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Body size, cell membranes and tissue metabolism in mammals

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by

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1993
Declaration

This thesis is submitted in accordance with the regulations of the University of Wollongong in fulfilment of the requirements of the degree of Doctor of Philosophy. The work described in this thesis was carried out by me except for some of the final experiments, where technical assistance was required for enzyme assays and fatty acid analyses. No part of this thesis has been submitted to any other university or institution.

Patrice Couture

August, 1993
À tous mes mentors,
dont les premiers demeurent mes parents.
Summary

The relationship between body size and the rate of basal metabolism in mammals is the holistic basis of this thesis. In particular, the work presented investigates the relationships between body size, cell membrane phospholipid fatty acid composition, tissue metabolism and sodium pump activity. The species chosen were mouse, rat, rabbit, sheep and cattle, representing a 12 000-fold difference in body weight.

Heart, skeletal muscle and kidney cortex of larger mammals had less polyunsaturated membrane phospholipids than those of smaller mammals. The proportions of the various polyunsaturated fatty acids in heart, skeletal muscle and kidney cortex allowed to propose that the tissues of larger mammals generally display lower desaturase and elongase activities. A noteworthy exception is in brain, where membrane fatty acid composition remained remarkably constant in mammals of all size. The liver of larger mammals was less polyunsaturated than that of smaller mammals but rabbits showed the lowest extent of polyunsaturation of all species in this tissue. The proportions of saturated to unsaturated fatty acids in tissue phospholipids did not vary with body size.

Physiological measurements with liver and kidney cortex slices confirmed that the tissues of larger mammals are metabolically less active. A significant direct relationship between the rates of oxygen consumption and of potassium uptake (an indicator of sodium pump activity) was found.

This work provided evidence that body size, tissue metabolic rate, sodium pump activity and membrane polyunsaturation are related parameters.
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Chapter 1

General Introduction
1.1 Introduction

Body size appears to be a very influential factor for the determination of metabolism. While larger organisms obviously consume more energy in toto than smaller organisms, in every taxonomic group displaying a wide range of body sizes it has been found that the larger the organism the lower its mass-specific metabolic rate (Schmidt-Neilsen, 1990). This has been shown for unicellular organisms, for poikilotherms in general as well as for homeotherms and even for some trees (Hemmingsen, 1960).

In this thesis, an investigation of the mechanisms involved in the determination of metabolic rate will be carried out. In particular, this work will address aspects of metabolism found at the cellular and cell membrane level. Eutherian mammals are ideal for an investigation into metabolism and body size since the range of body size that they display is one of the greatest for living animals and their tissues are readily available. This comparative system (mammals of varying body size) will then provide us with a very useful experimental tool to investigate mechanisms or strategies that, at the cell membrane level, have been previously observed in vertebrates to correlate with metabolic rate.
1.2 Body Size and Metabolism in mammals

The well-known shrew to elephant allometric relationship of basal metabolic rate\(^1\) (Figure 1.1) is the holistic basis of this thesis. Although it is obvious that *in toto* a cattle consumes more oxygen than a mouse, per unit mass the mouse has a 11-fold higher rate of aerobic metabolism (Table 1.1.). The following sections summarize what is known about this relationship and defines the rationale of the research undertaken in this thesis.

\[\text{Figure 1.1} \quad \text{Relationship between mass-specific metabolic rate and body size in mammals (From Schmidt-Neilsen, 1990).}\]

\(^1\) Metabolic rate refers to the energy metabolism per unit time. Metabolic rate can be measured by calculating the difference in energy content between food taken and excreta, by measuring heat production or oxygen consumption rate. Temperature, time of day, exercise, stress, health condition, age, food intake are some of the many factors influencing the metabolic rate of an individual (see Schmidt-Neilsen, 1990). When measuring metabolic rate on a whole animal, "basal metabolism" is usually defined as the metabolic rate of a fasting animal in a resting state. In this condition an animal is expected to spend most of its energy only for the maintenance of the system (Blaxter, 1989). When measuring the metabolic rate of tissues, "resting metabolism" refers to experimental conditions such that the cells should not be performing any metabolic work other than what is necessary for their maintenance.
Table 1.1

Body weight and mass-specific rate of metabolism in a range of mammals (calculated from Kleiber, 1961)

<table>
<thead>
<tr>
<th>Species</th>
<th>Body weight (g)</th>
<th>Metabolic rate (kcal/kg/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>21</td>
<td>171</td>
</tr>
<tr>
<td>Rat</td>
<td>282</td>
<td>100</td>
</tr>
<tr>
<td>Rabbit</td>
<td>3565</td>
<td>45</td>
</tr>
<tr>
<td>Dog</td>
<td>14100</td>
<td>38</td>
</tr>
<tr>
<td>Sheep</td>
<td>46600</td>
<td>28</td>
</tr>
<tr>
<td>Human</td>
<td>56000</td>
<td>23</td>
</tr>
<tr>
<td>Cow</td>
<td>454000</td>
<td>15</td>
</tr>
</tbody>
</table>

1.2.1 The determination of size

Berrill (1955) stated that the regulation of size is essentially a matter of rate and duration of growth. All mammals were believed to have similar sized cells, the larger species being simply made out of more cells. Nevertheless, this author reported a doubling in the linear dimensions of cells from homologous tissues between a mouse and an elephant, or an approximately 8-fold difference in volume. Interestingly, Berrill also points out the finding of Smith (1912) that the peribranchiate urodeles, whose cells are many times the size of those of their metamorphic cousins, are also characterised by a comparatively low rate of metabolism. This suggests that metabolic rate may be a function of cell size, as well as of body size.

Other examples provided by Berrill (1955) are the findings by Whitaker (1933) and Tyler (1935) who measured the metabolic rate of eggs of various
sizes. It was proposed from their findings that eggs before fertilisation or activation are in a state of "abnormal" (low) metabolism compared with the adults and that the low metabolism of the very large oocytes compared to the cells of adult is in some way the basis of the attainment of large cell size.

Since then, an intraspecific comparison by Falconer et al. (1978) has provided further evidence that larger animals, displaying lower metabolic rate, also have larger cells. These authors selected for body size in mice over many generations and measured the organ size, cell size and cell number in the small and large mice thus produced. There was a 2-fold difference in 6-week body weight between the large and small strains. The organs of the larger strain were 1.8, 2.4, 2.4 and 2.2-fold larger than in the small strain for lung, liver, spleen and kidney, respectively. They found that the larger mice had more and larger cells than the smaller ones, in homologous tissues. In the lung and spleen, the higher cell number accounted for around 70% of the difference in organ weight, with the remaining 30% being due to larger cellular volumes. In liver and kidney, 50% of the organ size difference was found to be due to larger cellular volumes. When the large and small strains were compared at the same size (ie. at different ages), these differences were not present. This relationship certainly merits further investigation.

To my knowledge, there are currently no theoretical limits on the size to which an organism can grow. According to Berrill (1955), "the final limit to growth, and therefore size, is genetically inherent in the species and in general not subject to experimental modification other than a degree of stunting through some form of malnutrition".
Evidence for a genetic basis of adult size in intraspecific comparisons has since been demonstrated experimentally: it is possible, through selective breeding, to modify adult body size. The example cited above of selective breeding of mice initially from the same litter yields a remarkable divergence in body size: by selecting for size at each generation, after 15 generations the adult size of one strain reached double that of the other strain (Falconer, 1973; Falconer et al., 1978). Other classic examples are the small breeds of dog and the miniature horses, obtained by selectively breeding smaller offspring. Garland and Adolph (1991) mention that body mass shows “relatively high heritability” and is therefore candidate for selection in variable environments.

1.2.2 The influence of body size on basal metabolism

The differences in weight-specific basal metabolic rate between species can be best appreciated by comparing animals of very different body size: only small differences can be observed between species of similar weight, such as mouse and rat (or sheep and pig). Once the weight-specific basal metabolic rate of a wide range of mammals (Figure 1.1) is plotted on a double logarithmic plot against their weight however, the relationship between metabolism and body size is obvious. In 1963, the Third Symposium on Energy Metabolism adopted the 0.75 mass exponent of basal metabolism (Kleiber, 1965), yielding a mass-specific allometric relationship for metabolism with an exponent of -0.25. This exponent implied that a doubling in body weight results in a decrease in weight-specific basal metabolism of 16%. The value of this exponent has recently been challenged. Heusner (1991) offers a review of the arguments and suggests the adoption of the exponent 0.68. From the data that he collected from the literature, it appears that the allometric relationship of basal metabolism could be made up of two parallel regression lines, with the larger
mammals on the higher line. Including all mammals in a regression would artificially increase the slope of the regression. From this exponent, the mass-specific coefficient is -0.32, implying that a doubling in body mass yields a decrease in mass-specific metabolism of about 20%. The coefficient of 0.68 is not significantly different from the theoretical 2/3 exponent, calculated from the changes in surface/volume ratios associated with changes in mass (Heusner, 1991).

In the present thesis, the species chosen were mouse, rat, rabbit, sheep and cattle. From the rates of *in toto* basal metabolic rate reported by Kleiber (1961) for these species, the allometric exponent in this comparison is 0.76, or mammals in this range decrease their metabolism by 15% for a doubling of body size. This allometric exponent will be used for the following discussion.

The allometric exponent of basal metabolism will represent the sum of all the factors influencing this relationship. There is some evidence, presented below, for considering non-metabolic explanations of this relationship. However, simple calculations suggest that this relationship also has a cellular basis.

Metabolic heat comes mainly from internal organs. In humans for example, while internal organs (kidney, heart, lungs, brain and splanchnic organs) represent only 8% of the total body weight, they are responsible for approximately 72% of heat production (Aschoff *et al.*, 1971). Brody (1945) showed that some of the organs of smaller mammals are proportionally greater in size than those of larger mammals. His estimates of the allometric relationships of organ size were similar to those reported by Else and Hulbert (1985), with exponents of 0.87 for liver and 0.78 to 0.85 for kidney. The latter
authors also reported similar allometric relationships for brain and lung (the exponents were 0.69 and 0.90, respectively). Skeletal muscles did not show an allometric decrease in relative size. Else and Hulbert (1985) confirmed Brody's report that the relative size of this tissue did not vary with size in mammals (the slope of their allometric relationship was 1.01).

Interestingly, Else and Hulbert (1985) reported that mammals, which are four to five times more metabolically active at the same body temperature than reptiles of comparable size, also have relatively larger liver, kidney, brain, heart and skeletal muscle (1.9, 2.5, 3.7, 1.7 and 1.3-fold increase, respectively). These findings lend support to the hypothesis that relatively large organ size is often associated with high metabolic rates.

It is important to point out that these interspecific relationships differ from the intraspecific comparison presented by Falconer et al. (1978). By selecting for size in mice, the larger mice exhibited relatively smaller lungs, but larger livers, spleens and kidneys (allometric exponents: 0.89, 1.28, 1.27 and 1.17 respectively). Thus, the decrease in organ size in larger mammals possibly only applies to interspecific comparisons of some homologous tissues.

The decrease in organ size for larger mammals cannot explain all of the observed decline in mass-specific metabolic rate with increasing body size. Holliday et al. (1967) proposed that organ size and mass-specific metabolic rate both contribute to the allometric decrease in mass-specific basal metabolic rate observed among mammalian species. Brody (1945) has shown that a doubling in body size will result in a 9% decrease in the relative size of liver and kidney (his allometric exponents are 0.867 and 0.846 for liver and kidney cortex, respectively). If all tissues showed a similar decrease in relative size in larger
mammals, about half the allometric decrease in mass-specific basal metabolic rate measured for the whole organism in the mouse to cattle comparison (15% decrease for a doubling in body size or 11-fold between mouse and cattle) could be explained by differences in relative tissue size.

Considering all tissues, the decrease in relative size of some organs in larger mammals will account for substantially less than half of the lower mass-specific basal metabolic rate in these animals. For example, the skeletal muscle is the major organ in mammals in terms of weight and as seen above the proportions of this organ remain unchanged across a range of body size. However, since this tissue is not a very important contributor to basal or resting metabolic rate (in humans, while skeletal muscle constitutes 42% of body mass, it is responsible for about 16% of resting metabolic rate: Aschoff et al., 1971), the impact of the allometric decrease in the relative size of tissues which are major contributors to basal metabolic rate (such as liver and brain) will remain substantial.

1.2.3 Influence of body size on metabolic rate at the tissue and cellular level

Accounting for the aforementioned interspecific decrease in the relative size of some internal organs in larger mammals, there would remain more than 40% of the whole organism decrease in metabolism with body size to be explained by other means. Part of the decrease of mass-specific metabolic rate in larger mammals could be due to these animals containing proportionally more structural components, which would not be very metabolically active. The proportions of connective tissue do not vary in mammals of different body mass (the allometric exponent is 0.98) (Calder, 1984). However, larger mammals
have proportionally more skeleton than smaller mammals (the allometric exponent is 1.07), this parameter thus contributing to the scaling of metabolic rate. The other part of explanation for the scaling of basal metabolic rate should be found in the mass-specific metabolic rate of the major contributors to basal metabolic rate, the internal organs.

Krebs (1950) carried early investigations on the relationship between body size and tissue metabolism by measuring the oxygen consumption of tissue slices. His data (which I have plotted and calculated the allometric equations, see Figure 1.2), suggest that there are tissue-specific allometric relationships of tissue metabolic rate. Unlike Brody's data on organ weights discussed above, however, these results must be treated with care as there is no way to verify that the metabolic rate measured was "resting" (see footnote 1) to the same extent for each tissue and species. If differential damage occurred in different species' tissue cells during the slicing process, some slices could display a higher resting metabolic rate if the cells were recovering, or a lower rate if they were moribund. The methods in Chapter 3 have been designed to address this question, by monitoring cell damage in the slices used for the measurements of metabolic parameters.

These data (Krebs, 1950) support the hypothesis of Holliday et al. (1967) that part of the decrease in mass-specific metabolic rate with increasing body size is due to tissue metabolic activity. The allometric exponents for mass-specific metabolic rate determined from Krebs' data range from -0.07 for the kidney and brain to -0.14 for the spleen. The lung and liver displayed intermediate exponents (-0.10 and -0.12, respectively). If Krebs' data are correct, they imply that for a doubling in body size there is a 5 to 9% decrease in mass-specific tissue metabolic rate, accounting for approximately one third to two
Figure 1.2

Allometric relationship of oxygen consumption of tissue slices for a range of mammals (mouse, rat, guinea pig, rabbit, cat, dog, sheep, cattle and horse) as measured by Krebs (1950).

Legend:
- **Kidney**: $Y = 52.5X^{-0.07}$
- **Brain**: $Y = 40.7X^{-0.07}$
- **Liver**: $Y = 30.9X^{-0.12}$
- **Spleen**: $Y = 27.5X^{-0.14}$
- **Lung**: $Y = 14.8X^{-0.10}$
thirds of the change in mass-specific metabolic rate of the whole organism if the metabolic rate of all tissues is in the range encompassed by kidney and spleen.

Considering the values presented above, it is possible that the scaling of metabolism in mammals can be accounted for by (i) a decrease in organ size, in parallel with (ii) a lower mass-specific rate of metabolism in the tissues of larger mammals. This also raises the question as to whether the lower metabolism in larger mammalian tissues can be related to cell volume-specific differences in metabolism or to these tissues having less cell volume per unit weight, or to both. This question will be addressed in this thesis, by measuring the intracellular volumes per unit mass in mammalian liver and kidney cortex slices as well as the rate of oxygen consumption, in an allometric comparison.

1.3 Membrane-related aspects of metabolism

The remainder of this introduction will be concerned with cell membrane composition, function and the possible relationships of these parameters with metabolic rate. Membranes not only compartmentalise the cell, but are the site of many physiological processes, such as ion and metabolite transport, enzyme reactions, hormone recognition and signal transduction. The role of membrane composition, structure and function in cell metabolism has been the subject of many investigations. Since Green and Fleisher (1963) discussed the essentiality of the presence and unsaturation of phospholipids in restoring mitochondrial activity after delipidation of mitochondrial proteins, a great deal of evidence has been presented for relations between cell metabolism, membrane function and composition (Singer and Nicholson, 1972; Fettiplace and Haydon, 1980; White and Somero, 1982; Brenner, 1984; Hulbert, 1985 and 1993; Stubbs and Smith, 1984; Hazel, 1988; Garg et al., 1990; Clandinin
et al., 1991; Hoch, 1992). The enormous variability found in cell membrane composition and the many functions of membranes have so far precluded a clear understanding of their relationships with each other and with cell metabolic rate in general. Nevertheless, some patterns have emerged and I devote the following sections to a brief review of our current understanding of this aspect of metabolism.

It is the purpose of this thesis to enlarge our knowledge of how cell membrane composition may be related to metabolic rate. It is in this perspective that I have analysed the cell membrane fatty acid composition as well as membrane permeability to potassium, the activity of the sodium pumps, a membrane enzyme, and the rate of aerobic metabolism in the tissues of mammals displaying greatly different rates of metabolism.

1.3.1 Description of phospholipid structure

The basic structural component of phospholipid molecules is a glycerol or a sphingosine backbone. In technical terms, the former are called "phosphoglycerides" and the latter "sphingomyelins" (Sylver, 1985). Figure 1.3 shows that in phosphoglycerides the carboxyl end of the fatty acid chain is attached to each of the first two carbons of a glycerol backbone through an ester link (except for ether lipids where the second fatty acid is attached to the glycerol backbone through an ether bond). The third carbon binds various alcohols through a phosphate group. These alcohols are usually choline, ethanolamine, serine or inositol. Cardiolipin (diphosphatidylglycerol) is an unusual phosphoglyceride in that the phosphate group binds two glycerol groups, each carrying two fatty acid chains. Sphingomyelins have only one fatty acid attached to the amino group of the sphingosine. The sphingosine is
an amino alcohol containing a long hydrocarbon chain (Figure 1.3).

The proportions of various phospholipids differ between species, tissue and cell membrane. Phosphatidylcholine and phosphatidylethanolamine are the most common phospholipids in mammals. Cardiolipin is found in important proportions in liver mitochondrial inner membranes (around 20% in rat liver mitochondrial inner membranes). Sphingomyelin is abundant in plasma membranes (it makes up to 20% of rat liver plasma membranes). Although ether lipids (plasmalogens) do not usually occur in high proportions in mammalian membranes, they are the major constituents of certain membranes. In canine myocardial sarcoplasmic reticulum for example, 53% of the phospholipid is ether lipid (Evans and Graham, 1989).

![Phospholipid structure](image)

**Figure 1.3** Phospholipid structure (From Evans and Graham, 1989).
Most naturally occurring fatty acids are chains of even-numbered carbon atoms (Lehninger, 1982). Odd-numbered chain fatty acids have until now been considered of so minor importance as membrane constituents that no mention is made of them in common textbooks (Lehninger, 1982; Stryer, 1988). In ruminants, these fatty acids are known to be derived from the elongation of the products of microbial fermentation of carbohydrates, yielding propionic acid, a three carbon molecule. They occur in important proportions in some plant tissues and marine organisms (Lehninger, 1982).

The fatty acid chains in phospholipids vary in length and unsaturation. The length of a fatty acid, often referred to in this thesis, considers the number of carbon atoms in the chain and not the physical length, which depends on unsaturation. In a saturated fatty acid chain, each carbon is attached to two adjacent carbons as well as binding two hydrogen atoms, except the first carbon (the methyl end), which binds three hydrogens, and the last one (the carboxyl end), which binds a hydroxyl group and an oxygen atom. These chains are thus saturated with hydrogen. Unsaturated fatty acids have had two hydrogen atoms from adjacent carbons removed, and the available electrons on these carbon atoms form a second bond, yielding a double bond. Double bonds are rigid, compared to single bonds about which rotation can take place (Atkins, 1990). As introducing double bonds in a saturated chain makes kinks in this chain, the resulting unsaturated chain is physically shorter than the saturated straight chain.

