) found lower body fat accumulation in rats which were fed a safflower oil diet as compared to rats consuming isoenergetic amounts of a tallow diet. They attribute the differences to increased diet-induced thermogenesis and fat oxidation rates in the former diet-fed rats. Despite the fact that hyperphagia was observed when the high saturated fat diets were fed in studies 1 and 2, these findings concur with my results which show the rapid development of DIO in rats fed high saturated fat diets. Although dietary intakes in DIO study 1 were not isoenergetic, the finding that the diet which was moderately high in polyunsaturated fat resulted in no significant increases in adiposity is consistent with the results of these previous studies.

In a related study, Gong, Stern and Horwitz (1990) examined the effects of

eight days of high fat feeding (Crisco shortening and corn oil, 49 and 16% energy, respectively) on BAT GDP binding in lean (Fa/?) and obese (fa/fa) Zucker rats. Despite isoenergetic intakes of the high fat and low fat (control) diets, increased binding was observed in lean rats, but not in the obese rats. They conclude that the alterations appeared to reflect the amounts of dietary fat and carbohydrate and were independent of energy intake (Gong *et al*, 1990). The results of this and previous studies as well as the results described in this chapter, suggest that the type of dietary fat, the amount of dietary fat, the total energy intake, and the genotype of the species which is studied are important factors in the activation of diet-induced thermogenesis and the development of DIO.

2) Assessments of adiposity:

The development of obesity was observed in the high saturated fat diet-fed rats in both studies. The determinations of total body fat and lean body weights in the first study (Figure 4.1.4) showed that HSF rats were on average significantly fatter than MPF or C rats, while lean body weight remained unchanged. While these data supported the conclusions that we expected, much of the other data gathered using TOBEC techniques were clearly inaccurate (*eg.* total body fat determinations of rats following 12 weeks of dietary restriction in Figure 4.2.6).

Several investigators have encountered problems with data reproducibility and accuracy of serial determinations with the use of this methodology in small laboratory animals. It has been reported that although good correlations between TOBEC

measurements and carcass assessments can be obtained at a single time, the validity of serial measurements has been questioned, particularly during periods of weight change (Novak, Bell, Levitsky *et al*, 1991). Further studies on the use of the EM SCAN™ body composition analyser with revised LBM equations are currently being conducted and are being funded by EM SCAN™. Therefore, although it was hoped that the use of this new technology during long term diet studies would obviate the need to kill large numbers of animals in order to monitor changes in body composition, it is apparent that a suitable non-invasive method for serial determinations of body composition remains to be developed.

At each of the time points at which it was assessed, the correlation of carcass homogenate-determined total body fat weight with the sum of the weights of the epididymal and perirenal fat pads (Figures 4.2.8 - 4.2.10) was much better than the correlation of the latter with the TOBEC-determined total body fat weight ($r = 0.675$; Figure 4.2.7). Therefore if adiposity needs to be assessed only at the time that the rats are killed, then weighing fat depots is a much less expensive and time consuming procedure than either carcass analysis or TOBEC.

Similar to my results are the recent results of Newby, DiGirolamo, Cotsonis *et al*, (1990). Newby *et al* found a strong linear correlation between the weight of male Wistar rat retroperitoneal (*ie.* perirenal) fat pads and the total body lipid weight ($r = 0.967$). They concluded that total body fat weight can be estimated with an approximate variation of ±3-4% by simply weighing the retroperitoneal fat pads.

3) Na^+,K^+ pump activity:

a) Erythrocyte Na^+,K^+ pump activity: In both of the studies included in this chapter, Na^+,K^+ pump activities in erythrocytes from the rats which became obese (*ie.* HSF group in study 1 and HF1 and HF2 in study 2) were not significantly different from control determinations. These results support the findings of Davis and Bernardis (1984) who found no changes in Na^+,K^+ ATPase activity in erythrocyte membrane preparations from male Sprague Dawley rats which were fed cafeteria diets. The results are however in contrast with their findings for female rats; Davis and Bernardis (1984) found increased erythrocyte Na^+,K^+ ATPase activity which was correlated with total body adiposity. To my knowledge there are no published results on the effect, or lack of effect, of defined high fat diets on Na^+,K^+ pump activity in intact erythrocytes. My results in this cell type therefore support neither the hypothesis that lower Na^+,K^+ pump activity may contribute to the development of obesity or the hypothesis that increased Na^+,K^+ pump activity may act as an energy buffer in the attenuation of obesity development. However, as discussed earlier, erythrocyte results sometimes do not reflect the results obtained from other cell types. Moreover because the data are expressed in terms of cell volume little or no change in the "specific activity" could be observed while in actuality a change in total Na^+,K^+ pump activity could occur if there was an increase in the total number of erythrocytes.

Similar to the rat erythrocyte Na^+,K^+ transport results of Zhao and Willis (1988) who observed decreased Na^+,K^+ pump activity following 7 - 14 days of starvation, I also found decreased Na^+,K^+ pump-mediated K^+ influx following 12 weeks of dietary restriction in two of the three diet groups in DIO study 2. Decreased Na^+,K^+ pump activity would be in keeping with the hypothesis that Na^+,K^+ pump

activity increases and decreases when dietary energy is abundant and scarce, respectively.

b) Thymocyte Na^+,K^+ pump activity: The results of DIO study 1 demonstrate that while thymocyte Na^+,K^+ pump activity in the HSF diet group was not significantly different from that in the control diet group, it was significantly greater than the activity in the MPF diet group. This greater activity is directionally opposite to changes in activity that potentially could contribute to the increased metabolic efficiency and development of DIO observed in this group. These results are more consistent with the idea that Na^+,K^+ pump activity is stimulated during the development of DIO.