The fatty acids found in membrane phospholipids come from the diet or from de novo synthesis. The cells have the ability to synthesize palmitate, a 16-carbon saturated fatty acid (16:0), before it is incorporated in the membranes and sometimes further modified. The synthesis of phospholipids mostly takes
place in the endoplasmic reticulum (although the last step in the formation of cardiolipin occurs in the mitochondrial inner membrane) (Evans and Graham, 1989). The initial reactions involve the attachment of fatty acid chains to the glycerol backbone. The newly formed phospholipids are then modified by elongating or desaturating the fatty acid chains by two groups of membrane enzymes: elongases add two carbons at a time at the carboxyl end of the chains and desaturases add double bonds by transferring two hydrogen atoms from adjacent carbons to molecular oxygen, thus forming water. Transferases are also present, which as their name indicates replace some fatty acids on the glycerol backbone with others (Stryer, 1988).

The terminology of the fatty acids that will be used throughout this thesis describes their length, unsaturation and position of the terminal double bond from the methyl end of the fatty acyl chain (the methyl end is situated in the middle of the bilayer). For example, 16:0 indicates a saturated chain that is 16 carbons long, while 22:6\(\Delta3\) indicates a 22 carbon chain with 6 double bonds, the last of which is found between the third and fourth carbons from the middle of the bilayer. From surveying all the literature cited in this thesis and that deals with membrane composition and consistent with the results reported in Chapter 2, in mammalian cell membranes the longest membrane fatty acid reported is 24:1\(\Delta9\) and the most polyunsaturated is 22:6\(\Delta3\).

Depending on the position of the terminal double bond, referred to in this thesis as \(\Delta\), fatty acids can be classified in different groups. In mammalian membranes, \(\Delta3\), \(\Delta6\), \(\Delta7\), \(\Delta9\) and \(\Delta11\) are found. Mammalian desaturases only form double bonds in fatty acid chains above the seventh carbon from the methyl end (Stryer, 1988). For this reason, in vertebrates all fatty acids with double bonds found in the \(\Delta3\) or \(\Delta6\) position must have been incorporated
from the diet and not synthesised *de novo*. They are thus termed "essential fatty acids", as they have to come from the diet. In practical terms, only two fatty acids are said to be "essential": linoleate (18:2\(\Delta6\)) and linolenate (18:3\(\Delta3\)). Once incorporated, these fatty acids can be further elongated and desaturated. They are not only important for their incorporation in the membranes, but some of them also serve as precursors for the synthesis of various messenger molecules (eg. eicosanoids, that are made from 20:4\(\Delta6\) and other polyunsaturated fatty acids. Evans and Graham, 1989).

As Stubbs and Smith (1984) report in their review, the desaturases have a preference for some groups of fatty acids as substrates, in the order \(\Delta3 > \Delta6 > \Delta9\), leading to the preferential incorporation of \(\Delta3\) fatty acids in the membranes, if they are available. It is worth mentioning here that the studies from which these conclusions were drawn have been carried out primarily with small mammals such as rats or guinea pigs (Van Golde *et al.*, 1969; Brenner, 1984; Stubbs and Smith, 1984; Garg *et al.*, 1990) or with fish (Wodtke, 1978; Hazel, 1984). I am not aware of comparative studies addressing the scaling of substrate preferences for the elongases and desaturases in mammals. That the desaturases of larger mammals do not show the same order of preference or affinity for various fatty acid groups remains a possibility.

There are an impressive variety of different lipid molecules in cell membranes. Van Deenen (1969) placed a conservative estimate for erythrocyte membranes at 150 to 200 distinct types. These lipids are the only structural elements of the cell that respond to changes in temperature by fundamentally altering their composition (Hazel, 1988). The changes include the modification of the proportion of the various phospholipid classes (with various head groups) and the replacement or modification of the fatty acid side chains, generating various molecular species.
1.3.2 Description of membrane structure and composition

In aqueous solutions, phospholipids assemble in a gel (solid) state or in a liquid crystalline state, depending on temperature, pressure and salinity. A liquid crystal is a substance having liquid-like imperfect long-range order but some crystal-like aspects of short-range order (Atkins, 1990). Thus, while the bilayer in which they assemble forms a stable structure in shape, the constituents have free movement in the plane of the bilayer.

Having observed that most membranes are an assembly of proteins and phospholipids, researchers were initially faced with the dilemma of deciding "which is the brick and which is the mortar" (Singer and Nicolson, 1972). Proteins, known to have the ability to form rigid structures, were ideal candidates as "bricks".

Green and Fleisher (1963), having observed that (inner) mitochondrial membranes are rich in proteins, proposed the disk hypothesis, a model in which the phospholipids occur inside protein cylinders. An alternative arrangement described by these authors was one in which the "structural proteins" formed rigid networks with phospholipids intercalated in rows in the gaps. In both models, the hydrophobic fatty acid chains of the phospholipids stood side by side, with head groups pointing in both directions. In both cases too, the proteins were the essential structural components.

Luzzatti (1968) proposed that the most common conformation of lipids in biomembranes is the bilayer. Later, the now generally accepted "Fluid Mosaic Model" was formulated by Singer and Nicolson (1972). In this model, the phospholipids form bilayers with intercalated membrane proteins. The
novelty in this model was the proposition that most animal cell membranes are dynamic, fluid structures and that phospholipids, and not proteins, are the essential constituents (the "mortar"). These authors wrote that "...the (membrane) proteins have been shown to be grossly heterogeneous in molecular weight. There is no convincing evidence that there exists one predominant type of membrane protein that is specifically a structural protein".

The major impetus to the proposal of the fluid mosaic model of cell membranes was the discovery that membrane proteins are mobile in the plane of the phospholipid bilayer (Singer and Nicolson, 1972). These authors had also hypothesised that if the fluid mosaic model was correct, the proteins should be randomly distributed throughout the plasma membranes. The model has generally stood the test of time, but their hypothesis about the random distribution of membrane proteins has since proven to be incorrect. In each type of membrane, the constituents are organised in domains where lipid-protein and lipid-lipid interactions are highly specific and organised to provide the required structural and functional characteristics (Clandinin et al., 1991). Singer and Nicolson (1972) disregarded the possibility of functionally-related protein assemblages and interpreted the electron micrograph evidence of the tightly packed and ordered array of rhodopsin pigments in retinal receptor disk membranes as an artefact due to "optical tricks...to enhance the apparent order".

Nonetheless, by testing this model Singer and Nicolson (1972) gathered evidence that the lipid environment of a biomembrane is fluid enough to allow proteins to move about laterally. For example, the receptor disk membranes of the frog retina contain rhodopsin as the main, if not the only, protein. The authors discuss evidence that the orders of reflections of the rhodopsin
molecules by x-ray diffraction, corresponding to the spacing of the molecules, indicated a non-crystalline, aperiodic arrangement of the particles in the plane of the bilayer. As well, temperature-induced changes in the x-ray diffraction maxima of rhodopsin proteins were indicative of the liquid-like nature of the membranes. Finally, the arrangement of these proteins could be disrupted by the addition of bovine serum albumin, which binds weakly to the pigment, demonstrating that these molecules are not static in the membranes but could move with respect to each other.

The work of Papahadjopoulos, who pioneered the preparation of reconstituted membrane vesicles, or liposomes, demonstrated that phospholipids are the only essential structural components of biological membranes (Papahadjopoulos, 1971b; Kimelberg and Papahadjopoulos, 1972; Kimelberg and Papahadjopoulos, 1974). These studies have shown that under appropriate conditions phospholipids self-assemble in bilayers that display typical biomembrane properties without the need of adding cholesterol or proteins.

This is not to say that the presence of membrane proteins and cholesterol does not alter the physiological properties of membranes. The three volumes of Membrane Fluidity in Biology (edited by Roland C. Aloia, 1983) give extensive examples. Indeed in many membranes the density of nonaggregated proteins is such that they are separated from their neighbours by only a few rows of phospholipid molecules. The weight ratio of protein to lipid in most membranes ranges from 0.25 in myelin membranes to 3.6 in mitochondrial inner membranes. For most animal cell plasma membranes the ratio is close to 1.0 (Evans and Graham, 1989). A discussion of the lipid-protein interactions is found in Section 1.3.5.
As for cholesterol, a sterol which is a structural element in many cell membranes, because of the way this molecule interacts with the phospholipids, it acts as a buffer on membrane fluidity. Since the sterol group of cholesterol bonds with a phospholipid head group and the hydrocarbon chain plunges into the hydrophobic bilayer, in sufficient concentrations its effect is to eliminate the phase transition temperature and to slow the movements of phospholipids (Yeagle, 1985; Hazel, 1988; Lee, 1991). This is an aspect of membrane composition for which I have not investigated the consequences.

1.3.3 The concept of homeoviscosity

Since Baranska and Wlodawer (1969) reported that ambient temperature influenced membrane composition in frogs, it has become well established that membrane phospholipid fatty acid composition in the cell membranes of multicellular organisms can be altered by various environmental factors. "Homeoviscosity" is used to describe the ability of cells to regulate and maintain the viscosity of their membranes at a state appropriate for their functions by altering their composition. This hypothesis was first proposed by Sinensky (1974), after he showed that, during temperature acclimation, *Escherichia coli* would modify their cell membrane phospholipid fatty acid composition in such a way that membrane fluidity was maintained: colder growth temperatures would stimulate these organisms to accumulate more polyunsaturated fatty acids.

In biochemical terms, homeoviscosity involves the regulation of membrane composition such as the number and position of double bonds in the fatty acid chains of membrane phospholipids and possibly also involve changes

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2 Fluidity is defined as the inverse of viscosity, measured in "poises".
in other membrane constituents affecting fluidity, such as cholesterol content. Cholesterol may not play an important role in homeoviscosity. One study of thermal acclimation with fish found that warm-acclimated carps (32°C) had a 37% higher cholesterol to phospholipid ratio than carps acclimated at 10°C (Wodtke, 1978), while a second study failed to find any such changes (Wodtke, 1981). In his review on homeoviscous adaptation, Hazel (1988) concluded that although thermal acclimation sometimes influences cholesterol content of biological membranes no consistent pattern can be found.

In their review, Stubbs and Smith (1984) ranked the effects of double bonds on the melting point of phospholipid mixtures as follows. One or two double bonds depress the melting point to the maximal extent. Adding double bonds near either end of the fatty acid chains has decreasing effects. The authors report that in biological membranes the first double bond is usually added around the middle of the chain and the subsequent ones in increasing distance from the middle of the chain.

Homeoviscous adaptation is possible thanks to the desaturases, elongases and possibly also to the transferases which could regulate differential incorporation of particular dietary fatty acids in the membranes, although there is at present little evidence that the latter mechanism is involved in the control of membrane composition (Stubbs and Smith, 1984). Those enzymes, concentrated mainly in the endoplasmic reticulum (Evans and Graham, 1989), can react very rapidly to changes in membrane fluidity. Brenner (1984) details a series of experiments showing the complexity of the biofeedback mechanisms that regulate the action of these enzymes. The enzymes adjust membrane composition under the complex influence of fluidity, temperature and composition itself. For example, desaturases are multienzyme complexes that
involve the NADH-cytochrome b<sub>5</sub> microsomal electron transport system, including cytochrome b<sub>5</sub> and its reductase. The propensity of these proteins to transfer electrons from NADH to the desaturase is increased by a decrease in membrane fluidity (apparent microviscosity) of the microsomal membranes (Brenner, 1984). This relationship may be the basis of the regulation of homeoviscosity.

Much of the literature on the modification of membrane composition is concerned with ectotherms. In his review on the subject, Hazel (1988) thoroughly discusses the mechanisms by which the cell membranes of ectothermic organisms adapt to environmental changes such as temperature, pressure and salinity. Since these factors are not of importance in normal conditions for most endotherms, only a brief overview of their effects on membrane composition is presented here.

Temperature affects both the "phase (gel vs. liquid crystalline) state of membrane lipids and within a specific phase, the rates of molecular motion and/or the order of membrane constituents" (Hazel, 1988). The report above for *E. coli* (Sinensky, 1974) is consistent with the reports reviewed by Hazel (1988) that decreasing environmental temperature induces an increase in membrane phospholipid fatty acid unsaturation in ectotherms.

A good example of the effects of hydrostatic pressure on cell membrane composition is the work of Cossins and MacDonald (1986). Deep-sea fish have a lower saturated to unsaturated fatty acid content in their liver mitochondrial membranes than fish from shallow water whilst cardiolipin shows the opposite trend. The Unsaturation Index (an indicator of the number of double bonds per 100 fatty acid molecules in tissue phospholipids) was not, however,
significantly correlated with depth.

Salinity which Hazel (1988) describes as a factor of paramount importance for influencing the structure and function of those biomembranes facing salinity variations, has not been thoroughly studied for its effects on membrane composition. The effects reported so far are to alter the proportion of negatively charged phospholipid species in bacteria and euryhaline crabs (see Hazel, 1988).

Hazel (1988) concludes that although the adaptations of cell membranes to environmental variables cannot, with the current knowledge, lead to a simple concept of compositional membrane response to physical factors in terms of "fluidity", the "concept of homeoviscous adaptation, in its broadest application, remains a useful one in adaptational biology."

To increase their control over their metabolic activity, multicellular organisms have evolved mechanisms to isolate their cells from the vagaries of the environment. Metazoans in general have isolated their cells from fluctuations in external salinity. Homeothermy is thought to also have evolved in this perspective. Through the evolution of homeothermy, some groups of vertebrates overcame the next environmental challenge by developing an ability to maintain a constant internal temperature independent of changes in environmental temperature.

Is there then still a role for homeoviscous adaptation in homeotherms? The presence of regulatory mechanisms for homeoviscosity in mammals is revealed by experiments of dietary manipulations. Feeding mammals diets containing different types and amounts of fatty acids imposes changes in
membrane composition that are opposed by the action of the elongases and desaturases (I did not find any comprehensive review on the regulation of membrane composition in mammals by these membrane enzymes but Stubbs and Smith (1984) and Clandinin et al. (1991) in their reviews on dietary manipulations of membrane composition in mammals both acknowledge that internal regulatory mechanisms are present).

In ectotherms, variations in environmental factors are responsible for changes in both membrane composition and metabolic capacity. Although the cells of endotherms live in a much more stable environment than those from ectotherms, the metabolic demands that they face vary greatly, in particular depending on the size of the organism (Section 1.2). Environmental temperature also influences basal metabolic rate in mammals, but the variations induced by this factor remain minor in comparison to the differences in basal metabolic rate observed in mammals of greatly different body size. In fact, Garland and Adolph (1991) give some examples of reported interpopulation differences in basal metabolic rate in mammals but state that “whether basal metabolic rate shows climatic adaptation within species of small mammals is still controversial”. Nevertheless, outside the thermoneutral zone homeotherms increase their metabolic rate by activating either heat production or heat dissipation mechanisms (Blaxter, 1989). In the present study, it was assumed that all mammals used had spent their recent lives in a thermoneutral environment.

The choice of different-sized homeotherms for investigating the relationships between membrane composition and metabolic rate is thus appropriate as it provides a quite direct system to study relationships between metabolic activity and membrane composition, a system in which temperature
need not be considered. The work undertaken in this thesis has the potential to enlarge the current concept of homeoviscosity beyond the need for regulating membrane composition in response to the physical effects of external factors on membrane fluidity by providing evidence that membrane composition is also related to metabolic demands.

Stubbs and Smith (1984), in their review on mammalian membrane fatty acid composition, fluidity and function, state that while homeoviscous adaptation in ectotherms involves the modification of the proportions of saturated and unsaturated fatty acids, these remain remarkably constant following dietary manipulations in mammals. While these authors discuss the complex effects of the various types of phospholipids present in biological membranes on their physical properties, the emphasis in their review is put on dietary modifications of membrane phospholipid fatty acid composition more than on the modifications caused by the actions of elongases and desaturases. This reflects the abundance of literature on the former aspects for mammals. The interspecific comparison of cell membrane phospholipid fatty acid composition of homologous cells in tissues of mammals with different metabolic rates was not addressed and the data for such a comparison remains scattered throughout the literature.

In a more recent review, Clandinin et al. (1991) addressed the question of membrane composition and function in mammals, concentrating mainly on the effects of dietary lipids. The evidence that they present is consistent with the proposal that metabolic activity is intimately linked to cell membrane composition. For example, they reported studies suggesting that modifications of membrane phospholipid fatty acid composition alter hormone binding or responsiveness, thus modifying the regulation of metabolic pathways such as
protein synthesis.

Clandinin et al. (1991) also presented experimental evidence that dietary fats can modify nuclear activity. High dietary linoleic acid (18:2Ω6) in mice liver increased the incorporation of 18:2Ω6 and 20:4Ω6 in the nuclear envelope phospholipids and increased release rates of RNA from the nuclei. This finding suggests a link between cell activity and membrane composition, and it becomes tempting to also suggest that dietary fat intake may be linked to metabolic activity in a broad sense: diet preference could be related to the requirements of membrane composition for a given level of metabolic activity as well as to the metabolic needs for fuel.

Gavrilova et al. (1992) showed that varying the composition of rat liver plasma membranes by modifying the proportions of some phospholipids induced changes in protein kinase activity. From these results, they suggested that rat liver protein kinase activity is influenced by membrane fluidity. Protein kinases are major mediators in the response of cells and tissues to hormonal influence as well as in the control of cell growth and development (Hunter, 1987) and may therefore be related to differences in metabolic rate.

Another factor known to influence both cell membrane composition and metabolic rate is the activity of the thyroid gland. Hulbert et al. (1976), in their comparisons of normal vs. thyroidectomized rats, found that lower levels of thyroid hormones are accompanied by a decrease in membrane unsaturation. This is of interest in view of the well known decrease in metabolic rate following thyroidectomy (Sokoloff, 1970).
Finally, some evidence has been presented that there is a genetic basis for membrane composition and membrane metabolic function. The Clandinin team found that in mice from different strains specific gene differences alter nuclear envelope phospholipid fatty acid profile, nuclear envelope triphosphatase activity, RNA efflux from isolated nuclei and binding of L-triiodothyronine (T₃) to the liver nuclear envelope (see Clandinin et al., 1991). The level at which membrane fluidity and composition in general are set may have a genetic basis but the organism could retain the capacity to adjust this setting. For example, according to Hoch (1992) there is enough evidence in the literature to suggest that thyroid hormones control the genetic expression of the enzymes that synthesize and desaturate fatty acyls-CoA. Thyroid hormone regulation alters metabolic activity as its up- or down-regulation change mitochondrial phospholipid composition and alter oxidative phosphorylation. Thyroid hormones levels thus relate directly with State 4 respiration and to a lesser extent with State 3 respiration (Hoch, 1992). The experiments mentioned above on thyroid hormone (T₃) binding to the nuclear envelope also suggest that responsiveness to thyroid hormones can be altered genetically.

1.3.4 Metabolic roles and cost of the transmembrane sodium gradient and effects of phospholipid composition

Among a multitude of roles, plasma membranes maintain high potassium and low sodium concentrations inside the cell, against strong concentration gradients. The Na⁺-K⁺ ATPase, or sodium pump, is in charge of maintaining the sodium and potassium gradients. By expelling three sodium ions for each two potassium ions transported into the cell (at the cost of one ATP), this pump is also electrogenic. The gradients may have originally
evolved as a mean to control cell volume by balancing the osmotic effects of the large number of organic molecules within the cell. In modern cells, they also serve as an energy source for most membrane transport systems (for transport of glucose and amino acids into cells against their concentration gradients, for co- and countertransport of ions across the cell membrane, for the bulk extrusion of Ca\textsuperscript{2+} and for transepithelial transport in intestine, kidney and secretory glands), for the formation of the membrane potential and, in muscles and nerves, for de- and repolarization of membrane potential (Lamb, 1990; Skou, 1992). The aspects of the sodium gradients related to metabolic function and cost are discussed in the following sections.

1.3.4.1 Cotransport of sodium and metabolites

Guidotti et al. (1978) reviewed the regulation of amino acids in animal cells. They reported that the transport of more than half of the amino acids is sodium-dependent. The authors also suggested that the gradient of neutral amino acids established by a sodium-dependent uptake could provide the energy needed for the transport of other (sodium-independent cationic) amino acids via a process termed heteroexchange.