In the first study thymocyte total K^+ influx decreased in all groups during the nine week feeding period. Approximately 94% of the decrease in total K^+ influx apparently was due to the decrease in ouabain-sensitive K^+ influx while the remainder was due to a lower residual K^+ influx. No changes were observed in bumetanide-sensitive K^+ influx. In the second study, there were temporal decreases in each of the K^+ influx pathways which were studied. I am unaware of reasons for these temporal decreases in thymocyte K^+ influx. There are however many age-related changes in thymic physiology including maturation and differentiation of the various T cell subsets, and thymic atrophy (Schulof, Naylor, Sztein *et al*, 1987; Robson-MacDonald and Lees, 1990). While only speculative, it is possible that there are age-related changes in the expression of Na^+,K^+ pump isoforms; it could be that

the expression and synthesis of Na^+,K^+ pumps or of the highly ouabain-sensitive isoform(s) of the Na^+,K^+ pump decreases with age.

In the second study, as compared to the control data, there were significant increases in Na^+,K^+ pump activity in thymocytes from both groups which were fed the high saturated fat diets. However there seem to be differences between the two high fat diet groups in the time over which the increases occur; at 12 weeks pump activity was higher in the group which was fed the lard-based diet than in the other two diet groups, but at 24 weeks pump activity was higher in the group which was fed the tallow-based diet than in the other two diet groups. In this study, similar to the first study, the direction of the observed changes in Na^+,K^+ pump activity is consistent with the idea that activity is stimulated, at least in the early stages, of high saturated fat diet-induced obesity.

The mechanisms underlying the stimulation of thymocyte Na^+,K^+ pump activity by a high saturated fat diet are unknown, and could involve local, humoral or neural factors. As the stimulation was observed only in thymocytes which, unlike erythrocytes, are innervated by sensory and sympathetic neurons (Weihe, Müller, Fink *et al*, 1989), neural stimulation may be involved. Previous studies have shown that a cafeteria diet will stimulate sympathetic nervous system dependent Na^+,K^+ ATPase activity in rat BAT and soleus muscle (Swann, 1984).

It is also possible that no effect is observed in erythrocytes because, unlike the thymocytes, they do not possess the cellular "machinery" to respond to a humoral

stimulus. For example, if thyroid hormones were responsible for the increased Na^+,K^+ pump activity observed in the thymocytes, erythrocyte Na^+,K^+ pump activity would be unlikely to respond similarly as these cells are anucleated and therefore have no nuclear thyroid hormone receptors to respond to the humoral stimulus.

Potential local factors might include the effects of altered plasma membrane lipid composition; changes in membrane composition can be achieved through alterations in dietary lipid which can alter the activities of enzymes associated with cellular membranes (Wahle, 1983; Innis and Clandinin, 1981; Neelands and Clandinin, 1983). Murray and Patrick (1986) found that thymocytes of Sprague Dawley rats fed four weeks on a diet which was high in polyunsaturated fat (corn oil; 40% total energy) had lower Na^+,K^+ pump-mediated Na^+ efflux rate constants than those from rats fed a high saturated fat diet (coconut oil; 40% total energy). There was no dietary control group in this study. The results of my first study are thus very similar to those of Murray and Patrick (1986) in that Na^+,K^+ pump activity rates were highest in HSF diet- followed by control diet- and MPF diet- fed rats. The studies described in this chapter are, to my knowledge, the first controlled studies involving defined diets which demonstrate diet-induced increases in Na^+,K^+ pump activity in intact nucleated cells.

c) Hepatocyte Na^+,K^+ pump activity: As a result of the high variability in the hepatocyte Na^+,K^+ transport determinations, few significant differences were observed between the groups of rats studied. The only significant difference in

Na^+,K^+ pump activity was a decrease observed following dietary restriction of rats which had been fed the HF2 diet. As mentioned in the results section, the overall pattern of the hepatocyte Na^+,K^+ pump data (Figure 4.2.15) is similar to that for thymocyte Na^+,K^+ pump data (Figure 4.2.14) and the statistical analysis of correlation coefficients for the hepatocyte and thymocyte Na^+,K^+ pump data showed that hepatocyte and thymocyte Na^+,K^+ pump data are significantly correlated in diet groups HF1 and HF2 at the 24 week time point. Thus it seems possible that if the hepatocyte ion flux techniques could be refined so as to reduce this variation within groups then differences between groups similar to those observed using thymocytes might be detected. It might be possible to reduce the variation within groups by using a larger total number of cells per ion flux determination for each rat, similar to the techniques of Nobes, Lakin-Thomas and Brand (1989). Moreover the use of [^{3}H]ouabain binding techniques would determine whether or not there was an increased number of Na^+,K^+ pumps per cell in the high saturated fat diet-fed rats.