It is now generally accepted that the electrochemical sodium gradient across the plasma membrane provides the driving force for secondary active, sodium-coupled transport of a variety of solutes, including certain amino acids and bile acids through symports and antiports (Aronson, 1981; Freel and Goldner, 1981; Van Dyke and Scharschmidt, 1983). As well, fatty acids with a carbon chain length of less than 14 are absorbed actively by a sodium-dependent mechanism (in Blaxter, 1989). Stryer (1988) states that "most symports and antiports in animal cells are driven by Na\textsuperscript{+} gradients that are
generated by the Na\(^+\)-K\(^+\) ATPase”.

Van Dyke and Scharschmidt, (1983) measured the effects of sodium-coupled solute transport on intracellular sodium concentration and on Na\(^+\)-K\(^+\) ATPase-mediated cation pumping in rat hepatocytes under various conditions. Their results suggested that in those cells the activity of the sodium pumps is increased by an increase in cotransport of solutes and by a concomitant increase in intracellular sodium concentration. They report that the relationship of sodium pump activity to intracellular sodium concentration is sigmoidal in shape and that the normal intracellular sodium concentration is similar to the apparent K\(_{Na}\) of cation pumping\(^3\). This means that even small changes in the normal intracellular sodium concentration will have a strong impact on the activity of the sodium pumps which will oppose the changes and thus tend to maintain intracellular sodium homeostasis.

The liver is the major site of amino acid metabolism in the body. This includes both catabolic reactions (converting them into urea, carbon dioxide and water through the citric acid and urea cycles) which are energy-generating processes and anabolic reactions (leading to the formation of proteins and of a broad spectrum of biologically important metabolites for export to the other tissues), energy-consuming processes (See Lehninger, 1982 or Stryer, 1988). Kilberg (1982) has elaborated on the sodium dependence of amino acid transport in isolated rat hepatocytes. The evidence he reviewed suggest that amino acid transport (and thus a good part of the sodium influx) is dependent on hormones, especially insulin, glucagon, glucocorticoids and catecholamines in hepatocytes. In this light, it is not surprising that Skou (1992) stated insulin

\(^3\) The K\(_m\), or Michaelis constant, is defined as the substrate concentration for which the enzyme has reached half of its maximal activity or, from Stryer (1988), as the concentration of substrate for which half of the active sites are filled.
and catecholamines as stimulators of sodium pump activity and that Rossier et al. (1987) considered that catecholamines and insulin (along with thyroid hormones) modulate the activity or the number of sodium pumps. The link between these hormones and sodium pump activity could be through a stimulation of amino acid (protein) metabolism.

Sugars can also be carried across the plasma membranes of some cells by cotransport with sodium. Freel and Goldner (1981) proposed a model involving membrane proteins for the cotransport of glucose and sodium. In intestinal and kidney cells, glucose and sodium bind to a membrane protein (a symport) and penetrate together the plasma membranes. Other cell types rely on sodium-independent glucose transporters (Stryer, 1988). This latter mechanism is probably operative in liver cells, as to my knowledge no cotransport system involving sodium with glucose has been reported for this tissue.

Hulbert and Else (1981) have conducted a comparison between a mammal and a reptile of similar size and preferred body temperature but with different basal metabolic rates and levels of thyroid activity. Using ouabain as a specific inhibitor of the sodium pumps, they found that the mammals, which displayed higher thyroid activity, also spent more of their energy on the sodium pumps in their liver, kidney and brain than the equivalent reptilian tissues.

In 1988, Haber et al. reported that thyroid hormones increase sodium and potassium permeability in cultured rat liver cells. In their experiments, the thyroid hormone-induced increase in pump activity (measured as rubidium uptake) came after the increase in metabolic activity (as assessed using lactate production) and the increase in uptake of solutes such as deoxyglucose and
amino acids (here leucine); Guidotti et al. (1978) reported that, although leucine transport is sodium-independent, it belongs to an exchange system with other, sodium-dependent amino acids. Haber et al. (1988) also showed that actinomycin D (an inhibitor of RNA synthesis) or cycloheximide (an inhibitor of protein synthesis) abolished the effects of thyroid hormones on deoxyglucose uptake and lactate production.

If the results of Haber et al. (1988) are correct, they can be interpreted in terms of a sequence of events that tie thyroid hormones to sodium and potassium fluxes. In liver cells, a primary effect of thyroid hormones could be, after binding to nuclear receptors, to induce the synthesis of RNA and proteins. This would then require the transport of amino acids and of solutes for the synthesis of the peptides and to provide energy to the process. These would be largely transported using the sodium and potassium gradients, thereby activating the sodium pumps rapidly as the intracellular sodium concentration rises. Haber et al. (1988) could not detect any change in [Na]_i and suggested that the changes preceding the activation of the sodium pumps may have been too small to be detected with the sensitivity of the method that they used.

This interpretation is consistent with the evidence presented above, that sodium pump activity in rat hepatocytes is related to solute transport (Van Dyke and Scharschmidt, 1983) and that other hormones, especially insulin, glucagon, glucocorticoids and neurotransmitters such as catecholamines in hepatocytes influence both the rate of uptake of amino acids (Kilberg, 1982) and the activity of the sodium pumps (Rossier et al., 1987; Skou, 1992).

The roles of potassium gradients in metabolic function has not been much investigated. The paucity of information concerning the relationship
between metabolic activity, solute transport and potassium efflux in the scientific literature is surprising given its importance (the stoichiometry of the sodium pumps suggest that the rate of net potassium efflux must be two thirds of that of the net inward sodium movements). Even the comprehensive technical review by Aronson (1981), where the mathematics of secondary active transport are detailed, only mentions potassium flux in relation to the balance of charge distribution across the plasma membranes. Potassium efflux may be mostly involved in feedback mechanisms of transmembrane ion gradients and in the maintenance of membrane potential. Indeed, the permeability of phospholipid membranes is much greater for potassium than for sodium. Papahadjopoulos (1971) has showed that under optimal conditions liposomes made of pure phospholipids are up to 10 times more permeable to potassium than to sodium. Thus, sodium influx could be mostly coupled to metabolic functions whereas potassium efflux could be more regulated by physical and chemical processes than by physiological processes.

1.3.4.2 Permeability of plasma membranes in relation to membrane phospholipid composition

Studies using intact cells, where ion gradients are involved in various metabolic processes, have lead to the proposition that sodium pump activity, membrane permeability and composition may be related (Solomonson et al., 1976; Hulbert and Else, 1990). There is sufficient evidence in the literature to suggest that membrane phospholipid fatty acid composition per se influences permeability. Following are examples of studies performed with protein-free reconstituted liposomes.

In 1968, De Gier et al. reported that liposomes of different phospholipid fatty acid composition differed in their permeability to glycerol and erythritol.
They discovered that the introduction of double bonds in the fatty acid chains induced an increase in liposome permeability to these molecules. They also observed that decreasing the hydrocarbon chain length had a similar effect and that both factors had a greater influence at low temperature. They were among the first to realise that cholesterol, a common plasma cell membrane constituent, decreased liposome permeability.

Moore et al. (1969) observed differences in sodium permeability of lecithin (phosphatidylcholine) membranes of different fatty acid composition. They observed that manipulating the ω3 and ω6 fatty acid content of liposomes (referred to by the authors as “membrane particles”), prepared from various sources, had a major impact on their passive permeability to sodium ions. At 25 and 37°C, the liposomes containing lower levels of ω3 and ω6 fatty acids (prepared from EFA-deficient rats) were more permeable to sodium. At 50°C, the liposomes rich in ω3 and ω6 fatty acids were more permeable to sodium. The proportions of the families of fatty acids present in liposomes (ω6 and ω3) was more important to sodium permeability than the proportions of individual fatty acids from these families.

Recently, I have been involved in experiments demonstrating that liposomes prepared from mitochondrial phospholipids of rat liver are both more polyunsaturated and more permeable to protons than those from the bearded dragon (Amphibolurus vitticeps) (Brand, Couture and Hulbert, submitted). Liposomes from rat liver mitochondrial phospholipids were twice as permeable to protons, whereas in a previous study we had reported that intact rat mitochondrial inner membranes were about 4-5 fold more permeable to protons at the same membrane potential than those from the lizard (Brand et al., 1991). However, the liposomes, and especially those from rats, were less
polyunsaturated than the mitochondrial membranes, which could explain at least partly the differences in the results between liposomes and mitochondrial preparations.

This work is similar to earlier studies which linked the proton permeability of the inner mitochondrial membrane phospholipid bilayer and fatty acid composition. Nobes et al. (1989) had reported that mitochondria from isolated rat hepatocytes with different thyroid status displayed up to 7-fold difference in proton permeability. Thyroid hormones are known to affect membrane fatty acid composition, higher levels of thyroid hormones leading to more polyunsaturated membrane fatty acids (eg. Hulbert et al., 1976). Brand et al. (1992) then investigated the proton permeability in liposomes prepared from liver mitochondrial inner membranes of hypo- to hyperthyroid rats. The liposomes from hyperthyroid rats were found to be 3 times leakier to protons than those from hypothyroid rats.

Stillwell et al. (1993) have recently investigated the permeability characteristics of membranes of various fatty acid composition to three molecular species. They used the chromium ion (Cr\(^+\)), the small molecule erythritol (C\(_4\)) and the larger molecule carboxyfluorescein (C\(_{21}\)). Liposomes and cultured tumour cells containing various amounts of 22:6\(\Omega3\) or 18:1\(\Omega9\) in phosphatidylcholine (one of the two fatty acids in this phospholipid was saturated, as usually found in membranes) were compared to controls containing saturated phosphatidylcholine (18:0) instead. Their results make it clear with both tumour cells and liposomes that adding the more unsaturated fatty acids greatly increased the permeability of the membranes. They also compared their results with previous reports and concluded that the major effect of adding 22:6\(\Omega3\) to membranes is an increase in permeability beyond
what is observed when adding less unsaturated fatty acids to membranes. This contrasts with the effects reported for membrane fluidity, as membranes containing monounsaturates, 18:3ω3 or 22:6ω3 exhibit similar fluidity (measured with fluorescent probes) (Popp-Snijders et al., 1986; Ehringer et al., 1990).

The accumulation of evidence has left little doubt that the permeability of cell membrane phospholipid bilayers to various ions and molecules can differ according to membrane composition. The remainder of the differences in membrane permeability found in various cells could be accounted for by processes involving membrane proteins.

1.3.4.3 The importance of sodium and potassium pumping in cell metabolism and the possible effects of ouabain on cell metabolism.

There is some controversy as to whether or not sodium and potassium transport is an important cost to cell metabolism. Much of this controversy has been brought about by the methods used for the estimation of the oxygen consumption associated to the action of the sodium pumps. Before the availability of sensitive oxygen electrodes, the common way to estimate the contribution of the sodium pumps was by using a Warburg manometer. This method involved the measurement of oxygen consumption over time periods as long as 45 minutes in sealed flasks in the presence or absence of ouabain, a specific inhibitor of the sodium pumps (Rossier et al., 1987; Skou, 1992). Such experiments typically yielded very high estimates of the contribution of sodium pumps to cell metabolism, usually 30% or more (see Hulbert and Else, 1981 and Else and Hulbert, 1987 for example). Whittam (1961) suggested that the maintenance of sodium and potassium gradients across the plasma membrane
can be an important cost for the cells.

The early claims by Whittam (1961), Smith and Edelmann (1979) and Else and Hulbert (1987) that an important proportion of the liver cell ATP turnover was consumed by the sodium pumps have been criticised recently by Clausen et al. (1991). It appears from their review that in liver, under normal physiological conditions, around 5% of the cell energy is devoted to the sodium pumps. Using an oxygen electrode and taking measurements of oxygen consumption rates 6 minutes after the addition of ouabain, Nobes et al. (1990) found that 6 to 10% of the total oxygen consumption of resting rat liver hepatocytes was inhibited by ouabain and we reported that in lizard hepatocytes the sodium pumps were responsible for 10% of the oxygen consumption (Brand et al., 1991). Higher values are obtained in epithelial cells such as kidney cortex (Clausen, 1991) when these cells are involved in reabsorption and transport of nutrients and ions, processes usually coupled to sodium transport (Skou, 1992). Lote (1987) proposed that the filtering kidney cortex can devote around 50% of its energy for sodium transport, as sodium influx is high in these cells due to reabsorptive processes of the kidney. Thus the high sodium transport rates found in some tissues can be associated with a specific metabolic function that makes use of the sodium gradients.

We have already discussed the role of the sodium gradient and sodium influx in cell metabolism and the influence of plasma membrane phospholipid fatty acid composition in affecting sodium permeability. From the evidence presented above, it seems that sodium pumping (and thus membrane permeability to sodium and potassium) only becomes a major contributor to the total cell metabolic rate in situations where the sodium gradient is involved in physiological work. The passive permeability of plasma membranes (ie not
associated to any metabolic function other than the consumption of ATP and consequent heat production) associated to sodium would be a minor contributor to cell metabolic rate. In liver cells at least, in the improbable case that most of the sodium influx would occur as a pure cost to the cells this leak could represent perhaps 5% of their oxygen consumption.

Mechanistically, the cost of this plasma membrane cation leak can perhaps be compared to the cost of the proton leak across the inner mitochondrial membrane in state 4 respiration. The proton gradient, maintained at the cost of energy and oxygen consumption, is used in state 3 respiration to drive ATP synthesis, just as the sodium and potassium gradients are used for various metabolic processes. Even in the absence of ATP synthesis (ie during state 4 respiration) protons still leak across inner mitochondrial membranes. This passive leak, which results in oxygen consumption and heat production, is estimated to be responsible for 20-25% of the resting oxygen consumption of rat hepatocytes (Nobes et al., 1990; Brown et al., 1990) and it has therefore been proposed as a significant contributor to basal metabolism (Brand, 1990).

The "leaky membranes as a source of heat" hypothesis was proposed by Else and Hulbert (1987) as a mechanism by which mammals could have increased their basal metabolism over that of their ectothermic ancestors partly by developing plasma membranes more leaky to sodium and potassium, thus spending additional energy (and producing extra heat) for maintaining the gradients. For the allometric comparison of mammalian metabolism, I propose in this thesis to see the relationship from another perspective. Sodium influx may be mostly associated to cellular metabolic activity and in cells such as hepatocytes, which are not involved in large scale reabsorptive processes,
sodium influx and thus sodium pump activity may be closely related to cell metabolic activity. Simply put, since the sodium gradients are used by cell metabolism and restored by the sodium pumps a match between cell metabolism and sodium pump activity is expected. To test this hypothesis, I have measured the rates of potassium uptake (an indicator of sodium pump activity) and of oxygen consumption in liver and kidney cortex slices from a range of mammals displaying different metabolic rates.

As we have seen, long-term exposure of cells to ouabain (such as in experiments with a Warburg manometer) leads to larger decreases in oxygen consumption rates than when using other methods such as oxygen electrodes and short incubation periods (Brand et al., 1991; Clausen et al., 1991). I propose here a mechanism by which long-term exposure to ouabain can lead to an important inhibition of cell metabolism as well as a possible physiological significance of this decline in oxygen consumption in cells such as liver cells that are not involved in large-scale reabsorptive processes. Once ouabain has inhibited the sodium pumps, the transmembrane sodium and potassium gradients collapse slowly. When these gradients have disappeared, the part of the cell metabolism that is directly dependent on them will be inactivated. Thus, the early experiments where ouabain was used with cells or tissue slices incubated in a Warburg manometer for extended periods may have actually yielded estimates of the sodium gradient-dependent oxygen consumption. Such metabolic processes may be, for example, those that depend on amino acid transport such as protein synthesis and they, not the sodium pumps, may be an important cost to non-epithelial cells. Deprived of such processes, the cells will eventually die. It could be the disappearance of the sodium gradient more than the presence of high intracellular sodium or low potassium concentrations that is most detrimental to the cells.
Since the blocking of the sodium pumps leads to a gradual decline in cell metabolism any choice of incubation time after the addition of ouabain for the estimation of the cell energy devoted to the sodium pumps remains arbitrary, although choosing the shortest time after which maximal inhibition of the sodium pumps has been achieved will yield the most realistic estimates. For this reason, in this thesis I have sought means to estimate the contribution of the sodium pumps to cell metabolism that did not involve the use of ouabain and the concomittant gradual collapse of the sodium and potassium gradients across the plasma membranes.

1.3.5 Impact of membrane phospholipid fatty acid composition on protein function

Aside from inducing changes in membrane permeability, modulating membrane phospholipid fatty acid composition can also affect the function of membrane proteins. In 1963, Green and Fleisher postulated that the interactions of phospholipids with membrane proteins are of two types: ionic interactions take place between acidic phospholipids and basic proteins, while hydrophobic interactions occur throughout the bilayer. The fluid mosaic membrane model proposed by Singer and Nicolson (1972) was based on the evidence that the lipid environment of the membranes allows the movements of membrane-bound proteins. Since then, strong evidence has been found to support the hypothesis that the physical state of membrane lipids alters protein function. Some of it is presented below.

Kimelberg (1975) studied the activity of the sodium pumps from rabbit kidney outer medulla, then compared their activation energy in the presence or absence of cholesterol. He also investigated the effects of two phospholipid
classes, phosphatidylserine (PS) and phosphatidylcholine (PC) on sodium pump activity and the concentrations of Mg^{2+} and ATP required for maximal activity of the sodium pumps. The first step in this experimental approach consisted of extracting the sodium pumps from the cell membranes using deoxycholate, a detergent. The membrane enzymes were then relipidated by sonication in the presence of various proportions of PS, PC and cholesterol, to form liposomes. The Arrhenius plots of enzyme activity at different temperatures were then established. This approach allowed the determination of the phase transition temperature of the liposomal membranes and of the Arrhenius activation energy of the sodium pumps.

From these findings, it was suggested that sodium pump activity is altered by the fluidity of the reconstituted liposome lipid environment. Adding cholesterol, for example, abolished the temperature-related phase transition of the lipid bilayers and increased the activation energy of the membrane-bound enzymes. Sonicating the sodium pumps with phosphatidyl serine, but not with phosphatidyl choline, reactivated the enzymes. These findings were taken as evidence that the activity of the sodium pumps is affected by membrane fluidity and that the class of phospholipids present in the vicinity of membrane-bound enzymes affect their activity.

Solomonson et al. (1976) described changes in sodium pump activity measured in partially purified plasma membranes of Ehrlich ascites tumour cells with different phospholipid fatty acid composition. These changes were induced by growing tumour cells in mice fed diets in which the fats varied in their unsaturation. The sodium pumps were less active in membranes where 18:1\omega9 and 20:3\omega9 were more abundant and 18:2\omega6 and 20:4\omega6 less abundant.
Several reviews on the above topics have been written (Stubbs and Smith, 1984; Hazel, 1988; Clandinin et al., 1991). These reviews cover different aspects of the roles and impact of membrane phospholipid fatty acid regulation for membrane-bound protein and membrane and cell function. It has been incontrovertibly demonstrated that polyunsaturated fatty acids are required in biological membranes if their proper functioning is to occur (Stubbs and Smith, 1984).

It is now apparent that, just as the type of lipids in a membrane affect protein function, the presence of membrane proteins affects the physical properties of membranes (Stubbs and Smith, 1984). Given the ratios of proteins to lipids in some biomembranes (approx. 1:4 to 4:1. Evans and Graham, 1989), this is not surprising. The early notion that membrane proteins are surrounded by lipids somewhat distinct from the bulk membrane lipids is inconsistent with the evidence that the exchange rate between annular and bulk lipids is rapid (see Stubbs and Smith, 1984). According to Stubbs and Smith (1984), this means that the modulation of both protein mobility and conformation in the membrane will be due to all of the lipids in the area of the protein and not only to those in temporary contact with them. These authors also reviewed the extensive evidence that the activity of several membrane enzymes is affected by membrane composition. The literature reviewed by them supports the early findings reported above that ATPases are greatly affected by fatty acyl chain length as well as by phospholipid head group interactions. Although no definite relationship between membrane fluidity and ATPase motion and conformation has been demonstrated, it can be said that in general increased order in the membrane phospholipid bilayer is correlated with decreased ATPase activity.
This section would be incomplete without mentioning the contribution of Lee (1991) in the discussion of the interactions of membrane phospholipid fatty acids and proteins. He argues that it is unnecessary to imply a direct relationship between fluidity and membrane protein activity in normal physiological conditions. He concluded that while it was clear that many membrane enzymes could only function properly if the membrane was in a fluid state, the modulations in cell membrane phospholipid fatty acid composition could not be thought as tight regulators of membrane protein activity. Lee argues that experiments to determine the effects of fluidity on membrane protein activity have failed to yield convincing evidence. The factors acknowledged by Lee to affect membrane protein activity are the physical state of the membrane (the extent of order of the phospholipids, gel or liquid crystalline) and the phospholipid head groups, whose charges interact with embedded proteins.

I agree with Lee (1991) that it is unrealistically simplistic to attribute the regulation of membrane protein activity solely to membrane fluidity. His propositions support well the conclusions of other reviewers, discussed above, who addressed the relevance of the concept of membrane fluidity to membrane function (Stubbs and Smith, 1984, for example). Physiologists may misuse the word fluidity when discussing the importance of membrane composition on their function.

1.3.6 Reported correlations of cell membrane phospholipid fatty acid composition and metabolic rate in interspecific comparisons

Changes in cell membrane composition have often been associated to changes in metabolic activity. For example, I have already mentioned the effects
of thyroid hormones on these parameters in rats (Hulbert et al., 1976). Other examples have also been briefly mentioned which are the subject of extensive reviews, such as thermal acclimation in ectotherms (Hazel, 1988 for example). Membrane enzymes such as the sodium pumps can also increase in number if the work load exceeds their capacity (Lamb, 1990 offers a brief review on the subject). This other aspect of adaptation of membrane composition will not be discussed in this thesis.

Interspecific comparisons considering both metabolic activity and membrane composition have also consistently yielded similar relationships, although such reports are rare in the literature. Still today, to my knowledge most of such evidence comes from two sets of experiments, one of them between vertebrates of two classes, reptiles and mammals, the other one, less detailed, among mammalian species of different body sizes. These are introduced here to put in context the hypotheses described at the end of this chapter. They will be further encountered in the discussion of Chapter 2.

There is an important difference in the basal metabolic rate of mammals and reptiles, even when body temperature and body weight are kept constant. A rat for example has a basal metabolic rate that is 7-fold that of a bearded dragon (Amphibolurus vitticeps) (Brand et al., 1991). Hulbert and Else (1989) reviewed the evidence (that they had mainly contributed to build) that mammals have a higher proportion of polyunsaturated fatty acids in their membranes than reptiles of the same size and body temperature. They reported that although the mammalian phospholipids contained significantly less total unsaturated fatty acids, these unsaturated fatty acids were significantly more polyunsaturated.
In homeotherms, Gudbjarnason and Oskarsdottir (1975), in an investigation of heart stress tolerance and membrane fatty acid composition, injected isoproterenol to cause physiological stress in heart tissue. They found that this drug caused the accumulation of longer and more polyunsaturated fatty acids in heart tissue as well as an increase in heart rate. Curious about this relationship, they measured the phospholipid composition in the heart muscle of animals displaying large differences in heart rate. They reported a decrease in polyunsaturation of the heart phospholipids with increasing body size (and decreasing heart rate).

Continuing this investigation, Gudbjarnason et al. (1978) reported that, in a range of mammals, the amount of 22:6Ω3 is a key to the decrease of polyunsaturation with increasing heart rate (and body size) in the heart cell membranes. They proposed that this fatty acid could participate in the formation of cation-conducting (possibly Na⁺) transmembrane channels and that more of them could be required in more metabolically active cells. The findings of Stillwell et al. (1993), reported above, agree with this possibility as they found that membrane permeability to substances such as Cr⁺, erythritol (C₄) or carboxyfluorescein (C₂₁) could be increased if high amounts of 22:6Ω3 were present.

The findings by the Gudbjarnason team, reported above, are the most direct evidence to date for a relationship between metabolic rate and tissue polyunsaturation in different-sized mammals. The investigations in this thesis aim at enlarging this relationship found in heart by analysing the detailed composition of heart muscle phospholipid fatty acids in different-sized mammals and by including four other tissues in the study: the liver, the kidney cortex, the brain and another striated muscle, skeletal muscle.
1.4 Summary and hypotheses

The ubiquity of the phospholipid membrane throughout the living world argues for the versatility of this structure when cells impose different constraints on them. Membranes are partitioning structures capable of providing a suitable environment for the proper functioning of the molecules and processes that occur on, in or through them. The myriad cell types that have evolved vary greatly in their metabolic demands and so to in their membrane composition.

Cell membrane phospholipids, whose fatty acids are either synthesized in the organisms themselves or incorporated from the diet, react to the cellular metabolic activity by adjusting their composition through a yet not completely understood feedback process involving a battery of enzyme systems, the elongases, desaturases and transferases; in the above sections we found evidence that membrane composition is associated with differences in cellular metabolic activity. It is difficult with most of the interspecific studies reported so far to discriminate between the effects of metabolism itself and other factors, such as genetic distance, on membrane composition.

The case of body size in mammals comes forth as a good candidate for the investigation of this question. It is known that the mass-specific metabolic rate in mammals is a function of their body mass (Figure 1.1). The mass-specific metabolic rate of the mouse is 11 times that of a cattle (Table 1). To decrease the genetic distance between the species chosen, as it increases the chances of observing variations in body composition unrelated to metabolism, only eutherian mammals were chosen. Among those eutherians, I chose two rodent species, mouse (Mus musculus) and rat (Rattus norvegicus), as well as a
lagomorph, the rabbit (*Oryctolagus cuniculus*), and two ruminants, sheep (*Ovis aries*) and cattle (*Bos taurus*). These animals were also chosen because of the even spread of their body weights on a logarithmic scale, from 30 to 300 000g. As well, their choice was favored by the relative easy availability of their tissues and their substantial research histories.

This thesis has been divided in three parts. Part 1 is concerned with the analysis of tissue phospholipid fatty acid composition, Part 2 with physiological measurements and Part 3 examines relationships between the first two parts: membrane composition vs. tissue metabolic rate, sodium pump activity and plasma membrane permeability to potassium. In Part 1 (Chapter 2), the composition of the tissue phospholipid fatty acids from mammalian species presenting a range of size and metabolic rates will be examined for liver, kidney cortex, heart, skeletal muscle and brain. The following hypothesis will be examined:

1- **Mammals with a higher mass-specific basal metabolic rate possess more polyunsaturated cell membranes.**

Part 2 (Chapter 3) will verify if the scaling of basal metabolic rate is partly due to larger mammals having less metabolically active tissues and less active transport of potassium by the liver and kidney cortex in these species. The first step in this investigation will be to quantify the importance of the allometric variations in tissue metabolic rate, using tissue slices, given that differences in relative organ size cannot fully account for the lower rate of basal metabolism in larger mammals. The differences in mass-specific oxygen consumption rates in the tissues of different-sized mammals will be assessed.
Variations in intracellular water space (dextran-excluding water space) per unit mass that could explain differences in mass-specific tissue metabolic rate will also be considered. The hypothesis to be tested in these experiments will be:

2- The scaling of mass-specific basal metabolic rate is partly due to the scaling of mass-specific liver and kidney cortex metabolic rate.

I have also discussed that the sodium gradient is essential for cell metabolism and that the sodium influx associated with metabolic functions may be more important for cell energetics than the passive membrane permeability. Since the activity of the sodium pumps is mainly controlled by variations in intracellular sodium concentration, a greater use of the sodium gradient will be associated to greater sodium pump activity. The rate of $^{86}$Rubidium (a substitute for potassium) uptake by liver slices and kidney cortex slices from different-sized mammals will be used to assess the extent of coupling between sodium pump activity and metabolic activity. The working hypothesis will be:

3- Sodium pump activity is directly proportional to metabolic activity.

Part 3 (Chapter 4) will be concerned with possible relationships between tissue phospholipid fatty acid composition, metabolic rate and membrane permeability. This chapter will mainly consider membrane polyunsaturation and indicators of cell metabolism in liver and kidney cortex of different-sized mammals. The first step in this comparison will be to compare the rates of
oxygen consumption measured with tissue slices to indicators of membrane polyunsaturation. The hypothesis will be:

4- Mammalian tissues displaying higher mass-specific metabolic rate possess more polyunsaturated cell membranes

Since evidence has been presented that membrane composition affects membrane-bound enzyme activity, the possible association of membrane polyunsaturation to metabolic activity can be in part due to relationships between metabolic demands and the activity of such membrane-bound enzymes as the sodium pumps. Thus, the relationship between membrane polyunsaturation and sodium pump activity will be investigated. To this end, the following hypothesis was formulated:

5- Mammalian tissues whose cell membranes are more polyunsaturated also display higher sodium pump activity.

Finally, since correlations have been proposed between membrane polyunsaturation (and in particular the proportions of highly polyunsaturated fatty acids such as 22:6\(\omega_3\)) and permeability to protons and various molecules, the last hypothesis aimed at investigating the possible relationship between membrane permeability to potassium and polyunsaturation, with special reference to 22:5\(\omega_3\) and 22:6\(\omega_3\). The hypothesis is:

6- Tissues whose cell membranes contain higher levels of highly polyunsaturated fatty acids display higher rates of potassium efflux.
Chapter 2

Allometric Comparison of Mammalian Tissue Phospholipid Fatty Acid Composition
2.1 Introduction

This chapter aims to test the first hypothesis of this thesis, that mammalian tissues displaying higher mass-specific metabolic rate possess more polyunsaturated cell membranes. To this end, the complete phospholipid fatty acid composition of five tissues (liver, heart, kidney, brain and skeletal muscle) from five mammalian species of very different size and metabolic rate (mouse, rat, rabbit, sheep and cattle) was determined.

Total tissue phospholipid fatty acid was used in this study instead of various isolated membranes as this is the first investigation on the subject. Although the fluxes of sodium and potassium and their active transport takes place across the plasma membrane, various metabolic processes occur in other cell membranes. Thus as a first step in the investigation of the relationships between membrane composition and metabolism a pooled sample of all cell membranes was considered appropriate.
2.2 Materials and Methods

2.2.1 Animals and tissues

All animals used were adults and appeared to be in good health. The mice (*Mus musculus*, breed CBA/H), rats (*Rattus norvegicus*, breed Wistar) and rabbits (*Oryctolagus cuniculus*, breed New Zealand White) were killed by concussion. Mice and rats were fed *ad libidum* Rat and Mouse Cubes (Allied, NSW). Rabbit pellets were from Sieldorf, NSW. All laboratory animals had free access to water. The sheep (*Ovis aries*) and cattle (*Bos taurus*) tissues were collected within 30 minutes after death at Yallah abattoir and transported back to the laboratory on ice. Liver samples were taken randomly from the main lobe. Kidney cortex samples were taken randomly for all animals except in mice where the whole kidney cortex was used. Heart samples were excised from the ventricular muscles in sheep and cattle while the whole cardiac muscle was kept for smaller mammals. Brain samples were collected from the cortex. Skeletal muscles were the gastrocnemius for mice, rats and rabbits while for sheep and cattle samples from the inner thigh were obtained. Tissues were frozen within 30 minutes after death except for sheep and cattle tissues, frozen within 90 minutes after the death of the animal. The tissues were kept at -80°C for a period of time not exceeding 12 weeks. Phospholipid fatty acid composition remained stable for up to 6 months under those conditions (Kerry Withers, pers. comm.).

Mice, rats and rabbits were weighed after death and their mean (±s.e.) weight was 42.1±1.2g, 581±44g and 4100±737g, respectively. The weight of individual animals could not be obtained for sheep and cattle, so an average of carcass weight from the experiments in Chapter 3 was used to estimate the weight of a whole animal, using carcass weight as 55% of total body weight.
The weights used for calculations are $32.9 \pm 0.8$ Kg for sheep and $369 \pm 34$ Kg for cattle.

2.2.2 Chemicals

All solvents used in the extraction and purification of the fatty acids from tissue phospholipids were of nanograde purity and were purchased from Mallinckrodt, except for methanol, which was obtained from BDH. Various sources of commercial fatty acid methyl ester standards were used. Individual fatty acid methyl esters were purchased from Sigma Chemicals, except 22:1Ω9, purchased from Nucheck Prep. Inc. (USA). Commercial mixtures of fatty acid methyl esters used were PUFA 2 (Supelco), Standard Lipids Mixture ME82 and Qualmix Fish (Larodan).

2.2.3 Analysis of total membrane phospholipid fatty acid composition

A- Extraction of total lipids

To prevent contamination, only very clean glassware and tools were in contact with the samples. All solvents, except hexane, contained 0.01% butylated hydroxytoluene (BHT), an antioxidant. This prevents the degradation of unsaturated fatty acids by atmospheric oxygen. The samples can be stored in solvent at -20°C at the end of any stage, provided that the atmosphere in the tube is replaced by nitrogen.

To extract the total lipids from tissue samples, approximately 100 mg of tissue was placed in a 50 ml erlenmeyer flask and 21 ml of chloroform:methanol (2:1)\(^1\) was added. The sample was then homogenized using an Ultra Turrax,

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\(^1\) When extracting lipids from aqueous suspensions such as mitochondria, 20 volumes of chloroform:methanol are added instead of 21 ml and step 3 is omitted.
increasing speed gradually until maximal speed is reached. Homogenization was stopped 30 seconds after the clumps of tissue had disappeared. The probe was then checked for pieces of tissues stuck to the tip, these were removed with tweezers and the sample rehomogenized for an additional 20 seconds.

Maximal extraction of the lipids was ensured by leaving the homogenate at room temperature for 20 minutes. After that time, the sample was filtered (Whatmann #1) into a separating funnel, as illustrated in Figure 2.1. The erlenmeyer flask and filter were washed with 2-3 ml of chloroform:methanol (2:1). When filtration was completed, 5 ml of 0.73% NaCl in water was added to the separating funnel. After the funnel had been vigorously shaken, the phases were left to separate for at least 10 minutes. The top (hydrophilic) phase contained water, methanol, salts, sugars, nucleic acids and proteins. Lipids were in the bottom (hydrophobic) phase.

Once the phases had separated, the bottom (chloroform) phase was
collected by filtering (Whatmann #1) through anhydrous sodium sulphate, as in Figure 2.1. The extraction was repeated by three successive additions of 2 ml chloroform and the chloroform phases combined. The sample was collected in a Rotavapor flask (preweighed if the weight of the total lipids was desired).

Chloroform was evaporated off to a convenient volume, around 3-5 ml (evaporated dry if the weight of total lipids was required) using a Rotavapor R (Büchi) with the water bath at 37°C.

B- Separation of phospholipids and neutral lipids

This step (modified from Hirsch and Ahrens, 1958) was performed using hand-made chromatography columns, illustrated in Figure 2.2. The procedure was accelerated by applying a gentle vacuum to the column.

To prepare the silicic acid column, just enough glass wool to block the bottom opening of the column was inserted and packed using a glass rod. The amount of silicic acid required to give the desired column length was measured by adding the powder to the column. Maximal separation was achieved with a 7 x 0.3 cm column (refer to preliminary experiments, below). This powder was transferred to an erlenmeyer flask and a slurry made by addition of chloroform. The slurry was then added to the column and silicic acid allowed to settle, ensuring that the column did not run dry by adding more chloroform when necessary. Once the level of chloroform was a few millimeters from the top of the column, column was ready to use.

The sample was then added to the column with a pasteur pipette, taking care not to disturb the surface of the column. When the sample had almost
completely penetrated the column, 10 ml of chloroform was added. The neutral lipids were thus eluted, as they do not adhere to silicic acid in the presence of a non-polar solvent.

The elution of neutral lipids was complete when the added chloroform had almost completely passed through the column (refer to preliminary experiments, below). The collection test tube was then changed for a 10-15 ml pyrex test tube with a screw top and 10 ml of methanol (a polar solvent that releases the phospholipids) was added. The column was let run dry.

Figure 2.2
Glass columns for column chromatography.

C- Methylation of fatty acids

During this procedure, the bonds between fatty acids and the glycerol backbone are broken. The carboxyl end of the fatty acids is methylated to yield methyl esters of the fatty acids.
The purified phospholipids were dried with a stream of nitrogen (the tubes can be immersed in 35°C water to speed the procedure). 2 ml of 20% boron trifluoride in methanol was then added to redissolve the phospholipids. The tubes were tightly sealed and heated in a water bath at 75°C for 60 minutes, then cooled on ice.

The fatty acid methyl esters were then extracted as follows: 3 ml of water and 3 ml of petroleum ether (BP 40-60°C) were added and the sample mixed. The top (petroleum ether) phase, containing the fatty acid methyl esters, was transferred to a clean screw top test tube. Two more additions of petroleum ether were made to ensure maximal recovery of the fatty acid methyl esters. The extracts were combined.

D- Purification of fatty acid methyl esters

The same chromatography columns as for the separation of neutral lipids from phospholipids (Figure 2.2) were used for this step and they were prepared the same way, except that petroleum ether was used instead of chloroform and Florisil (containing 7% water w/w) instead of silicic acid. After the sample was added to the column (the fatty acids will adhere to the Florisil in the presence of petroleum ether), BHT and other possible contaminants were removed by washing the column with 10 ml of petroleum ether. The fatty acid methyl esters were then eluted by the addition of 10 ml of petroleum ether containing 5% diethyl ether. The purified fatty acid samples were dried and redissolved in hexane. They were now ready for gas chromatography.
Analysis of fatty acid composition

The fatty acid analysis was performed using a Varian 3300 gas chromatograph with a 25 m BPX70 fused silica capillary column (internal diameter: 0.22 mm and 0.25 μm coating thickness) purchased from SGE Scientific. The column was connected to a flame ionisation detector coupled to a Shimadzu C-R3A chromatopac integrator. Depending on the split ratio, 0.1 to 1 μl of sample was injected. The temperature program (initial temperature 100°C and 4°C rise per minute until 235°C, no plateau) was sufficient to elute all fatty acids.
Identification of peaks

All fatty acids were identified by matching their retention time to that of standards. The only available standard for 17:1 did not mention its class (Ω). This was determined using retention times of sample peaks as in Figure 2.3.

Following the identification of peaks, only those peaks that were 0.1% of total or more were kept for the analysis of the data. Each of the unidentifiable peaks constituted less than 0.75% of the total peak area except for an unknown appearing once in a sheep and once in a cattle liver and which represented 2.9 and 1.0% of the sample respectively. Unknowns were ignored in the analysis of the results.

\[ y = 39.998 - 5.684x + 0.235x^2 \quad R^2 = 1.000 \]

**Figure 2.3**
Identification of an unknown by polynomial regression. The 3 fatty acids for which the retention times known from standards are plotted with the unknown, which is assumed to be a Ω7 monounsaturate with 17 carbons. The perfect fit includes the unknown. The data are in quadruplicate (superimposed) (from cattle muscle).
2.2.4 Calculations

The integrator calculated the relative peak area of each fatty acid. The area of a peak is proportional to the weight of the molecules ionised together. These percentages were used directly in older literature as weight %, after normalization to 100% once the unidentified or undesired peaks, such as traces of BHT and the solvent peak, had been substracted.

In this thesis, all the results are expressed in mol %. This unit indicates the ratios of fatty acid molecules, instead of the ratios of their weights. To convert weight % to mol %, each weight % in a sample was divided by the molecular weight of that particular fatty acid (from the Handbook of Chemistry, 1973). These relative molar ratios were divided by the total relative molar ratios of a sample and multiplied by 100.

The unsaturation index (UI) represents the average number of double bonds per 100 membrane fatty acid molecules. It was calculated by summing the products of the proportion (mol %) of each unsaturated fatty acid multiplied by its number of double bonds.