4) BAT activity and growth:

a) BAT GDP binding: The measurement of GDP binding, which provides an indication of uncoupling protein activity and therefore an indication of the thermogenic activity of the tissue, was conducted only in the second study. The results (Figure 4.2.17) suggest that uncoupling protein activity is stimulated in both high saturated fat diet groups at least during the first 24 weeks of dietary treatments. At

36 weeks GDP binding is greater in the lard-based diet group as compared to both the tallow-based diet group and the controls. However, in view of the generally unchanged mitochondrial content (cytochrome c oxidase activity; Figure 4.2.18) and increased UCP content (Figure 4.2.19), the results may reflect an increased concentration of UCP in mitochondria rather than increased thermogenic activity, specifically.

These findings are in agreement with the findings of others which have shown that diets which are moderately high in fat will, at least initially, increase BAT thermogenesis (Hogan, Sullivan and Triscari, 1985a; 1985b) and sympathetic nervous system activity (Levin, Triscari, Hogan *et al*, 1987; Levin, Triscari and Sullivan, 1983a; 1986).

In the previous studies conducted by Levin and colleagues it was found that once the DIO is established following approximately 12 weeks of the consumption of a moderately high fat diet (15.8% of the total energy content as fat - mainly as corn oil and bovine milk fat) BAT tends to atrophy and to have lower sympathetic nervous system activity (Levin *et al*, 1983a,b, 1985). It is unclear why GDP binding remains higher in the lard-based diet group and not in the tallow-based diet group after 36 weeks of treatments. The obesity in these rats fed the high saturated fat diets develops rapidly, and these GDP binding data show that BAT can still be acutely activated by the diet even after 36 weeks of a high fat (lard) diet.

Consistent with the idea that BAT thermogenesis acts as an energy buffer, my

results show that any dietary activation of BAT GDP binding is reversed to the level of the controls following dietary restriction.

b) Cytochrome c oxidase activity: Drawing conclusions from the cytochrome c oxidase activity results is difficult. In the first study, the results showed no significant differences between the HSF, MPF and C groups after nine weeks of dietary treatments. In the second study the only significant differences between diet groups were observed following a slightly longer dietary treatment period of 12 weeks. At this time point cytochrome c oxidase activity is greater in the groups fed either of the high saturated fat diets as compared to the data from the control group. At 24 weeks, similar to the results of uncoupling protein content, any differences between the three diet groups have disappeared. At 36 weeks, again similar to the uncoupling protein results, cytochrome c oxidase activity appears to be increased in the lard-based diet group as compared to the data from the other two diet groups (although for the cytochrome c oxidase data this difference does not reach statistical significance). The high amount of variation in results within groups precludes any conclusions based on the data gathered at the 24 week and 36 week time points. It appears that there is no effect of dietary restriction on cytochrome c oxidase activity and therefore on mitochondrial content.

c) Uncoupling protein (UCP) content: The uncoupling protein content results reveal that the consumption of a high saturated fat diet increases, at least initially, the thermogenic capacity of interscapular BAT. The data from both studies support this

conclusion. In the second study, similar to the GDP binding results, it was found that, as compared to the control data, even after 36 weeks of dietary treatments UCP content remains higher in the lard-based diet group and but not in the tallow-based diet group. The reasons for the absence of any differences between the three diet groups after 24 weeks of dietary treatments are unknown. Again similar to the GDP binding results, any dietary-induced increases in UCP content are reversed to the level of the controls following dietary restriction.

In view of the finding of no dietary-induced alterations in mitochondrial content in the first study and at the 24 and 36 week time points in the second study the observed changes in UCP content indicate a selective increase in UCP content and not just in the proliferation of mitochondria.

The finding of dietary-induced increases in UCP content in the rats which were fed the high saturated fat diets is in keeping with the postulated energy buffering role for BAT thermogenesis during the development of DIO. The specific mechanisms underlying these diet-induced changes are unknown. However, it is likely that at least some of the observed increases were due to diet-induced increases in activity of the sympathetic nervous system. Increased sympathetic activity has been observed during the development of DIO in young SD rats fed a moderately high fat diet (15.8% of the total energy content as fat - mainly corn oil and bovine milk fat) (Levin, Triscari and Sullivan, 1986). As earlier mentioned, Swann (1984) demonstrated that a "cafeteria" diet induced Na^+,K^+ ATPase activity in rat brown

adipose tissue and soleus muscle; this activation depended upon ß-noradrenergic receptor stimulation (Swann, 1984). However, further studies are needed to more clearly identify the mechanisms possibly underlying the changes observed in the DIO observed in the studies described in this chapter.

d) BAT total protein content: As the measurement of total protein content provides a general estimate of BAT growth and atrophy, the data from both studies indicate that feeding a high saturated fat diet induces tissue growth. In the first study this induction occurred over a nine week feeding period, and in the second study the increase occurred over the first 12 week period. The results from the second study at the 24 week and 36 week time points indicated that, while there was no atrophy of BAT in the rats fed the high saturated fat diets, there were no longer any differences in the total amount of BAT protein between the three diet groups. At the 24 week time point it is evident that 12 weeks of dietary restriction resulted in atrophy of BAT; at 36 weeks, however, it appears that BAT did not respond through atrophy to dietary restriction as might be expected. This lack of response after 24 - 36 weeks of dietary treatments may in some way be related to the decreased density of ß-adrenergic receptors and the lower response of adenylate cyclase to catecholamines in older rats (Scarpace, Mooradian and Morley, 1988). However the rats used in this study (Scarpace *et al*, 1988) were older (24 mo.) than those used in my studies. The rats were also of a different strain (Fisher 344) and fed a chow diet.

e) BAT DNA content: The DNA content results of both the first and second studies

involving the consumption of a high saturated fat diet for nine weeks and 12 weeks, respectively, showed that there were no differences in BAT cellularity between the high saturated fat diet groups and the control groups. Similarly after 24 weeks and 36 weeks of dietary treatments in the second study there were no distinguishable differences in BAT cellularity between the three diet groups. There were no effects of dietary restriction.

5) Plasma T_3 and T_4 concentrations:

Effects of the high fat diets and of dietary restriction upon plasma T_3 concentration were observed in the second study. In general the changes which were observed are consistent with the idea of the occurrence of metabolic adaptation in states of dietary energy surfeit and deficit. That is, significant increases in plasma concentrations were detected only in the hyperphagic, high fat diet-fed groups of rats, and dietary restriction resulted in significant decreases in serum thyroid hormone concentrations in all instances except one.

Thyroid hormones have long been known to influence thermogenesis in humans and animals. However, the changes in plasma T_3 levels (Figure 4.2.21; 24 week values) were apparently insufficient to significantly alter resting metabolic rate (compare with 4.2.4) in the *ad libitum*-fed rats. The decreases in T_3 in the diet restricted groups do however appear to correlate with decreases in the resting metabolic rate.

As discussed in Chapter 1 thyroid hormones are known to be involved directly

in the stimulation of Na^+,K^+ pump activity and indirectly in the stimulation of BAT thermogenesis. However the role of thyroid hormones in Na^+,K^+ pump activity or BAT thermogenesis in DIO in laboratory rats has not as yet been studied. The present results are the first reported that demonstrate high fat diet-induced increases in plasma T_3; it has been reported that increases with overfeeding are found with carbohydrate and mixed-diet overfeeding but not with dietary fat (Norgan, 1990; Danforth, 1989).

In a recent study by Katzeff (1990) the effects of aging upon the thyroid hormone response to mixed-calorie overfeeding were examined in Sprague Dawley rats. Prior to this study it was unknown whether, as compared to younger rats, older rats had similar responses in thyroid hormone metabolism to mixed-calorie overfeeding. The diet used to induce overfeeding was chow which was supplemented with a cafeteria diet; although the total energy consumption is reported, the proportion of dietary energy from the cafeteria diet is not. The distribution of energy from protein, carbohydrate and fat is not reported. The effects of 28 days of overfeeding were studied in 4 and 14 month old rats; as compared to the chow fed controls, the rats fed the mixed diet were hyperphagic at both time points.

Similar to my results, Katzeff found that overfeeding had no effect on serum T_4 concentrations. Serum T_3 concentrations however were increased 24% by overfeeding in the four month old rats. My results were similar; at approximately 4.5 months of age (*ie.* 12 weeks of dietary treatment), plasma T_3 concentrations were

increased 30% in the hyperphagic rats fed the lard-based high fat diet. Why increased plasma T_3 concentrations were not observed in the hyperphagic rats fed the tallow-based diet is unclear.

Katzeff found that overfeeding at 14 months of age resulted in no increases in T_3; at this time point the mixed-diet-fed rats were still hyperphagic. The average daily energy intake of the 14 month old rats (both cafeteria and chow groups) in Katzeff's study was however decreased significantly from the four month values. My results showed that after 36 weeks (or nine months) of overfeeding plasma T_3 concentrations were the same in the three diet groups and the high fat diet-fed rats were still hyperphagic. Katzeff theorizes that the observed changes in T_3 clearance and production rates, which in turn alter the serum T_3 concentrations, are at least partially due to the decreased energy intakes of the rats. My results at both the 12 and 24 week time points, however, show no difference in daily energy intake between the two high fat diet fed groups, and yet plasma T_3 concentrations were greater in HF1 as compared to HF2. Moreover, my daily energy intake data show that over the 32 weeks that intake was analysed (Figure 4.2.2) there were no significant decreases in energy intake over time, as observed by Katzeff. My data thus do not support Katzeff's theory and suggest that lard- and tallow- based diets have different effects on plasma T_3 concentrations. It may be that hyperphagia above a certain "threshold value" is necessary for diet-induced changes in plasma T_3 concentrations, but that effects of other factors can override the effects of hyperphagia.

6) Dietary fat and BAT activity:

My finding of high saturated fat diet-induced activation of BAT but no activation of BAT in rats which were fed a moderately high polyunsaturated fat diet is opposite to the findings of Mercer and Trayhurn (1987). It is also opposite to what one might expect from other results in the literature which support the idea that dietary polyunsaturated fat is more effective in the stimulation of BAT activity (Vander Tuig and Romsos, 1984; Laury, Goubern, Razanoelina *et al*, 1988). For sympathetic stimulation and BAT growth it may be that overeating a high fat diet is more important than the diet's composition. Hyperphagia was not observed in any of these previous studies. It has been shown that even a high fat cafeteria diet may not activate BAT when the rats do not overeat (Rothwell, Stock and Warwick, 1985).