To calculate the average chain length, the chain length of each fatty acid was multiplied by its proportion (mol %) and the sum of these products divided by 100.

The other indexes were calculated simply by adding the proportions (mol %) of the relevant fatty acids.
2.2.5 Statistics

The differences between species for the indexes reported in Tables 2.1 to 2.5 were tested for significance using a Mann-Whitney U test (Porkess, 1988) and a significance level of 5% for a two-tail test.

All allometric relationships were plotted the conventional way, on double logarithmic plots and the linear regressions were obtained using Cricket Graph (version 1.3.1) software. The correlations with body weight were tested using the Pearson product moment correlation coefficient (r) and a two-tail test. The level of significance has been indicated in each case. These regressions were tested for significance using the mean values for each species, giving only 3 degrees of freedom. Including the individual data (20 for each regression) would allow 18 degrees of freedom and many more regressions would be significant. This very demanding method (three degrees of freedom) was chosen as it highlighted only the most significant allometric relationships.

The slope of a logarithmic relationship can be interpreted in terms of the % change of a given parameter y for a doubling of x. In the allometric comparison, it is calculated as follows:

\[ \text{% change for a doubling in body size} = (1-2^{\text{slope}}) \times 100. \]
2.3 Results

The presentation of the results has been partitioned to highlight the relationships observed between the various parameters measured. At the end of the results section, Table 2.7 summarizes the major statistically significant allometric trends observed in each tissue. It may be convenient to refer to this table when allometric variations are described and discussed throughout this chapter.

2.3.1 General differences in tissue membrane phospholipid fatty acid composition

Tables 2.1 to 2.5 describe all fatty acids found in mammalian tissue phospholipids that make up at least 0.1% of the total fatty acid composition. The data for liver and kidney are presented first, as these tissues were also examined in Chapter 3. They are followed by heart, for which an allometric decrease of unsaturation (Gudbjarnason and Oskarsdottir, 1975) and 22:6\(\alpha_3\) (Gudbjarnason et al., 1978) have previously been reported (see Chapter 1). The fatty acid composition for the other muscle examined, skeletal muscle, is then presented, followed by brain.

The tables are organized by presenting first the non-essential fatty acids (NEFA: includes saturated, \(\omega_{11}, \omega_9\) and \(\omega_7\) fatty acids), followed by the essential fatty acids (EFA: includes \(\omega_6\) and \(\omega_3\) fatty acids). This organization also reflects the position of the last double bonds in the bilayer. Within each group, the fatty acids were organized in increasing length and/or degree of unsaturation. This organization highlights the fact that in mammalian membranes the NEFA included all the saturated as well as monounsaturated fatty acids, while the EFA comprised all the polyunsaturates except one, 20:3\(\omega_9\).
**TABLE 2.1**

Total membrane phospholipid fatty acid composition in the LIVER of five mammalian species. Results are average mol % ± s.e. (n=4). Indexes in a row with common exponent letters do not differ significantly (p < 0.05).

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>MOUSE</th>
<th>RAT</th>
<th>RABBIT</th>
<th>SHEEP</th>
<th>CATTLE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saturated</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16:0</td>
<td>17.0 ±0.8</td>
<td>14.3 ±0.6</td>
<td>12.8 ±0.5</td>
<td>12.0 ±0.7</td>
<td>10.7 ±0.7</td>
</tr>
<tr>
<td>17:0</td>
<td>0.3 ±0.0</td>
<td>0.6 ±0.0</td>
<td>0.5 ±0.1</td>
<td>1.1 ±0.1</td>
<td>0.7 ±0.0</td>
</tr>
<tr>
<td>18:0</td>
<td>16.2 ±0.3</td>
<td>22.9 ±1.6</td>
<td>16.8 ±0.5</td>
<td>29.2 ±0.9</td>
<td>29.2 ±0.2</td>
</tr>
<tr>
<td>20:0</td>
<td>0.2 ±0.0</td>
<td></td>
<td></td>
<td></td>
<td>0.3 ±0.3</td>
</tr>
<tr>
<td>22:0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O11</td>
<td>0.4 ±0.0</td>
<td>0.2 ±0.0</td>
<td>0.2 ±0.1</td>
<td>0.1 ±0.1</td>
<td></td>
</tr>
<tr>
<td>O9</td>
<td>1.1 ±0.0</td>
<td>0.9 ±0.1</td>
<td>0.8 ±0.2</td>
<td>0.3 ±0.2</td>
<td>0.3 ±0.1</td>
</tr>
<tr>
<td>18:1</td>
<td>9.1 ±0.2</td>
<td>3.8 ±0.4</td>
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<td>16.0 ±1.5</td>
<td>10.1 ±0.3</td>
</tr>
<tr>
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<td>0.2 ±0.0</td>
<td>0.2 ±0.1</td>
<td>0.1 ±0.1</td>
<td>0.1 ±0.1</td>
<td></td>
</tr>
<tr>
<td>20:3</td>
<td>1.1 ±0.0</td>
<td>0.4 ±0.0</td>
<td>1.0 ±0.1</td>
<td>0.7 ±0.1</td>
<td>0.5 ±0.1</td>
</tr>
<tr>
<td>22:1</td>
<td></td>
<td>0.2 ±0.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O7</td>
<td>0.3 ±0.0</td>
<td>0.3 ±0.0</td>
<td>0.2 ±0.1</td>
<td>0.3 ±0.2</td>
<td>1.8 ±0.4</td>
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<td>0.9 ±0.1</td>
<td>0.8 ±0.2</td>
<td>0.3 ±0.2</td>
<td>0.3 ±0.1</td>
</tr>
<tr>
<td>16:1</td>
<td>0.3 ±0.0</td>
<td>0.5 ±0.0</td>
<td>0.2 ±0.1</td>
<td>0.8 ±0.0</td>
<td>0.9 ±0.1</td>
</tr>
<tr>
<td>18:1</td>
<td>3.6 ±0.1</td>
<td>3.2 ±0.2</td>
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<td>0.2 ±0.1</td>
<td>0.9 ±0.1</td>
</tr>
<tr>
<td>O6</td>
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<td>0.1 ±0.0</td>
<td>0.1 ±0.1</td>
<td>0.1 ±0.1</td>
<td>0.1 ±0.0</td>
</tr>
<tr>
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<td>6.7 ±0.4</td>
<td>9.0 ±0.7</td>
</tr>
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<td>18:3</td>
<td>2.2 ±0.1</td>
<td>0.8 ±0.0</td>
<td>1.0 ±0.1</td>
<td>0.4 ±0.1</td>
<td>3.0 ±0.3</td>
</tr>
<tr>
<td>20:3</td>
<td>23.2 ±0.5</td>
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<td>10.3 ±0.7</td>
<td>9.5 ±0.3</td>
<td>15.0 ±1.5</td>
</tr>
<tr>
<td>20:4</td>
<td>0.5 ±0.0</td>
<td>0.6 ±0.0</td>
<td>1.0 ±0.2</td>
<td>0.1 ±0.1</td>
<td>1.7 ±0.5</td>
</tr>
<tr>
<td>22:4</td>
<td>1.2 ±0.1</td>
<td>0.6 ±0.1</td>
<td>0.9 ±0.1</td>
<td></td>
<td>0.4 ±0.2</td>
</tr>
<tr>
<td>22:5</td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>O3</td>
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<td>0.1 ±0.0</td>
<td>0.1 ±0.1</td>
<td>4.3 ±0.3</td>
<td>2.8 ±1.8</td>
</tr>
<tr>
<td>18:3</td>
<td>0.2 ±0.0</td>
<td>0.2 ±0.0</td>
<td>0.2 ±0.1</td>
<td>0.3 ±0.2</td>
<td>7.0 ±0.5</td>
</tr>
<tr>
<td>22:5</td>
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<td>0.8 ±0.1</td>
<td>0.6 ±0.1</td>
<td>0.1 ±0.1</td>
<td>0.3 ±0.1</td>
</tr>
<tr>
<td>22:6</td>
<td>11.8 ±0.4</td>
<td>6.9 ±0.6</td>
<td>0.5 ±0.0</td>
<td>0.7 ±0.3</td>
<td>3.8 ±0.1</td>
</tr>
</tbody>
</table>

**INDEXES**

<table>
<thead>
<tr>
<th>Unsat. Index</th>
<th>MOUSE</th>
<th>RAT</th>
<th>RABBIT</th>
<th>SHEEP</th>
<th>CATTLE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unsaturated</td>
<td>221 ±4 a</td>
<td>216 ±3 a</td>
<td>156 ±5 c</td>
<td>186 ±4 b</td>
<td>189 ±2 b</td>
</tr>
<tr>
<td>% unsat.</td>
<td>66.2 ±0.5 b</td>
<td>62.2 ±1.1 b c</td>
<td>69.9 ±1.0 a</td>
<td>57.7 ±0.5 d</td>
<td>59.0 ±1.0 cd</td>
</tr>
<tr>
<td>% monouns.</td>
<td>15.1 ±0.2 a</td>
<td>8.9 ±0.5 b</td>
<td>16.4 ±1.3 a</td>
<td>17.9 ±1.4 a</td>
<td>14.2 ±0.7 a</td>
</tr>
<tr>
<td>% polyunsat.</td>
<td>51.1 ±0.7 a</td>
<td>53.3 ±0.6 a</td>
<td>53.5 ±1.9 a</td>
<td>39.8 ±1.5 c</td>
<td>44.9 ±0.4 b</td>
</tr>
<tr>
<td>Ch. length</td>
<td>18.7 ±0.0 a</td>
<td>19.7 ±0.0 b</td>
<td>18.1 ±0.0 b</td>
<td>18.6 ±0.0 a</td>
<td>18.7 ±0.0 a</td>
</tr>
<tr>
<td>% O9</td>
<td>10.4 ±0.2 b</td>
<td>4.3 ±0.4 c</td>
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<td>16.9 ±1.5 a</td>
<td>10.6 ±0.3 b</td>
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<tr>
<td>% O7</td>
<td>5.4 ±0.1 a</td>
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<td>2.3 ±0.3 c</td>
<td>1.6 ±0.3 c</td>
<td>4.0 ±0.4 b</td>
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<tr>
<td>% O6</td>
<td>37.4 ±0.3 b</td>
<td>44.7 ±1.0 a</td>
<td>50.2 ±1.8 a</td>
<td>16.8 ±0.9 d</td>
<td>29.1 ±3.0 c</td>
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<tr>
<td>% O3</td>
<td>12.6 ±0.4 b</td>
<td>8.1 ±0.7 c</td>
<td>2.3 ±0.2 d</td>
<td>22.3 ±0.9 a</td>
<td>15.2 ±2.6 ab</td>
</tr>
</tbody>
</table>
TABLE 2.2
Total membrane phospholipid fatty acid composition in the KIDNEY CORTEX of five mammalian species. Results are average mol % ± s.e. (n=4). Indexes in a row with common exponent letters do not differ significantly (p < 0.05).

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>MOUSE</th>
<th>RAT</th>
<th>RABBIT</th>
<th>SHEEP</th>
<th>CATTLE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saturated</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16:0</td>
<td>12.9 ± 0.2</td>
<td>18.8 ± 0.2</td>
<td>10.3 ± 0.4</td>
<td>18.5 ± 0.6</td>
<td>20.7 ± 0.5</td>
</tr>
<tr>
<td>17:0</td>
<td>0.3 ± 0.0</td>
<td>0.4 ± 0.0</td>
<td>0.4 ± 0.1</td>
<td>1.0 ± 0.0</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>18:0</td>
<td>15.8 ± 0.4</td>
<td>18.5 ± 0.1</td>
<td>12.1 ± 0.5</td>
<td>16.8 ± 0.4</td>
<td>12.9 ± 0.2</td>
</tr>
<tr>
<td>20:0</td>
<td>0.1 ± 0.0</td>
<td>0.1 ± 0.0</td>
<td>0.1 ± 0.0</td>
<td>0.4 ± 0.0</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>21:0</td>
<td>0.1 ± 0.0</td>
<td>0.1 ± 0.0</td>
<td>0.1 ± 0.0</td>
<td>0.4 ± 0.0</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>22:0</td>
<td>0.1 ± 0.0</td>
<td>0.1 ± 0.0</td>
<td>0.1 ± 0.0</td>
<td>0.4 ± 0.0</td>
<td>0.2 ± 0.1</td>
</tr>
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<td>Ω11</td>
<td>0.2 ± 0.0</td>
<td>0.3 ± 0.1</td>
<td>0.2 ± 0.0</td>
<td>0.3 ± 0.1</td>
<td>0.2 ± 0.0</td>
</tr>
<tr>
<td>Ω9</td>
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<td></td>
<td></td>
</tr>
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<td>18:1</td>
<td>7.8 ± 0.1</td>
<td>6.8 ± 0.1</td>
<td>12.2 ± 0.5</td>
<td>16.0 ± 0.9</td>
<td>15.7 ± 0.2</td>
</tr>
<tr>
<td>20:3</td>
<td>0.4 ± 0.0</td>
<td>0.3 ± 0.0</td>
<td>0.8 ± 0.0</td>
<td>0.6 ± 0.1</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td>22:1</td>
<td>0.2 ± 0.1</td>
<td>0.2 ± 0.1</td>
<td>0.2 ± 0.1</td>
<td>0.2 ± 0.1</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>24:1</td>
<td>0.1 ± 0.0</td>
<td>0.2 ± 0.0</td>
<td>0.1 ± 0.0</td>
<td>0.4 ± 0.0</td>
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<td>Ω7</td>
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</tr>
<tr>
<td>15:1</td>
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<td>2.3 ± 0.1</td>
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<td>3.1 ± 0.2</td>
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<tr>
<td>16:1</td>
<td>0.6 ± 0.0</td>
<td>0.7 ± 0.0</td>
<td>0.5 ± 0.0</td>
<td>0.6 ± 0.1</td>
<td>0.8 ± 0.2</td>
</tr>
<tr>
<td>17:1</td>
<td>1.8 ± 0.1</td>
<td>1.9 ± 0.1</td>
<td>1.5 ± 0.1</td>
<td>1.7 ± 0.2</td>
<td>1.1 ± 0.0</td>
</tr>
<tr>
<td>18:1</td>
<td>2.8 ± 0.0</td>
<td>2.7 ± 0.0</td>
<td>1.7 ± 0.1</td>
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<td>2.3 ± 0.1</td>
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<tr>
<td>Ω6</td>
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<tr>
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<td>8.6 ± 0.2</td>
<td>33.6 ± 0.9</td>
<td>10.1 ± 0.7</td>
<td>15.6 ± 2.4</td>
</tr>
<tr>
<td>20:3</td>
<td>2.0 ± 0.1</td>
<td>0.6 ± 0.0</td>
<td>0.9 ± 0.1</td>
<td>0.6 ± 0.0</td>
<td>1.5 ± 0.2</td>
</tr>
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<td>23.3 ± 0.5</td>
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<td>19.5 ± 0.6</td>
<td>13.4 ± 0.7</td>
<td>13.1 ± 0.4</td>
</tr>
<tr>
<td>22:4</td>
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<td>0.7 ± 0.0</td>
<td>0.8 ± 0.1</td>
<td>0.2 ± 0.1</td>
<td>0.3 ± 0.2</td>
</tr>
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<td>0.6 ± 0.1</td>
<td>0.6 ± 0.1</td>
<td>0.6 ± 0.1</td>
</tr>
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<td>Ω3</td>
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<td></td>
</tr>
<tr>
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<td>0.3 ± 0.0</td>
<td>2.0 ± 0.2</td>
<td>1.5 ± 0.5</td>
<td></td>
<td></td>
</tr>
<tr>
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<td>0.2 ± 0.1</td>
<td>0.1 ± 0.1</td>
<td>0.1 ± 0.1</td>
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</tr>
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<td>5.2 ± 0.3</td>
<td>3.7 ± 1.4</td>
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</tr>
<tr>
<td>20:5</td>
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<td>0.2 ± 0.0</td>
<td>0.7 ± 0.1</td>
<td>3.7 ± 0.2</td>
<td>3.2 ± 0.4</td>
</tr>
<tr>
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<td>0.1 ± 0.0</td>
<td>0.2 ± 0.0</td>
<td>0.7 ± 0.1</td>
<td>3.7 ± 0.2</td>
<td>3.2 ± 0.4</td>
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<tr>
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<td>0.8 ± 0.1</td>
<td>3.1 ± 0.2</td>
<td>1.3 ± 0.0</td>
</tr>
</tbody>
</table>

INDEXES
Unsat. Index 249 ± 4<sup>a</sup> 185 ± 1<sup>b</sup> 186 ± 3<sup>b</sup> 172 ± 3<sup>c</sup> 163 ± 5<sup>c</sup>
% unsat. 71.0 ± 0.6<sup>b</sup> 62.1 ± 0.2<sup>d</sup> 77.0 ± 0.9<sup>a</sup> 63.4 ± 0.5<sup>cd</sup> 65.3 ± 0.5<sup>c</sup>
% monouns. 16.5 ± 0.2<sup>c</sup> 16.5 ± 0.1<sup>c</sup> 18.6 ± 0.5<sup>b</sup> 24.4 ± 0.8<sup>a</sup> 24.3 ± 0.8<sup>a</sup>
% polyunsat. 54.6 ± 0.8<sup>a</sup> 45.6 ± 0.3<sup>b</sup> 58.4 ± 1.2<sup>a</sup> 39.0 ± 1.1<sup>c</sup> 41.1 ± 1.0<sup>c</sup>
Ch. length 18.9 ± 0.0<sup>a</sup> 18.3 ± 0.0<sup>b</sup> 18.3 ± 0.0<sup>b</sup> 18.2 ± 0.0<sup>c</sup> 18.1 ± 0.0<sup>c</sup>
% Ω9 8.3 ± 0.1<sup>c</sup> 7.2 ± 0.1<sup>d</sup> 13.2 ± 0.4<sup>b</sup> 16.6 ± 0.9<sup>a</sup> 17.4 ± 0.6<sup>a</sup>
% Ω7 8.4 ± 0.2<sup>b</sup> 9.6 ± 0.1<sup>a</sup> 5.9 ± 0.2<sup>c</sup> 8.5 ± 0.3<sup>b</sup> 7.4 ± 0.3<sup>b</sup>
% Ω6 36.5 ± 0.6<sup>c</sup> 43.1 ± 0.3<sup>b</sup> 55.4 ± 1.2<sup>a</sup> 24.2 ± 1.1<sup>d</sup> 30.6 ± 2.9<sup>cd</sup>
% Ω3 17.7 ± 0.7<sup>a</sup> 2.3 ± 0.0<sup>c</sup> 2.2 ± 0.1<sup>c</sup> 14.1 ± 0.5<sup>b</sup> 10.0 ± 2.4<sup>b</sup>
TABLE 2.3

Total membrane phospholipid fatty acid composition in the HEART of five mammalian species. Results are average mol % ± s.e. (n=4). Indexes in a row with common exponent letters do not differ significantly (p < 0.05).

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>MOUSE</th>
<th>RAT</th>
<th>RABBIT</th>
<th>SHEEP</th>
<th>CATTLE</th>
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Total membrane phospholipid fatty acid composition in the **SKELETAL MUSCLE** of five mammalian species. Results are average mol % ± s.e. (n=4). Indexes in a row with common exponent letters do not differ significantly (p < 0.05).

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<th>RABBIT</th>
<th>SHEEP</th>
<th>CATTLE</th>
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**INDEXES**

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<th>RABBIT</th>
<th>SHEEP</th>
<th>CATTLE</th>
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### TABLE 2.5

Total membrane phospholipid fatty acid composition in the BRAIN of five mammalian species. Results are average mol % ± s.e. (n=4). Indexes in a row with common exponent letters do not differ significantly (p < 0.05).