The degree of obesity induced by high fat diets and the extent to which the diet activates BAT depends on many factors including: strain of rat (Levin *et al*, 1986; Fisler *et al*, 1987), length of dietary treatment (Hill *et al*, 1989), composition of dietary fat (Mercer and Trayhurn, 1987), and the extent of the hyperphagia (Rothwell *et al*, 1985). These factors, as well as differing end points used for measuring BAT response, have made it difficult to generate theories about the effects of dietary fat on BAT thermogenesis.

My results, moreover, indicate that the effects of the high lard and tallow diets upon both Na^+,K^+ transport and BAT activity are different. The dietary effects of these saturated fats upon either Na^+,K^+ transport or BAT activity had not been

compared prior to these studies. The reasons underlying the differences between these two saturated fats, in terms of the activation of Na^+,K^+ pump and BAT activities, are unknown and warrant further study.

In conclusion, the results from these two studies in which obesity in a large number of Sprague Dawley rats was induced using defined high saturated fat diets do not lend support to the hypothesis that decreased energy expenditure attributable to Na^+,K^+ pump activity can contribute to the development of some forms of obesity. Na^+,K^+ pump activity was not lower in erythrocytes, thymocytes or hepatocytes of the obese high fat diet-fed rats. The increases in thymocyte Na^+,K^+ pump activity with high fat diet-feeding are directionally opposite to changes that might contribute to the increased metabolic efficiency and development of obesity observed in these animals. Similarly the increased BAT activity and growth would counter metabolic efficiency and the development of DIO. Both mechanisms, Na^+,K^+ pump activity and BAT thermogenesis, are stimulated and therefore both mechanisms could potentially contribute as energy buffers in the prevention of DIO. However, despite their activation these mechanisms did not produce detectable changes in RMR and were insufficient in the prevention of DIO in these high saturated fat diet-fed rats.

Chapter 5: General Discussion

The Na^+,K^+ pump and metabolic adaptation Until there is a method of directly determining the proportion of total cellular energy expenditure attributable to Na^+,K^+ pump activity that does not depend on the use of the inhibitor ouabain, with its other non-specific and secondary effects, the accurate experimental determination of this value is not possible. However theoretical estimates can be derived through the use of Na^+,K^+ pump rate data and the estimate of one ATP molecule expended per transport of 3 molecules of Na^+ or 2 molecules of K^+. When such an approach is taken the estimated energy required to fuel Na^+,K^+ pump activity throughout the body does amount to a significant proportion of whole body energy expenditure. A similar type of approach was taken by Gill *et al* (1989); however, their estimation of whole-body heat production that is attributable to Na^+,K^+ ATPase activity in young sheep was predominantly based on proportions of ouabain-inhibitable tissue respiration. Using this approach Gill *et al* (1989) estimate that Na^+,K^+ ATPase accounts for 18%-23% of whole-body ATP use.

In addition, if the estimate of the cost of weight gain of approximately 20kJ (5 kcal) per gram of lean tissue and 40 kJ (10 kcal) per gram of adipose tissue is used (Blaxter, 1989), then even a small change in energy expenditure, which may, for example correspond to a few percent of one's daily energy intake, could, over the span of several years, lead to, or discourage, the development of moderate obesity.

Therefore a small change in Na^+,K^+ pump activity occurring throughout many of the various cell types of the body could potentially contribute to overall energy expenditure and therefore energy balance.

Energy expenditure by the Na^+,K^+ pump is a function of the transport rate and therefore can only change if there is also a change in sodium influx. If a cell is to maintain normal intracellular cation concentrations and cell volume, Na^+ and K^+ influxes must be counterbalanced by effluxes. Therefore a change in Na^+,K^+ pump-mediated ion transport could either be due to an increased "downhill" movement of Na^+ into cells (increased substrate for Na^+,K^+ pump activity) or to an increased concentration of active Na^+,K^+ pumps per cell. The former could be mediated through control mechanisms such as those occurring through the action of catecholamines, or insulin, while the latter could occur through the actions of steroid and thyroid hormones upon Na^+,K^+ ATPase gene transcription and translation.

The results of the earlier studies of Na^+,K^+ pump activity in blood cells of undernourished (marasmic) children, and in erythrocytes of starved rats support the "metabolic adaptation" idea in that decreased Na^+,K^+ pump activity coincides with decreased energy resources and increased Na^+,K^+ pump activity coincides with increased energy resources (*ie.* during nutritional rehabilitation). The results from my studies with undernourished cerebral palsied children do not support this idea; erythrocyte Na^+,K^+ pump activity was found to be higher in the undernourished children with CP as compared to well nourished children with CP and to control

children. However, as discussed in Chapter 3 involuntary muscular activity, seizures, seizure medication, and diet composition may have affected the results.

The results from my studies with diet-induced obesity in rats provide indirect support for the idea that altered Na^+,K^+ pump activity can contribute to metabolic adaptation. When Na^+,K^+ pump activity was altered through dietary alterations, it was increased during overfeeding and decreased during underfeeding. The fact that thymocyte Na^+,K^+ pump activity increased during overfeeding with a high fat (lard or tallow) diet while erythrocyte Na^+,K^+ pump activity was unchanged may provide some information as to the mechanisms involved in the observed changes in Na^+,K^+ pump activity. For example, it may be that the altered Na^+,K^+ pump activity involved increased cellular synthesis of pump molecules and alterations would therefore only be observed in nucleated cells. The diet-induced changes in T_3 would be in agreement with this type of control mechanism.

If an overall comparison is made of Na^+,K^+ pump results between the studies included in this thesis some incongruities are observed. The results from erythrocytes of children with CP during undernutrition and rehabilitation do not support the hypothesis that the Na^+,K^+ pump acts as an energy buffer. However, in rat erythrocytes, consistent with the hypothesis, Na^+,K^+ pump activity decreased during dietary restriction (DIO study 2). Moreover, in DIO study 1, while differences in Na^+,K^+ pump activity were observed in thymocytes between high saturated fat diet-fed rats and moderately high polyunsaturated fat diet-fed rats, no differences in

activity were observed in erythrocytes. Overall these results reveal the danger in drawing conclusions based on the results from one cell type about changes in Na^+,K^+ pump activity as they relate to whole-body energy expenditure.

BAT thermogenesis and metabolic adaptation

In laboratory rodents BAT is well known as having important functions in both thermal balance and energy balance. When BAT thermogenesis is defective, the development of obesity usually results. States of altered energy balance in laboratory animals, such as the genetic obesity observed in ob/ob mice or the leanness which is associated with hypothalamic lesions, are usually associated with altered thermogenic capacity of BAT (Himms-Hagen, 1990a). However the estimation of total energy expenditure during states of altered energy balance which is attributable to altered BAT thermogenesis has as yet not been possible. Despite the fact that several studies have shown diet-induced BAT hypertrophy and activation, the overall quantitative relevance of BAT thermogenesis to overall energy balance and obesity development has been questioned (Ma and Foster, 1989). It is only in cold-acclimated rats in which food intake and total energy expenditure are both greatly increased that BAT thermogenesis has been identified as a significant contributor to overall energy expenditure (Himms-Hagen, 1986; Foster, 1986). However, even a small change in overall energy expenditure resulting from altered BAT activity could potentially encourage or discourage the development of obesity.

The results of my studies of BAT activity and growth during diet-induced obesity in rats demonstrate marked increases in both BAT activity and growth during the excessive consumption of a high saturated fat diet. These results are in agreement with the hypothesis that BAT acts as an energy buffer during states of energy surfeit and deficit but they do not allow any quantitative estimate of its importance. My results show that lard- and the tallow- based diets have varied effects upon BAT activity; the lard-based diet appears to have been more effective in the induction of BAT activity. The reasons for the differences between the lard- and the tallow- based diets are unknown, but speculatively may involve the slight differences in the fatty acid compositions of lard and tallow (refer to Appendix 1).

Conclusion

The research described in this thesis involved the study of Na^+,K^+ pump-mediated ion transport and of the thermogenic activity and capacity of BAT under conditions of dietary energy surfeit and deficit. The overall objective was to determine if changes in the activities of the Na^+,K^+ pump and of BAT coincided with the hypothesis that both mechanisms can act as energy buffers to alter overall energy expenditure during states of energy surfeit and deficit. Based on the results of the studies described in Chapter 3 it is concluded that 1) erythrocyte Na^+,K^+ pump activity is not lower during the undernutrition that is associated with severe pediatric cerebral palsy and 2) Na^+,K^+ pump activity did not increase during clinical

nutritional rehabilitation as was expected based on the results of earlier studies in marasmic children in developing countries.

In laboratory rats I examined the effects of the excessive consumption of high saturated fat diets and the effects of food restriction upon both postulated mechanisms of energy expenditure, Na^+,K^+ pump activity and thermogenic activity of BAT. It was shown that the high saturated fat diets induced hyperphagia, increased metabolic efficiency and obesity. From a series of analyses of the validity of the use of presently available TOBEC equipment for the assessment of adiposity in rats it was shown that the methods are not suitable for serial determinations of adiposity. These studies also showed that, as compared to TOBEC determinations, weighing WAT fat pads, such as perirenal or epididymal pads, provides a more simple and accurate estimation of total carcass body fat weight.

While there were no detectable differences in the RMR between the high saturated fat diet-fed rats and the controls, the feeding of the high fat diets was associated with increases in thymocyte Na^+,K^+ pump activity and with increases in BAT activity (GDP binding) and thermogenic capacity (UCP content). Following a 12 week period of dietary restriction there were decreases in Na^+,K^+ pump activity in erythrocytes, thymocytes and hepatocytes from some of the diet groups which were studied. Similarly, significant decreases in BAT activity (GDP binding) were observed following dietary restriction. Plasma T_3 concentrations were increased in the lard-based diet-fed rats as compared to control rats, and decreases in concentrations were

observed following dietary restriction. While it is known that T_3 directly and indirectly acts in the induction of both Na^+,K^+ pump activity and BAT thermogenesis, respectively, it is unknown whether the observed changes in T_3 were involved in the changes in the activities of these two mechanisms of altered energy expenditure. It is concluded that during high saturated fat diet-induced obesity and dietary restriction Na^+,K^+ pump activity (in some cell types) and BAT thermogenic activity and capacity are altered and these mechanisms could therefore act as energy buffers in the maintenance of overall energy balance.

The finding that malnourished rats have a lower Na^+,K^+ pump activity in their erythrocytes whereas malnourished children have a higher Na^+,K^+ pump activity in their erythrocytes, both in comparison to the well nourished state, emphasizes the danger of extrapolating findings (and hypotheses) from one species to another. This discrepancy illustrates the need for studies with a variety of different species and for the validation of hypotheses by studies with human subjects whenever possible.