<table>
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<td>22:5</td>
<td>0.2 ± 0.0</td>
<td>0.1 ± 0.0</td>
<td>0.2 ± 0.0</td>
<td>1.1 ± 0.0</td>
<td>0.7 ± 0.0</td>
</tr>
<tr>
<td>22:6</td>
<td>17.7 ± 2.1</td>
<td>19.3 ± 0.9</td>
<td>11.7 ± 0.7</td>
<td>21.0 ± 1.2</td>
<td>14.7 ± 2.7</td>
</tr>
</tbody>
</table>

**INDEXES**

- **Unsat. Index**
- **% unsat.**
- **% monouns.**
- **% polyunsat.**
- **Ch. length**

<table>
<thead>
<tr>
<th>% Ω9</th>
<th>196 ± 15ab</th>
<th>228 ± 4a</th>
<th>233 ± 7ab</th>
<th>218 ± 7ab</th>
<th>182 ± 13b</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Ω7</td>
<td>62.2 ± 2.1c</td>
<td>69.2 ± 0.7ab</td>
<td>71.6 ± 0.6a</td>
<td>68.1 ± 0.9b</td>
<td>68.3 ± 1.8abc</td>
</tr>
<tr>
<td>% Ω6</td>
<td>29.2 ± 1.0b</td>
<td>29.1 ± 0.9b</td>
<td>27.3 ± 2.2ab</td>
<td>32.0 ± 1.3ab</td>
<td>39.5 ± 3.4a</td>
</tr>
<tr>
<td>% polyunsat.</td>
<td>33.0 ± 3.0b</td>
<td>40.0 ± 0.6a</td>
<td>44.3 ± 1.8a</td>
<td>36.1 ± 1.5b</td>
<td>28.8 ± 2.7b</td>
</tr>
<tr>
<td>% Ω9</td>
<td>18.6 ± 0.1b</td>
<td>18.9 ± 0.0ab</td>
<td>19.0 ± 0.0a</td>
<td>18.9 ± 0.1ab</td>
<td>18.6 ± 0.1b</td>
</tr>
<tr>
<td>% Ω7</td>
<td>19.0 ± 0.7b</td>
<td>19.1 ± 0.7b</td>
<td>17.7 ± 2.0ab</td>
<td>21.7 ± 1.0ab</td>
<td>27.3 ± 2.7a</td>
</tr>
<tr>
<td>% Ω6</td>
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<td>10.1 ± 0.2b</td>
<td>10.4 ± 0.4ab</td>
<td>11.0 ± 0.3ab</td>
<td>12.9 ± 0.7a</td>
</tr>
<tr>
<td>% Ω3</td>
<td>14.7 ± 1.0c</td>
<td>20.1 ± 0.3b</td>
<td>31.4 ± 1.4a</td>
<td>12.7 ± 0.8c</td>
<td>12.1 ± 0.5c</td>
</tr>
<tr>
<td>% % Ω9</td>
<td>18.2 ± 1.9a</td>
<td>19.8 ± 0.8a</td>
<td>12.1 ± 0.6b</td>
<td>22.8 ± 1.1a</td>
<td>16.1 ± 2.6ab</td>
</tr>
</tbody>
</table>
In Tables 2.1 to 2.5, indexes relating to the number of double bonds in the bilayer (Unsaturation Index, % unsaturated, % monounsaturates and % polyunsaturates) are presented first, with the average chain length. They are followed by the proportions of each class of phospholipid (%Ω9, %Ω7, %Ω6 and %Ω3). The %Ω11 were not reported there as i) only one phospholipid species represents this class (20:1Ω11), ii) it is only present in some tissues (liver, kidney and heart), iii) it was never found in cattle and iv) it was only present in small amounts (maximum 0.4% in mouse liver) when it was detected.

A general overview of Tables 2.1 to 2.5 shows that the membranes of mammalian tissues contained many different fatty acids and that various tissues varied in complexity. Considering only those fatty acids representing 0.1% or more of the total composition, 27 fatty acids were found in mammalian kidney cortex, while 26 were detected in liver, 25 in heart, 23 in brain and only 19 in skeletal muscle. A remarkable feature of the mammalian tissues examined is that although there were differences in the fatty acids accumulated by different species, the number of different fatty acids in a given tissue was quite conservative between species. Liver phospholipids contained 20 (rat) to 22 (rabbit) fatty acids, kidney cortex had 18 (mouse) to 23 (cattle) fatty acids, the heart ranged from 19 (rabbit) to 23 (cattle) fatty acids, while in all five species investigated 17 different fatty acids were found in skeletal muscle and 20 in brain.
2.3.2 Proportions of saturated to unsaturated fatty acids and extent of polyunsaturation

In all tissues and species investigated, about two thirds of the fatty acids (by weight) were unsaturated (from 58-60% in sheep and cattle liver to 79% in rabbit and sheep heart). This parameter was presented below for each tissue together with the degree of polyunsaturation, since these parameters were interrelated to some extent. There was no consistent allometric variations of the % unsaturates in mammalian tissue phospholipids.

Since around two thirds of the unsaturated fatty acids contained two or more double bonds, the membrane phospholipids of mammalian tissues were made of around 50% polyunsaturated fatty acids (from around 30% in cattle brain and skeletal muscle to 59% in rat and rabbit heart, Tables 2.1 to 2.5). Only skeletal muscle showed a significant allometric decrease in polyunsaturation. The extent of unsaturation of the polyunsaturates varied greatly, as described below for each tissue.

Given that number of double bonds varies greatly in polyunsaturates (between 2 and 6 were found in mammalian phospholipids), the extent of polyunsaturation of tissue phospholipids is best represented by the unsaturation index (UI) and not by the proportion of polyunsaturates. This index reflects both the proportions of saturated and unsaturated fatty acids and the extent of unsaturation of the fatty acids. The allometric relationships with the UI were clearly tissue specific, as illustrated in figure 2.4.
Figure 2.4

Unsaturation Index vs. body weight in the tissues of mouse, rat, rabbit, sheep and cattle. Standard errors on Unsaturation Index are indicated by the height of symbols. Equations for the logarithmic regressions and level of significance are given.
In liver, although a slow decrease in total unsaturation (indicated by the unsaturation index, UI) with increasing body size was apparent (Figure 2.4 A), the relationship was not significant. Since smaller mammals had significantly higher UI than larger mammals (Table 2.1, p < 0.05), the non-significance of the relationship was mainly due to rabbits displaying a very low UI in this tissue, reflecting the absence of the most polyunsaturated phospholipid species.

By contrast, rabbits had the highest amount of total unsaturates in liver phospholipids (Table 2.1). There was a tendency for smaller mammals to have more total unsaturates than larger mammals, although rats did not differ statistically from cattle. The liver of all species investigated showed similar amounts of monounsaturates, except for rats which had only almost half of what was found in other species. Larger mammals had significantly less polyunsaturates than the smaller mammals in this tissue (Table 2.1). The rabbit liver, which showed a very low UI, still contained high amounts of polyunsaturates because of the accumulation of 37% 18:2ω6, the highest amount in all tissues investigated. Thus, although in liver some interspecific differences in unsaturation-related parameters appeared, no significant allometric relationship emerged (Table 2.7).

Kidney showed a significant decrease (p < 0.05) in UI with increasing body size (Figure 2.4 B). The slope of this relationship indicated that a doubling in body size corresponded to a 2.9% decrease in the UI. There was no allometric trend in the proportion of total unsaturates in kidney cortex phospholipids. Instead, the decrease in UI in the larger mammalian kidneys was due to a combination of i) a significant increase (p < 0.05) in monounsaturates from 17 to 24% in the mouse to cattle comparison (Figure 2.5 A) and ii) a parallel significant decrease in polyunsaturates between smaller mammals and larger
mammals (Table 2.2), but with rabbits displaying the highest values because of the presence of 34% of 18:2ω6.

**Figure 2.5**
Relationship between the proportion of monounsaturates in membrane phospholipids and body weight in mammals. The data are mean ± s.e. (n=4).
In the heart, the relationship of the extent of membrane polyunsaturation (UI) to body size was highly significant \((p < 0.01, \text{Figure 2.4 C})\). A doubling in body size corresponded to a decrease of 3.0% in UI. The proportion of total unsaturated fatty acids did not show a clear allometric relationship, although the larger mammals (rabbit, sheep and cattle) possessed significantly more total unsaturates than mice (Table 2.3) and rats were intermediate. The proportion of polyunsaturates did not show any allometric trend (Table 2.3). On the other hand, there was a significant allometric increase \((p < 0.05)\) in the proportion of monounsaturates from 16 to 26\% in the mouse to cattle comparison (Figure 2.5 B). Thus, in heart the decrease in UI with increasing size is because larger mammals accumulated more monounsaturates and, as referenced in the following sections, that the polyunsaturates that were present were not as unsaturated.

The other type of muscle examined, skeletal muscle, showed the largest decrease in UI with increasing body size (Figure 2.4 D). It was also highly significant \((p < 0.02)\). A doubling in body size was accompanied by a 4.6\% decrease in UI. In this tissue, larger mammals had a significantly lower UI than smaller mammals, with the exception of cattle, which did not differ from sheep (Table 2.4). In this tissue, larger mammals had significantly more total unsaturates than the smaller mammals, although no statistically significant allometric trend was found. There was an increase in monounsaturates with body size (3-fold difference between mouse and cattle) in skeletal muscle \((p < 0.02, \text{Figure 2.5 C})\). A smaller but as significant decrease in polyunsaturates \((p < 0.02, \text{Figure 2.6})\) was also observed. As a result, larger mammals accumulated significantly more total unsaturated fatty acids in their muscles (Table 2.4).
The brain was an unusual tissue in terms of membrane fatty acid composition, as i) larger mammals did not exhibit significantly higher UI than smaller mammals, except between rat and cattle (Table 2.5) and ii) as illustrated in Figure 2.4 E, the intraspecific variability was greater in this tissue than in any other tissue examined in some species. For example, two mice brains had an UI of 170 and two of 220. Three cattle brains had an UI around 175 and one had 215. In all these cases this was mostly due to large differences in the proportions of the highly polyunsaturated 22:6\(\Delta 3\). Similarly, no allometric trend could be observed for the proportions of total unsaturates nor for the proportions of polyunsaturates. However, although no significant allometric increase in the proportions of monounsaturates was found in brain, cattle had significantly more monounsaturates than mice and rats (Table 2.5). Thus, brain and liver were the only tissues not to show any significant allometric trend of unsaturation-related indexes.
To summarize this section, in mammalian tissues the UI decreased with increasing body size to an extent that was tissue specific, from near 0% (brain) to 4.6% (skeletal muscle) for a doubling in body size. The proportion of total unsaturates varied intraspecifically between tissues as well as between species, but no significant allometric trend was found. Larger mammals had more total unsaturates in their muscles (heart and skeletal muscle), but less in liver than the smaller mammals. There was a significant increase in monounsaturates with increasing size in the three tissues for which there was a significant allometric relationship with the UI. Skeletal muscle was the only tissue to also show a significant allometric decline in the proportion of polyunsaturates (Figure 2.6). In the latter tissue, the combination of the strongest allometric increase in monounsaturates and of a decrease in polyunsaturates yielded the largest allometric change of UI observed in the tissues examined.

2.3.3 Length of the membrane fatty acid chains

As the standard errors in Tables 2.1 to 2.5 show, for a given tissue and species the average fatty acid chain length is the index that was found to be most constant. Mammalian cell membrane phospholipids had average chain lengths around 18.4 carbons long, ranging between 17.6 carbons in cattle skeletal muscle to 19.2 in mouse heart.

In mammalian tissues, the more polyunsaturated fatty acids also tended to be longer. This is illustrated in Figure 2.7, which shows that more polyunsaturated membranes contained longer fatty acids ($p < 0.01$). As a consequence, those tissues which showed an allometric relationship with the UI also displayed a relationship with fatty acid chain length (Figure 2.8). There was no allometric decrease in average chain length in liver and brain, but the
relationships with average chain length in heart and skeletal muscle were highly significant ($p < 0.01$). In the kidney cortex, the relationship was near statistical significance at the 5% level (the $r$ value was 0.876 while the critical value for 5% confidence level = 0.878).

**Figure 2.7**
Relationship between the Unsaturation Index and Average Chain Length in mammalian tissues. Average values ($n=4$) for each tissue (liver, kidney cortex, heart, skeletal muscle or brain) and species (mouse, rat, rabbit, sheep or cattle) are used.
Figure 2.8
Relationship between average fatty acid chain length (number of carbon atoms) and body weight in the mammalian tissues for which a significant allometric relationship was found. The data are presented as mean ± s.e.(hidden by symbols when not visible). (n=4).
2.3.4 Non-essential fatty acids

2.3.4.1 Saturated fatty acids

The data in Tables 2.1 to 2.5 show that the shortest saturated fatty acid detected was 15:0, only present in skeletal muscle where it made up to 3.7% of the total membrane fatty acids in rabbits. Another odd chain saturated fatty acid, 17:0, was detected in all tissues of all species, but always in very small amounts (maximum 1.1% in sheep liver). The longest odd chain fatty acid, 21:0, was detected only in rat and rabbit kidney and in sheep and cattle heart and was never more than 0.1% of the total composition. The longest saturated fatty acid, 22:0, was only present in liver, kidney cortex and heart and, although it was never more than 0.4% of the total composition, it tended to accumulate more in the larger mammals.

The even numbered chains 16:0 and 18:0 were the main saturated fatty acids in mammalian tissue phospholipids. The total amount of saturated fatty acids did not vary allometrically, as described above with the % unsaturates. There was no allometric relationship with the proportions of 16:0 or 18:0 either, except in liver. In this tissue, while the UI, the % monounsaturates and % polyunsaturates did not vary allometrically, there was a significant decrease in 16:0 (p < 0.01) and a proportional but non significant increase in 18:0 with increasing body weight (Figure 2.9). No such trend was observed in the other tissue examined.
Figure 2.9
Relationship between the proportions of 16:0 (•) and 18:0 (■) and body weight in mammalian liver phospholipids. Points are mean ± s.e. (n=4). Logarithmic relationships are given.
To my knowledge, this fatty acid has not been previously reported in mammalian tissues. It was only present in liver, kidney and heart, never in cattle and always in very small amounts (maximum 0.4% in mouse liver). This fatty acid tended to be present in higher proportions in smaller mammals, although no significant allometric trend could be found except in liver, where it decreased from mouse to sheep (p < 0.05).

Ω9 fatty acids

Five fatty acids of this group were present in mammalian tissues, but not all of them were found in every tissue. Skeletal muscle had only two Ω9 fatty acids (Table 2.4). Although the proportions of these fatty acids was variable, a significant allometric relationship was only observed in kidney cortex (Figure 2.10), where larger mammals accumulated more Ω9 monounsaturates. The Ω9 monounsaturates, and in particular 18:1Ω9, were responsible for the allometric increase in monounsaturates observed for this tissue (Figure 2.5 A).

In the Ω9 fatty acids, only 18:1Ω9 was found in large amounts in mammalian tissues (up to 25% in sheep and cattle skeletal muscle and cattle brain). Although larger mammals tended to accumulate more of this fatty acid in kidney cortex and skeletal muscle, there were no significant allometric trends in any tissue. The smaller mammals (mouse, rat and rabbit) had higher proportions of 18:1Ω9 in brain than in the other tissues.

The only other Ω9 fatty acid to be found consistently in all mammalian tissues was 20:3Ω9, but it never made up more than 1.1% of the total membrane fatty acid composition (in mouse liver). 20:1Ω9 was found in the brain of all
species, where it made up 1 to 2% of the total fatty acids. It was also present in liver, heart and kidney of some species, in no more than 0.3%, and was absent from skeletal muscle. 22:1Ω9 was the rarest fatty acid of this group. Small quantities of it were found only in rabbit liver, rabbit and cattle kidney cortex and in mouse brain. Very small amounts of 24:1Ω9 were also found in mouse and rat kidney cortex (0.1 and 0.2%, respectively). This fatty acid was also present in the brains of all species investigated. In the latter tissue, the proportions of this very long monounsaturated fatty acid (the longest found in mammalian tissues) reached 1.2 and 1.4% in sheep and cattle, respectively. This allometric trend was not statistically significant.

\[ y = 5.1x^{0.10} \]
\[ r = 0.887 \]

**Figure 2.10**
Relationship between the proportion of Ω9 fatty acids and body weight in mammalian kidney cortex. Data are mean ± s.e (hidden by symbols).
2.3.4.4 $\Omega 7$ fatty acids

The $\Omega 7$ fatty acids were particular in that they contained only monounsaturates and that the odd chains (15:1$\Omega 7$ and 17:1$\Omega 7$) were commonly more abundant than the even chains (16:1$\Omega 7$ and 18:1$\Omega 7$). As well, all four fatty acids were found in every tissue of every species, although there were usually less total $\Omega 7$ than other groups of unsaturates. An exception to this was in heart, where the larger mammals accumulated twice as much $\Omega 7$ (mainly odd chains) as they did $\Omega 9$.

In both heart and skeletal muscle, there was a highly significant increase of $\Omega 7$ fatty acids with body size ($p < 0.01$, Figure 2.11). In these striated muscles, cattle accumulated 9-10% of 15:1$\Omega 7$ and 5-7% of 17:1$\Omega 7$. These large increases were the cause of increase of the total monounsaturates in these muscles, reported in Figure 2.5 B and C.

The even chain $\Omega 7$ did not show major allometric trends. 16:1$\Omega 7$ was of minor importance in all tissues. Up to 2.5% of this fatty acid was found in cattle skeletal muscle, in which tissue it was higher in larger mammals than in smaller mammals. This small allometric trend in skeletal muscle was in contrast with that of liver, where mice had more 16:1$\Omega 7$, as well as 18:1$\Omega 7$. These allometric trends were not statistically significant. 18:1$\Omega 7$ was generally more abundant than 16:1$\Omega 7$, although the highest concentrations were only around 3.7%, in mouse and cattle brain. There was a significant allometric decrease ($p < 0.05$) of 18:1$\Omega 7$ in skeletal muscle.
Figure 2.11
Relationship between the proportions of Ω7 and body weight in mammalian heart and skeletal muscle phospholipids. Points are mean ± s.e. (n=4). Logarithmic relationships are given.
To summarize this section, in brain there were no significant allometric relationships in the proportions of saturated and monounsaturated fatty acids. In the liver, the smaller mammals accumulated 16:0 while the larger ones accumulated 18:0. The other three tissues showed an allometric increase in monounsaturates. In kidney cortex, this was due to increases in $\Omega_9$ fatty acids with size, while in heart and skeletal muscle larger animals accumulated $\Omega_7$ fatty acids, mainly odd chains.

2.3.5 Essential fatty acids

Typically, about half of the fatty acids in membranes came from the diet, as they were essential $\Omega_6$ and $\Omega_3$ fatty acids (Table 2.6). The rat and rabbit heart and the rabbit kidney had 58% essential fatty acids (EFA) while there was only around 30% in cattle brain and muscle. In general, larger mammals had less EFA in their tissues. The heart of all species was richer in EFA than the other tissues, except for mice where kidney had as much as heart. Brain was the tissue with the lowest amounts of EFA in smaller mammals. There was a significant allometric decline in EFA in skeletal muscle ($p < 0.02$). Given that all but one polyunsaturates were EFA, this reflects the relationship previously reported of the allometric decrease in polyunsaturates for this tissue (Figure 2.6).
TABLE 2.6

Total essential fatty acids (Ω6+Ω3) in mammalian tissue phospholipids. Results are average mol % ± s.e. (n=4). The mean values (± s.e.) for the five tissues is given.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>MOUSE</th>
<th>RAT</th>
<th>RABBIT</th>
<th>SHEEP</th>
<th>CATTLE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>50.0 ± 0.7</td>
<td>52.8 ± 0.6</td>
<td>52.5 ± 1.9</td>
<td>39.1 ± 1.6</td>
<td>44.3 ± 0.5</td>
</tr>
<tr>
<td>Kidney</td>
<td>54.2 ± 0.8</td>
<td>45.3 ± 0.3</td>
<td>57.6 ± 1.2</td>
<td>38.3 ± 1.2</td>
<td>40.6 ± 0.8</td>
</tr>
<tr>
<td>Heart</td>
<td>53.8 ± 0.1</td>
<td>58.3 ± 1.8</td>
<td>58.6 ± 3.4</td>
<td>53.7 ± 1.2</td>
<td>49.8 ± 3.2</td>
</tr>
<tr>
<td>Muscle</td>
<td>51.4 ± 0.8</td>
<td>49.6 ± 1.6</td>
<td>44.1 ± 1.3</td>
<td>30.5 ± 2.6</td>
<td>29.7 ± 1.5</td>
</tr>
<tr>
<td>Brain</td>
<td>33.0 ± 3.0</td>
<td>39.9 ± 0.6</td>
<td>43.5 ± 1.9</td>
<td>35.4 ± 1.5</td>
<td>28.1 ± 2.7</td>
</tr>
<tr>
<td>mean</td>
<td>48.5 ± 3.9</td>
<td>49.2 ± 3.1</td>
<td>51.3 ± 3.2</td>
<td>39.4 ± 8.7</td>
<td>38.5 ± 4.2</td>
</tr>
</tbody>
</table>

2.3.5.1 Ω6 fatty acids

The majority of the EFA in mammalian tissues were Ω6 (Tables 2.1 to 2.5). Larger mammals had less Ω6 than smaller mammals in their liver, kidney and skeletal muscle. Rabbits were exceptional in having more of this group of EFA than the other species in all tissues, rendering allometric trends non-significant. The accumulation of fatty acids from the Ω6 family varied between tissues and appeared to be a species characteristic unrelated to body size.

An important feature of the EFA in membranes is that, unlike the NEFA, they are easily desaturated (Stubbs and Smith, 1984). Thus an informative way to look at their occurrence in mammalian tissues is to visualize, with the help of histograms, the action of desaturases and elongases in animals of various size. This is illustrated for the Ω6 fatty acids in Figure 2.12. For each of the 25 histograms the individual fatty acids are presented in order of increasing length.
and unsaturation. This figure thus allows the action of the elongases and desaturases (as well as dietary intake and preferential incorporation) to be assessed by comparing the bars in each histogram.

Although six Ω6 fatty acids were detected in mammalian tissues, only three (18:2, 20:4 and 22:5) were found in substantial quantities (more than 3%), except in brain where 22:4Ω6 accounted for 3 to 6% of the total membrane fatty acid composition for all species. The brain of all species had lower amounts of Ω6 fatty acids than the other tissues, reflecting the low levels of EFA in this tissue (Table 2.6).

Linoleic acid (18:2Ω6) is a common diet component and it is the least unsaturated EFA. It accumulated in good proportions, at least 7%, in all tissues except in brain where rabbits, with only 1.7%, had about 3 times as much as the other species. Rabbits had accumulated very high amounts of 18:2Ω6 by comparison to other species in all tissues, up to 37% in liver. There was no significant allometric change for 18:2Ω6 in any tissue, although a trend for its increase in larger mammals was observed for heart.

Large amounts of arachidonic acid, or 20:4Ω6, were found in the tissues of smaller mammals. Rats had more of this fatty acid than the other species (up to 33% in liver and kidney) in all tissues, except in brain where rabbits had about the same amount. There was no significant allometric variation for 20:4Ω6 in any tissue.
Figure 2.12 Profile of ω6 fatty acids in mammalian tissues. The values are mean ± s.e. of mol %. The fatty acids in a graph are organized in increasing order of unsaturation and length.
The most polyunsaturated Ω6 fatty acid, 22:5Ω6, was only present in substantial amounts in mouse heart and skeletal muscle (5.5% and 4.2%, respectively) and in rabbit brain (9.8%). Thus rabbit brain had more of all major Ω6 fatty acids than the brains of the other species.

2.3.5.2 Ω3 fatty acids

Seven different Ω3 fatty acids were found in mammalian tissues, with up to six co-occuring in liver, kidney cortex and heart (Tables 2.1 to 2.5 and figure 2.13). Rabbits, in whose tissues very high amounts of Ω6 EFA were found, had remarkably low amounts of Ω3 fatty acids. There was no significant allometric trend in the total Ω3 fatty acids accumulated in any tissue, although the larger mammals had more in liver while mice showed higher levels in heart.

The most abundant fatty acid of this group in mammalian tissues was often 22:6Ω3 (top and right-hand histograms in Figure 2.13). By contrast to Ω6 fatty acids, there was a clear tendency for larger animals to accumulate shorter and less unsaturated chains in all tissues but brain. Larger mammals replaced large amounts of 22:6Ω3 with smaller amounts of many other Ω3 fatty acids.

Brain tissues generally contained more 22:6Ω3 than the other tissues. An exception to this was for mice, where the heart had higher levels. In the heart, as well as in skeletal muscle, there was a significant allometric decrease in 22:6Ω3 content (p < 0.05 and p < 0.02, respectively, Figure 2.14). Indeed, the allometric relationship observed for these muscles was the greatest one in range for an individual fatty acid, with amounts of around 20% in mice down to 0.5% or less in cattle muscles.
Figure 2.13  Profile of ω3 fatty acids in mammalian tissues. The values are mean ± s.e. of mol%. The fatty acids in a group are organized in increasing order of unsaturation and length.
Figure 2.14
Relationship between the proportions of 22:6ω3 and body weight in mammalian heart and skeletal muscle phospholipids. Points are mean ± s.e. (n=4). Logarithmic relationships are given.
Table 2.7
Summary of the statistically significant allometric changes in tissue phospholipid fatty acid composition. The direction of the allometric trend (↑ for increase and ↓ for decrease) as well as the level of significance are indicated.

<table>
<thead>
<tr>
<th></th>
<th>Sk. Muscle</th>
<th>Heart</th>
<th>Kidney</th>
<th>Liver</th>
<th>Brain</th>
</tr>
</thead>
<tbody>
<tr>
<td>UI</td>
<td>↓ (p &lt; 0.02)</td>
<td>↓ (p &lt; 0.01)</td>
<td>↓ (p &lt; 0.05)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% mono.</td>
<td>↑ (p &lt; 0.02)</td>
<td>↑ (p &lt; 0.05)</td>
<td>↑ (p &lt; 0.05)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% poly.</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ch. Length</td>
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<td>↓ (p &lt; 0.01)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Ω9</td>
<td></td>
<td>↑ (p &lt; 0.05)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Ω7</td>
<td>↑ (p &lt; 0.01)</td>
<td>↑ (p &lt; 0.01)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>22:6Ω3</td>
<td>↓ (p &lt; 0.02)</td>
<td>↓ (p &lt; 0.05)</td>
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<td></td>
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</tr>
<tr>
<td>16:0</td>
<td></td>
<td></td>
<td>↓ (p &lt; 0.01)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2.4 Discussion

The data found in the literature about total membrane phospholipids often concern the liver of rat (Moore et al., 1969; Hulbert and Else, 1989, for example) or mouse (Croset and Kinsella, 1989). Their data are in agreement with the values presented in this chapter. The fatty acid composition of rats and mice livers presented here was most comparable to those previously described for control conditions in the studies concerned with diet (Moore et al., 1969; Croset and Kinsella, 1989). In the data for rats from Hulbert and Else (1989) however, many parameters were lower than in the present study. The UI was 185 vs. 216 for liver but not different in kidney cortex. The proportions of unsaturated fatty acids that they reported were 54% vs. 62% for the data presented here for both tissues. The average chain lengths reported by these authors were 18.4 vs. 18.7 for liver but not different in kidney. To my knowledge, total tissue phospholipid fatty acid composition was not available for large mammals.

Before analysing the data in detail, it is important to point out the mechanisms of regulation of membrane composition. When discussing the interspecific differences in membrane composition, three factors can be thought to be influential. Given the importance of essential fatty acids in membrane phospholipids (Table 2.6), diet certainly plays a critical role in membrane composition. The second factor is the differential incorporation of dietary fatty acids. The regulation of this mechanism could be shared by the reabsorptive surfaces of the digestive tracts, extracellular transport systems and selection in the cell membranes themselves, where in the endoplasmic reticulum the phospholipids are assembled. The third factor, the action of the elongases and desaturases lies entirely in the cells themselves.
I have chosen to assume that each animal used in this study had an appropriate diet. This implies that when differences were found in the amounts of \( \Omega 6 \) and \( \Omega 3 \) fatty acids between species and tissues, it was considered that diet preference and differential incorporation were operative in regulating cell membrane composition. The selectivity of tissues for some EFA was obvious, with heart incorporating a higher proportion and brain a lesser proportion of them (Table 2.6).

It is also clear from the results that desaturases and elongases prefer EFA, as only one polyunsaturate from the NEFA group was found in mammalian tissue phospholipids. In cases where not enough EFA are available from the diet, higher than normal amounts of 20:3\( \Omega 9 \) (the only polyunsaturated NEFA in membranes) are often produced. Moore et al. (1969) reported that their EFA-deficient rats had 16% 20:3\( \Omega 9 \), whereas rats on a normal diet had around 1%. The very low levels of 20:3\( \Omega 9 \) fatty acids found here in mammalian membranes (maximum 1.1%) suggest that although interspecific differences in the proportions of EFA were found, all species had amounts appropriate for their normal functioning. Another sign of EFA deficiency is the increase in 18:1\( \Omega 9 \) (Moore et al., 1969; Léger et al., 1990). The levels of this fatty acid observed here in mammalian tissues were much lower than those reported by Moore et al. (1969) for EFA-deficient animals (31% in rat liver) and about half of the 9% they found in the liver, heart and kidneys of their control rats.

To be extensively desaturated, fatty acids need to be further elongated. While 18 carbon chains are not known to contain more than 4 double bonds, 20 carbon chains can contain up to 5 and 22 carbon chains up to 6 double bonds in mammalian tissues. Double bonds are not inserted below the seventh carbon
from the methyl end in mammalian tissues and double bonds are not found next to each other (Lehninger, 1982). As a result, to be highly polyunsaturated, fatty acids in membranes need to be long and to already have some double bonds below the seventh carbon before they are incorporated in phospholipids.

The correlation between the Unsaturation Index and chain length (Figure 2.7) is in agreement with this evidence. The negative correlations of chain length with body size in kidney cortex, heart and skeletal muscle thus suggest that for those tissues the larger mammals have less active elongases and that this may be part of the reason for the decrease in Unsaturation Index in larger mammals. The most polyunsaturated 18 carbon chain, 18:4Ω3, was only found (in very small proportions) in sheep and cattle liver, kidney cortex and heart. This is suggestive that for larger mammals a low elongase activity induced further desaturation of shorter fatty acid chains. This hypothesis is reinforced by the presence of substantial amounts of 20:5Ω3, the most polyunsaturated 20 carbon fatty acid, in larger mammals. Small mammals accumulated the longer 22:6Ω3 instead. This evidence suggests that at least part of the control of polyunsaturation in mammalian tissues may lie with the elongases.

The results presented in this chapter support the hypothesis that mammals with a higher mass-specific basal metabolic rate maintained generally more polyunsaturated membranes than mammals with lower metabolic rates. The data also show that not all tissues followed this allometric trend. In particular, even though brain maintained lower amounts of polyunsaturated (essential) fatty acids than other tissues (although the differences disappeared in larger mammals, which accumulated lower amounts of EFA in other tissues as well) mammals of all species showed a similar Unsaturation Index in this tissue. Brain tissue seemed conservative in
phospholipid fatty acid composition between species. Thus the scaling of polyunsaturation in mammalian tissues is a tissue-specific characteristic. The reverse trend, an increase of UI with body size, was never observed. The apparent increase in UI between mouse and rabbit in brain was not statistically significant (Table 2.5). Considering all tissues examined and their respective proportions in the body, with skeletal muscle being the most important contributor to weight in mammals, it is apparent that overall, the cell membranes of larger mammals are less polyunsaturated.

Both striated muscles examined (heart and skeletal muscle) showed a strong allometric response of UI and their overall response to changes in body size was clearly similar. This is consistent with the review by Stubbs and Smith (1984) in which they examined the modification of membrane fatty acid composition of these tissues to dietary changes together, as they found that they show comparable responses. Indeed, Charnock et al. (1989; 1992) showed that the fatty acid composition of cardiac muscle and skeletal muscle are almost identical and that the similarities remain following dietary manipulation. They went on to suggest that skeletal muscle biopsies could be reliable indicators of cardiac muscle fatty acid composition.

From my data, the skeletal muscle showed the highest specificity in composition (only 17 fatty acids were detected in this tissue for each species) and the strongest allometric response in membrane composition. Both heart and skeletal muscle of smaller mammals increased their UI by further desaturating polyunsaturates, suggesting a higher activity of the desaturases and elongases in these tissues. This was reflected in skeletal muscle by an allometric decrease in polyunsaturates (Figures 2.5 B and C and Figure 2.6). In the heart however, the remarkably constant proportions of polyunsaturates
between the species examined suggest that this muscle cannot compromise its level of polyunsaturates. Instead, while smaller mammals further desaturated their polyunsaturates, larger mammals accumulated monounsaturates. Thus in heart the trend of increase of total unsaturates in larger mammals (Table 2.3), was paralleled by a decrease in the extent of polyunsaturation (Figure 2.4) instead of a decrease in the amount of polyunsaturates. In both muscles, larger mammals preferred to accumulate \( \Omega 7 \) monounsaturates.

The allometric relationship of membrane polyunsaturation in kidney cortex was very similar to that observed in heart and skeletal muscle, apart from the preference of larger mammals to accumulate \( \Omega 9 \) instead of \( \Omega 7 \) monounsaturates, as in the muscles. The allometric decrease in UI in this tissue, although significant \((p < 0.05)\) was not as strong as for heart and skeletal muscle \((p < 0.01 \text{ and } p < 0.02, \text{ respectively})\). This may be why the scaling of average chain length, although obvious (Figure 2.8 A) was not statistically significant in kidney cortex while it was in the muscles.

Liver showed a substantially different and much smaller allometric decrease in membrane polyunsaturation than kidney cortex and striated muscles. While in some other tissues (heart and skeletal muscle) there was a tendency of the total unsaturates to increase in proportions in larger mammals, in liver the smaller mammals had higher proportions of them. In this tissue particularly, rabbits showed an unusual membrane composition. Thus, while these animals had an exceptionally low UI in liver, only equalled in mammalian tissues by skeletal muscle in the larger mammals, this was compensated by amounts of total unsaturates higher than that found in mice.
Another peculiarity of liver was the allometric changes in saturated fatty acids, with 16:0 decreasing and 18:0 increasing in larger mammals (Figure 2.9). A possible explanation for this is that smaller mammals did not let 18:0 accumulate in their membrane phospholipids and further desaturated it to \( \Omega_9 \) and \( \Omega_7 \) monounsaturates. This is not supported by the results presented in Table 2.1, where it can be seen that the decrease in 18:0 in smaller mammals is not matched by a proportional increase in these monounsaturates. Instead, smaller mammals tended to have more total unsaturates (Table 2.1) and more EFA (Table 2.6) in their liver phospholipids. This suggests that animals of smaller size may selectively accumulate higher amounts of EFA and 16:0 instead of 18:0 in this tissue.

The brain was certainly the most conservative tissue in terms of interspecific variations in membrane composition. Some requirement in this tissue for very high amounts of highly polyunsaturated fatty acids was clear in all species. While larger mammals had only small amounts of 22:6\( \Omega_3 \) in other tissues, all species, even rabbits which were notoriously low in \( \Omega_3 \) fatty acids, maintained high amounts of this fatty acid in brain (although not as much as the other species). The lower levels of 22:6\( \Omega_3 \) in rabbit brain were more than compensated for by very high proportions of 22:5\( \Omega_6 \). This is interesting, given that the review by Stubbs and Smith (1984) points out that in this tissue a reciprocal relationship between 22:5\( \Omega_6 \) and 22:6\( \Omega_3 \) exists when dietary manipulations are performed.

Docohexaenoic acid (22:6\( \Omega_3 \)) is considered to occur in high proportions in the phospholipids of excitable cells (Gudbjarnason, 1989). Thus, in addition to other functions, there may be a specific role of this fatty acid in cell excitability. There would be considerable implications from the results presented here. In
particular, this would strongly suggest that the brain of all mammals examined share a similar excitability. As well, this would suggest an allometric decline in muscle fibre excitability, not as pronounced in heart as in skeletal muscle. The results of Gudbjarnason et al. (1978) corroborate this relationship, as the relationship of 22:6Ω3 vs. heart rate (a physiological parameter associated to cell excitability) reported by them is stronger than that of 22:6Ω3 in heart vs. body weight (Figure 2.14).

A simultaneous examination of Figures 2.12 and 2.13 reveals that the preference of the desaturases for Ω3 fatty acids is apparent in all species examined. This is deduced by the higher desaturation of Ω3 fatty acids by comparison to Ω6 fatty acids in all tissues and species examined. This finding is consistent with the review by Stubbs and Smith (1984) for small mammals, in which the order of preference of desaturases for substrates has been presented as Ω3>Ω6>Ω9. Given that 18:1Ω9 is by far more abundant than 20:3Ω9 in mammalian membranes, this also supports the view that the desaturases do not normally desaturate Ω9 fatty acids if given the choice. This could be true of the elongases as well. Allometric comparisons of substrate preference of the desaturases have not been reported before and these results do not suggest any major differences between small and large mammals.

Given the preference of the elongases for Ω3 fatty acids, the scaling of desaturase activity is best represented in Figure 2.13. It is clear from this figure that in all tissues except brain the larger mammals had a lower desaturase activity. This potentially provides a substantial part of the explanation of the general decrease in UI in the tissues of these animals. Further studies will be required to test this hypothesis.
Because of the likely dependence of desaturation on chain length, discussed above, it is possible that much of the desaturase activity is controlled by the elongases. An indication that the desaturases, and not only the elongases, were less active in larger mammals is the accumulation of small amounts of 22:0 in liver, kidney and heart and only in some tissues and species. The presence of this very long saturated fatty acid could indicate either a high elongase activity or a low desaturase activity. Both these cases could be present in the data in Tables 2.1 to 2.3. Trace amounts of 22:0 were usually associated with a low UI. As well, the mouse heart, which has the highest UI of all tissues (264), would be expected given its UI to have very high elongase activity (Figure 2.7) and 0.1% 22:0 was found.

In the comparison of mammalian vs. reptilian membrane composition reported by Hulbert and Else (1989), the more metabolically active mammals displayed lower proportions of total unsaturates in their membranes, but these unsaturates were more polyunsaturated. The proportions of unsaturates varied in liver and kidney from 54% in rats to 61% in lizards. The values presented here in these tissues for rats were higher than theirs, but the interspecific differences were more important, ranging for liver from 58% in sheep to 70% in rabbits and for kidney from 62% in rats to 77% in rabbits. No relationships were found between the % unsaturates and body size.

On the other hand, the data presented here strongly reinforce Hulbert and Else’s hypothesis that animals with a higher metabolism maintain more polyunsaturated membranes. The scaling of UI in mammalian tissues is more important in all tissues except brain than had been reported by these authors for liver and kidney of reptiles and mammals. This is consistent with the larger differences in basal metabolic rate between mouse and cattle (11-fold, Schmidt-
Neilson, 1990) than between rats and bearded dragons (7-fold, Brand et al., 1991). A detailed analysis of the possible relationships between metabolic rate and polyunsaturation will be presented in Chapter 4.

The use of modern capillary columns and the increasing availability of standards has revealed that some membrane components are present in very small amounts. In this study, many fatty acids were reported in mammalian tissues that had previously been disregarded. Fatty acids such as 22:0, 20:1\(\Delta_{11}\), 22:1\(\Delta_{9}\), 15:1\(\Delta_{7}\), 17:1\(\Delta_{7}\) and even 18:4\(\Delta_{3}\) are such examples. Although often only present in trace amounts, these fatty acids may give clues on interspecific or between tissue differences in the regulation of membrane composition. Each of the major or minor fatty acids in biological membranes possibly has many functions. They may be required by some membrane proteins to function optimally, induce permeability to some molecules, help preserve membrane integrity and relative impermeability or act as a pool of precursor molecules for the synthesis of functionally important molecules such as the prostaglandins. Stubbs and Smith (1984) wrote that "it may in fact be necessary to regard the various polyunsaturated fatty acyl components as unique chemical structures, each able to contribute to the physical environment of membranes in a specific manner".