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Appendix 1

A comparison of the fatty acid compositions of lard and tallow (Vergroesen and Gottenbos, 1975)*:

Fatty acid	Lard (g% total fatty acids)	Tallow (g% total fatty acids)
Myristic; 14:0	1.0	3.0
Palmitic; 16:0	28.0	29.0
Stearic; 18:0	13.5	20.0
Arachidic; 20:0	-	1.0
Palmitoleic; 16:1n-9	3.0	2.0
Oleic; 18:1n-9	46.0	42.0
Linoleic; 18:2n-6	6.0	2.0
Linolenic; 18:3n-3	0.5	0.5
Gadoleic & Erucic; 20:1n-9, 22:1n-9	2.0	-

* Lard is a unique fat in that it has a preponderance of saturated fatty acids, mainly 16:0, at the 2 position on the glycerol backbone, instead of the 3 position as in most fats, including tallow.

Appendix 2

Suppliers of reagents:

atractyloside, potassium salt ..... Sigma
adenosine ..... Sigma
AIN mineral mixture ..... ICN
AIN vitamin mixture ..... ICN
Alphacel™ (non-nutritive bulk) ..... ICN
ascorbic acid ..... Sigma
bisbenzimidazole (Hoechst 33258) ..... Sigma
bovine serum albumin (fraction V) ..... Sigma
bumetanide ..... Sigma
calcium chloride ..... Fisher
casein ..... ICN
charcoal, activated (Norit) ..... Sigma
choline bitartrate ..... ICN
choline chloride ..... Sigma
collagenase, type IV ..... Sigma
corn oil (St. Laurent brand) ..... Loblaw's, Steinberg's or IGA grocers
corn starch (Canada brand) ..... Loblaw's Steinberg's or IGA grocers
cytochrome C (from horse heart, type III) ..... Sigma
Cytoscint, liquid scintillation cocktail ..... ICN
dibutyl phthalate ..... Sigma
DNA (from calf thymus) ..... Sigma
EDTA, potassium salt ..... Sigma
flame photometry [Na] and [K] standards ..... Instrumentation Laboratory
Freund's complete adjuvant ..... Sigma
Freund's incomplete adjuvant ..... Sigma
glucose ..... Fisher
glycerol ..... Baker
GDP, sodium salt ..... Sigma
[^{3}H]]GDP ..... Amersham
HEPES ..... Sigma
[carboxyl-^{14}C]carboxylinulin ..... New England Nuclear
lard (Maple Leaf brand) ..... Loblaw's, Steinberg's or IGA grocers
Lubrol WX ..... Sigma
magnesium chloride ..... Fisher
D,L-methionine ..... ICN
NCS (tissue solubilizer) ..... Amersham
ouabain (G strophanthin) ..... Sigma
petroleum ether ..... BDH

phenol reagent (Folin-Ciocalteau) ..... BDH
Pierce RBS 35 concentrate (detergent) ..... Chromatographic specialties
polyvinylchloride microtiter plates (Falcon 3912) ..... Falcon
potassium chloride ..... Baker
potassium phosphate
- monobasic ..... BDH
- dibasic ..... BDH
PPO ..... Sigma
[^{125}I]protein A ..... ICN
rotenone ..... Sigma
^{86}Rb ..... New England Nuclear
safflower oil (Tosca brand) ..... Loblaw's, Steinberg's or IGA grocers
^{22}Na ..... Amersham
sodium chloride ..... BDH
sodium hydroxide ..... Baker
sucrose ..... BDH
[^{14}C]sucrose ..... Amersham
sucrose (for diets) ..... Loblaw's, Steinberg's or IGA grocers
tallow ..... ICN
TES ..... Sigma
thyroxine (T_4),
- sodium salt ..... Sigma
- [^{125}I]T_4 ..... Amersham
D,L--α-tocopherol ..... ICN
trichloroacetic acid ..... Baker
tricine ..... Sigma
triiodothyronine, sodium salt ..... Sigma
TRIS ..... Fisher
Triton X-100 ..... Sigma
trypan blue ..... Fisher
Tween-20 ..... Bio-Rad
Universol, liquid scintillation cocktail ..... ICN
water, $^{3}H_2O$ ..... New England Nuclear

CURRICULUM VITAE

Mary-Ellen Harper
June 29, 1991

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DATE OF BIRTH: June 12, 1962

CITIZENSHIP: Canadian

EDUCATION:

1986-1991 Ph.D. Biochemistry (expected), University of Ottawa.
Supervisors: Jean Himms-Hagen, John Patrick.

1985-1986 Canadian Dietetic Association Internship, Ottawa Civic Hospital.

1981-1985 B.A.Sc. (with distinction), Applied Human Nutrition,
University of Guelph.

ACADEMIC AWARDS:

1991-1992 Natural Sciences and Engineering Research Council (NSERC) of Canada Postdoctoral fellowship. Location of tenure: Dr. M.D. Brand's laboratory, Biochemistry, University of Cambridge, Cambridge, U.K.

1991 National Institute of Nutrition Postdoctoral Fellowship (awarded but unable to accept after having received a NSERC fellowship).

1986-1990	Medical Research Council of Canada Studentship, and University of Ottawa Supplementary Scholarship.
1988	American Oil Chemists' Society graduate student award.
1985	University of Guelph Convocation Award, and a Natural Sciences and Engineering Research Council of Canada undergraduate summer research award.
1984	R.A. Stewart Scholarship, University of Guelph.
1983	R.A. Stewart Scholarship, University of Guelph.
1982	Class of 1930 Award, University of Guelph, and an Early In-course Scholarship.
1981	Hamilton Home Economics Association Scholarship to the University of Guelph.
1981	Ontario Scholar, Parkside High School, Dundas, Ontario.

SOCIETIES

Canadian Society for Nutritional Sciences - a member society of the Canadian Federation of Biological Sciences

Canadian Dietetic Association

Ontario Dietetic Association

VOLUNTEER ACADEMIC SERVICE:

1989-1991	Secretary for the Biochemistry Department on the Graduate Student Association Council.

RESEARCH EXPERIENCE:

Analysis of sodium and potassium transport in erythrocytes, thymocytes and hepatocytes
(Some of these techniques were acquired during a visit to Dr. J.S. Willis' laboratory in the Department of Physiology and Biophysics at the University of Illinois in June 1987.)

Analysis of brown adipose tissue function (Techniques including assays for uncoupling protein, GDP binding, cytochrome oxidase activity and DNA were learned in Dr. J. Himms-Hagen's laboratory.)

Isolation and analysis of membrane lipids (Experience with techniques including TLC and GLC was acquired during collaborative work with Dr. J.K.G. Kramer at the Animal Research Center, Agriculture Canada.)

Nutritional studies of rapid growth in undernourished children (These studies were carried out at the Children's Hospital of Eastern Ontario with severely cerebral palsied children who were admitted specifically for clinical refeeding.)

Large scale dietary studies with experimental rats (These studies with Sprague Dawley rats have involved diets aimed at inducing obesity, and have also involved dietary restriction regimes.)

ABSTRACTS & PRESENTATIONS:

1) Mary-Ellen Harper, John S. Willis and John Patrick. (1988) Active and passive transport of Na and K in erythrocytes of well and malnourished cerebral palsied children.

POSTER: Federation of American Societies for Experimental Biology (FASEB) convention;
Las Vegas, May 1988.

ABSTRACT: *FASEB J.* 2(6): A1629, 1988.

2) Mary-Ellen Harper, John K.G. Kramer and John Patrick. (1988) Erythrocyte membrane lipid alterations in children with cerebral palsy during rapid refeeding with Isocal™.

ABSTRACT AND SLIDE PRESENTATION:

Canadian section of the American Oil Chemists' Society convention;
Ottawa; October, 1988 (graduate student award received).

3) Mary-Ellen Harper, John K.G. Kramer and John Patrick. (1989) Erythrocyte membrane lipid alterations in undernourished cerebral palsied children during high intakes of a soy-oil based enteral formula.

POSTER: Federation of American Societies for Experimental Biology (FASEB) convention,
New Orleans, March, 1989.

ABSTRACT: *FASEB J.* 3(4):A944, 1989.

4) Mary-Ellen Harper, John Patrick and Jean Himms-Hagen. (1990) Diet-induced obesity and Na/K transport in erythrocytes and thymocytes of Sprague Dawley rats.

POSTER: Federation of American Societies for Experimental Biology (FASEB) convention;
Washington D.C., April, 1990.

ABSTRACT: *FASEB J.* 4(3):A377, 1990.

5) Mary-Ellen Harper, John Patrick and Jean Himms-Hagen. (1991) Altered Na/K transport and brown adipose tissue (BAT) activity in diet induced obesity (DIO) and food restriction.

POSTER: Federation of American Societies for Experimental Biology (FASEB) convention;
Atlanta, GA., April, 1991.

ABSTRACT: *FASEB J.* 5(6):A1654, 1991.

6) D. Welbourne, V.I.A. Montpetit, D.L. Keene, M.E. Harper and J.T. Hindmarsh. (1991) Correlation of muscle pathology, potassium and treatment in a case of familial hyperkalemic periodic paralysis (F.H.P.P.).

POSTER: American Assoiation of Neuropathologists convention; Baltimore, MD, June, 1991.

ABSTRACT: J. Neuropath. Exp. Neurol. 50:351, 1991

PUBLISHED PAPERS:

1) Mary-Ellen Harper, John Patrick and John S. Willis. (1990) Active and passive transport of Na and K in erythrocytes of well and malnournished cerebral palsied children. *Eur. J Clin. Nutr.* 44(8):549-558.

2) Mary-Ellen Harper, John Patrick, John K.G. Kramer and Mark S. Wolynetz. (1990) Erythrocyte membrane lipid alterations in undernourished cerebral palsied children during high intakes of a soy-oil based enteral formula. *Lipids* 25:639-645.

3) Mary-Ellen Harper, Jean Himms-Hagen and John Patrick. (1991) Diet-induced obesity and Na/K transport in erythrocytes and thymocytes of Sprague Dawley rats. *Nutr. Res.* 11(4):375-84.

LETTERS, NOTES AND COMMUNICATIONS:

1) Mary-Ellen Harper, John Patrick, John K.G. Kramer and Mark S. Wolynetz. (1991) *Letter to the Editor (response):* Erythrocyte membrane lipid alterations in undernourished cerebral palsied children during high intakes of a soy-oil based enteral formula. *Lipids* (In press. Accepted for publication June 25, 1991.)