The possible physiological significance of the scaling of membrane composition from the data presented here can only be conjectural. Two such roles are discussed, membrane permeability and membrane fluidity. The latter concept is still nebulous with respect to its significance in homeotherms, as in the range of composition usually displayed by mammalian membranes it seems that fluidity is appropriate for membrane function (Stubbs and Smith, 1984; Lee, 1991). Examination of the data presented in this chapter does not suggest
any dramatic change in parameters commonly associated with membrane fluidity. The importance of other membrane constituents in membrane fluidity was not considered in this study. Since cholesterol is known to influence membrane fluidity (Kimelberg and Papahadjopoulos, 1974; Kimelberg, 1975; Wiley and Cooper, 1975; Hazel, 1988; Lee, 1991) and permeability (de Gier et al., 1968; Papahadjopoulos, 1971; Wiley and Cooper, 1975; Fettiplace and Haydon, 1990), further studies investigating the scaling membrane composition should consider including this parameter in the investigation.

As the first and second double bonds appear to be the most important contributors of fluidity in membranes (Stubbs and Smiths, 1984; consistent with Ehringer et al., 1990) who report no difference in membrane fluidity in membranes containing 18:1\(\Omega3\), 18:3\(\Omega3\) or 22:6\(\Omega3\) considering the total % unsaturation may be the best available way to detect potential changes in fluidity. Lee (1991) stated that very major changes in phospholipid composition would be required to bring about significant changes in ATPase activity. No consistent allometric trend or the % unsaturates was observed, allowing me to propose that no allometric trends in membrane fluidity were present in the tissues of the species examined.

The changes in membrane composition associated with body size in skeletal muscle, heart and kidney cortex are likely to be more related to the modification of membrane permeability than to fluidity. This suggestion comes from the observations that the major changes in membrane composition were mainly brought about by modifications of the amounts of the highly polyunsaturated species such as 22:6\(\Omega3\) and that recent studies have linked the presence of this fatty acid to membrane permeability to a variety of substances (Jenski et al., 1991; Stillwell et al., 1993). The increase in monounsaturates in
larger mammals may have been a response to maintain membrane fluidity. Would these propositions be supported by further experimental evidence, they imply that the membrane enzymes of smaller mammals would work just as fast in the cell membranes of larger mammals, but that the smaller mammals' cells with membranes from larger mammals would not have a sufficient permeability to cope with the high demands of their metabolism.

In this study, the small mammals used were rodents, the intermediate-size animal a lagomorph and the larger mammals both ruminants. The choice of species used could have yielded differences in tissue membrane composition due to taxonomic differences instead of changes in body size. The fact that the relationship of 22:6Ω3 and heart rate discovered by Gudbjarnason et al. (1978) involved mouse, rat, rabbit, man and whale (no ruminants) support the case for a relationship of membrane polyunsaturation and body size independently of taxonomy in mammals. Nevertheless, future studies should include omnivores of different size to diminish the complications in interpreting the data due to the different diets of small and large mammals encountered in this study. The rodents and the lagomorph had on average 50% EFA in their tissue phospholipids while the ruminants had 40%. Nevertheless, since there was no increase in the NEFA polyunsaturate 20:3Ω9 the amount of EFA in the diet of the ruminants seemed appropriate to their needs regarding membrane composition.
2.5 Conclusions

The cell membrane phospholipid fatty acid composition in the tissues of different sized mammals supports the hypothesis that cell membrane polyunsaturation is associated with metabolic activity. The scaling of membrane polyunsaturation was a tissue specific characteristic, the extent of which it was possible to order as follows: skeletal muscle > heart > kidney > liver > brain (from Table 2.7). In the three tissues where the Unsaturation Index statistically significantly declined with increasing body size, EFA were involved in the decreasing parameters while possibly compensatory mechanisms from the NEFA increased in larger mammals (Table 2.7). This could not be taken as evidence of a lesser opportunity for larger mammals to accumulate EFA, since only in skeletal muscle was a significant decrease in EFA found and since no increase in the NEFA polyunsaturate 20:3Ω9 were found in larger mammals. Rather, lower elongase and desaturase activity in larger mammals and differential incorporation of the EFA in various tissues were proposed as the likely mechanisms acting to control the observed size- or metabolism-related changes in membrane composition.

The allometric decrease in polyunsaturation observed in mammalian skeletal muscle, heart and kidney cortex and the parallel increase in monounsaturates was suggestive that the physiological role of the scaling of membrane composition in these tissues is associated to the scaling of membrane permeability while membrane fluidity would remain relatively constant interspecifically in homologous tissues.
Chapter 3

Allometric Comparison of Metabolic Rate and Sodium pumping in Liver and Kidney Cortex of Five Mammalian Species
3.1 Introduction

The aim of this chapter is to measure indicators of cell metabolic activity and related cation fluxes and pumping in the liver and kidney cortex of mammals. The species chosen are the same as those examined in the previous chapter: mouse, rat, rabbit, sheep and cattle. The experiments have been designed to evaluate of the importance of potassium (and hence indirectly of sodium) pumping in metabolism and to allow for a discussion of their contribution to basal metabolism in mammals of various size.

The first step was to verify the allometric relationship between body size and tissue metabolic rate calculated from Krebs' (1950) data (Figure 1.1) using more modern techniques for the measurement of oxygen consumption while monitoring tissue slice quality. These experiments, also include the measurement of mass-specific intracellular volume, are aimed at testing the second hypothesis, that the scaling of mass-specific basal metabolic rate is partly due to the scaling of mass-specific liver and kidney cortex metabolic rate.

Another reason for the reassessment of oxygen consumption rates in different-sized mammals was to obtain estimates of intracellular volume, rate of potassium uptake and rate of oxygen consumption on the same slice preparations, hence allowing for more reliable estimates of the importance of cation pumping in the cell energy turnover in liver and kidney cortex slices. These comparisons are aimed at testing the third hypothesis, that sodium pump activity is proportional to metabolic activity.
Tissue slices are, in my view, the best available system through which to carry an allometric comparison of physiological parameters on liver and kidney cortex. While hepatocyte preparations have nowadays replaced liver slices of rat in metabolic studies, this method is impractical for comparative studies involving very large and very small mammals. Collagenase, usually used for the preparation of hepatocytes (although alternative methods are presented in Berry et al., 1983), is expensive and difficult to use on large or small tissues, as the best methods of hepatocyte preparation involve perfusion of the liver.

More importantly however, hepatocytes, or isolated liver cells, are known to differ in metabolic activity to liver cells in vivo (Clausen et al., 1991; Junge et al., 1976 and Berthou et al., 1989 provide examples and in Section 3.4.3 a comparison of respiration rates of hepatocytes and liver slices is conducted). Powis et al., (1989) compared rat, dog and human hepatocytes for their ability to metabolize foreign compound and concluded that liver slices may provide a better model than isolated hepatocytes for foreign compound metabolism studies with dog and human liver. Their study suggest that while hepatocytes from rats (with which animal this technique was mainly developed) may retain much of their in vivo properties, hepatocytes from larger mammals lose their in vivo properties to a large extent. This point is sufficient to discourage the use of hepatocytes in comparative metabolic studies.

Finally, for the measurement of cation fluxes and the estimation of their importance in vivo, it was thought more appropriate to use cell preparations that remain in the structural positions that they occupy in the tissue, bound to their neighbours so as to leave only part of the plasma membranes available for
exchange processes.

Two major criticisms are commonly brought forward concerning tissue slices. The first argument against the use of tissue slices in physiological studies is that slicing induces cell damage. This, of course, is true for all cell preparation techniques. Consequently, methods for assessing cell membrane integrity have been used throughout this chapter. These techniques are detailed in Section 3.3.1.1.

The second criticism to the use of tissue slices is that it is difficult to ensure and monitor proper diffusion through the slice. Elliott (1955) estimated that the maximal thickness for good diffusion in an average slice would be around 500 μm. These calculations were based on Warburg’s estimates, as well as from some experimental evidence he had collected. He recommends that “slices should not be cut very much thinner than the limiting thickness; this increases the proportion of damaged cells and the proportion of tissue subjected to high oxygen tension”. His estimates were considering systems where diffusion occurs passively. For the reasons elaborated in Section 3.3.1.2, 500 μm thick slices were chosen for the final experiments described in this chapter. Preliminary experiments (Section 3.3.1.3) enabled an estimation of diffusion rates in the system used and simple calculations have revealed that diffusion was not likely to be a hindrance in the experiments reported.
3.2 Methods

An account of the development of the methods used for the final experiments and the justifications for their choice are given in Section 3.3.1. The saline chosen for all the experiments in this chapter is a common medium for such studies. It is phosphate-buffered and contains glucose as substrate. The description of this saline in Appendix II is accompanied by a discussion of why it was chosen.

3.2.1 Chemicals

KCl, NaHCO₃, MgCl₂, NaCl, MgSO₄·7H₂O were obtained from BDH Analar. CaCl₂·2H₂O, Na₂CO₃, dimethylsulfoxide (DMSO) and trichloroacetic acid were purchased from AJAX Chemicals. Glucose, Bovine Serum Albumin (BSA), ouabain, KH₂PO₄, K₂HPO₄, Imidazole, Ethylenediamine tetraacetic acid (EDTA, sodium-free salt), Triton X-100, pyruvate, β-Nicotinamide adenine dinucleotide, reduced form (β-NADH), Tris, δ-ketoglutarate, (NH₄)₂SO₄ and adenosine diphosphate (ADP) were from Sigma Chemicals. All radioisotopes were purchased from Dupont/New England Nuclear. The scintillant (Emulsifier Safe) was from Packard Australia. Sodium metabisulphite (Wander, Australia) was purchased from local supermarkets.

3.2.2 Experimental animals

All animals used were young adults. The sheep and cattle tissues were obtained from Yallah, a local abattoir 20 km south of the University of Wollongong. Ruminants were fasted overnight before the slaughtering. Mice
and rats were obtained from the University of Wollongong breeding colonies, while rabbits were obtained from Tilside Rabbit Stud and kept in the animal house for at least 2 weeks prior to the experiments. These were not fasted. The body weights of these animals are reported in Table 3.2.

Killing of the animals and removal of tissues

Mice were killed by cervical dislocation, rats and rabbits were killed by concussion and all these animals were dissected immediately. Sheep were killed by cerebral electrocution and cattle by concussion and their tissues removed by normal abattoir procedures. Liver and kidney were removed and immersed in saline at room temperature until slicing, which was initiated within 10 minutes after the death of the animal, except for sheep and cattle, for which the tissues were not available before 15 and 25 minutes, respectively.

3.2.3 Preparation of the liver and kidney cortex slices

Liver samples of approximately 2x1x0.3 cm were excised from the whole tissues. For mouse, rat or rabbit kidney, the cortex was obtained by first cutting the kidney longitudinally in two halves, then by removing the medulla. In sheep and cattle, the kidneys were cut longitudinally in half and a stripe of cortex 3 mm thick and 15-20 mm long was taken from either side of the cut.

The manual slicer pictured in Figure 3.1 was used for the final experiments. For the slicing, a 5.5 cm #1 Whatman filter paper was folded in half and dipped in saline, then laid on the slicing platform. The piece of tissue to be sliced was then transferred to the platform with a large paintbrush, a few drops of medium were added to the sample and the stainless steel razor blade
(Wilkinson Sword, England) wetted. To make a slice, the blade-holding arm was lifted and the micrometer screw turned to push the slicing platform 500 \( \mu \text{m} \) further. The arm was released from 5 to 6 cm (this height was the minimum necessary to obtain good slices repeatably) above the platform and stopped on the lower stop, adjusted so that the blade cut only partly the first layer of filter paper. The slices were handled with a fine paintbrush (#0 or #1), by the extremities when possible.

Figure 3.1 The manual slicer
The average dimensions of tissue slices were: 6-10mm X 2-3mm X 0.5mm. Their wet weight was between 10 and 20 mg for all tissues and species, except for mouse kidney slices, which were smaller (2.5 to 6 mg). These slices were smaller as a result of the requirement to retain the orientation of the kidney cortex segment for slicing.

The liver samples were not oriented in any specific way for slicing. Kidney cortex is an assembly of glomeruli and tubules (refer to Lote, 1987 for histological details). The slices of this tissue were oriented perpendicularly to the plane of the cortex/medulla border. The proximal and distal tubules would not be joined in cortex slices as their connection, the loop of Henle, is situated in the medulla, which was removed. As the slices were around 500 µm thick, the tubules themselves, whose convoluted parts run roughly parallel to the cortex/medulla border, would also have been frequently sectioned. This would increase diffusion through tubules and capillaries.

The tissues obtained from the Yallah abattoir were sliced there and the slices were brought back to the University in 125 ml bottles filled with warm carbogen-saturated saline. The slices destined for the potassium efflux measurement were transferred to 10 ml vials containing 5 ml of saline and 30 µCi of $^{86}$Rb⁺ (rubidium). Before sealing the vials, the gas phase was replaced by carbogen (95% O₂ and 5%CO₂) kept under pressure in a 50 ml syringe. These sealed bottles were transported back to the laboratory in a 5 litre styrofoam water jug at 37°C (the temperature would drop 4°C in 2 hours and transportation lasted for 20 to 30 minutes).

Slicing was completed within 15 minutes. All the slices necessary for the manipulations were then transferred to saline at 37°C (after transport for
abattoir material), in the stirring wells described in Section 3.3.1.3 and Figures 3.5 and 3.6, 1 to 4 slices in each well.

3.2.4 Measurement of physiological parameters with tissue slices

The measurements were initiated 90 minutes after death (Table 3.1). This time was chosen as the minimal time by which slices from abattoir material were available, while giving all slices at least 30 minutes of stabilisation in the wells. Preliminary experiments on potassium uptake (Section 3.2.6 and Figure 3.2) initiated 90 minutes after death showed that sheep liver and kidney slices did not benefit from longer recovery periods. These slices had the lowest potassium contents (Tables 3.6 and 3.7). In Figure 3.2, a longer time is required for $^{86}$Rubidium to equilibrate with the intracellular potassium when the latter is high, as in mouse liver and kidney slices. In sheep liver and kidney slices, equilibrium was reached after 15 minutes with tissue potassium levels (estimated from $^{86}$Rubidium accumulation) much lower than that for mouse tissue slices. A subsequent increase of the intracellular potassium concentration would have been reflected by a slow increase of $^{86}$Rubidium accumulation. This was not observed.

The order in which the measurements were routinely performed is described in Table 3.1. During the manipulations, the saline in all open wells was constantly saturated with carbogen, as illustrated in Figure 3.6. Once the manipulations were completed, the slices were processed as described in section 3.2.9.2.
Table 3.1  Time-table of the manipulations on tissue slices. Except where specified, the time is the same for all animals. $t$ is time after death (minutes). Slices were given 90 minutes recovery and all manipulations were completed within 240 minutes after death.

$t=0$  Death

$t=30$  **Mouse-Rat-Rabbit**
Slicing completed. Transfer to wells & incubation in $^{86}\text{Rb}^+$

$t=50$  **Sheep-Cattle**
Transfer to bottles & incubation in $^{86}\text{Rb}^+$

$t=80$  **Sheep-Cattle**
Transfer to wells and incubation in fresh saline (including $^{86}\text{Rb}^+$)

$t=90$  Initiate oxygen consumption measurement, liver & kidney slices
Preparation of whole tissues ($1/100$ dilution)

$t=100$  Volume measurements (liver then kidney slices in triplicate)

$t=120$  Removal of first slices for oxygen consumption
Wells resealed for control respiration rate

$t=125$  Control slices collected (liver then kidney slices in triplicate)

$t=160$  Measurement of potassium efflux
Simultaneous measurement of potassium uptake for liver slices (in triplicate)

$t=200$  Initiate oxygen consumption measurement, liver & kidney slices

$t=210$  Measurement of potassium uptake for kidney slices (in triplicate)

$t=240$  Removal of second slices for oxygen consumption
Wells resealed for control respiration rate
Final saline pH measured
Samples homogenized
3.2.5 Oxygen consumption

Equipment

Oxygen consumption of tissue slices was measured using two oxygen electrodes (Strathkelvin Instruments oxygen meter model 781) fitted with standard membranes (18 seconds response-time for 90% change), coupled to a MacLab (Analog Digital Instruments) linked to a Macintosh SE computer running Chart/4 V3.1.3 (Analog Digital Instruments). The oxygen meter was left on standby continuously between experiments to eliminate the small drift of the signal in the first hour after turning it on.

Calibration

Calibration of the system was done at the end of each experiment. High purity water (air-saturated at 37°C) was added to the wells that were then sealed with the electrode, expelling any air bubbles through a hole in the electrode jacket. After a minute, any newly formed air bubbles were removed in the same method, to avoid a supersaturation of oxygen due to warming or bubbling. After a stable signal had been recorded for at least 10 minutes, all oxygen was absorbed by adding a few crystals of sodium dithionite. As the carbogen-saturated saline is about four times more concentrated in oxygen than air-saturated water is and the recordings are consequently performed above the calibration range, this calibration method recommended by the manufacturer assumes that the relationship between mV and nmol O₂·ml⁻¹ is linear.
Sterilisation of equipment

Bacterial contamination of the system can possibly yield a very important background oxygen consumption in the case of slices displaying slow metabolism and is thus of concern. To avoid this potential problem, saline was prepared fresh every 2 days and kept at 4°C, while grids, vials, stirring bars and electrode jacket were rinsed, then soaked in a 10g/litre solution of sodium metabisulphite for 3 hours at the end of each experiment, and after rinsing were kept in 2 litres of glass distilled water (to also ensure that any ouabain remaining from previous experiments would diffuse out). Possible bacterial contamination was regularly checked by measuring the oxygen consumption rate of carbogen-saturated saline.

Method of oxygen consumption measurement with tissue slices

The carbogen-saturated wells containing tissue slices were sealed with the electrode jacket and any gas bubbles were eliminated through a hole in the jacket. One liver and one kidney slice were monitored simultaneously in the separate systems. Oxygen consumption was monitored for 30 to 40 minutes. The slice was then removed and processed as described in Section 3.2.9.2. Only one slice was usually used per measurement, except for mouse kidney cortex slices that were very small (2.5 to 6 mg) and for sheep or cattle liver slices. In these cases, two slices were used since using only one slice gave too weak a signal.

After each measurement, a control of oxygen consumption in the saline was performed to be systematically subtracted. This control was typically low, less than 10% of the oxygen consumption rate of the slice. In liver slices
however, and especially in larger mammals, the second measurement (Table 3.1) commonly displayed a higher control respiration rate. Since the saline was replaced between measurements and the oxygen consumption of the electrode itself, assessed in distilled water or in fresh saline, was insignificant with the signal amplification used, an increase in control oxygen consumption was probably indicative that whole cells and/or mitochondria had been released from the slice and were not retrieved with it when the slice was removed.

**Calculations**

When using the MacLab to record the rates of oxygen consumption, converting mV to nmol O₂/ml could be done in one simple operation from within the saved recording. The oxygen concentration of air-saturated water was calculated from Walker (1987) to be 214.4 nmol O₂·ml⁻¹ at 37°C. Representative sections of 10 to 20 minutes were selected and the data transferred to Cricket Graph (version 1.3.1). The selected sections of recordings were chosen as representative of the slope for the entire recording. For the determination of slopes, given in nmol O₂·ml⁻¹·min⁻¹, a linear regression was computed from the trace (Cricket Graph v. 1.3.1).

The respiration rate was calculated as:

slice respiration rate (μmol O₂·g wet⁻¹·min⁻¹) = (nmol O₂·ml⁻¹·min⁻¹ · saline volume (ml))/mg wet slice.
3.2.6 Potassium uptake

Equipment and measurement of radioactivity

A five-well Packard Cobra Auto Gamma radiation counter was used for the determination of the amount of radioactivity in the samples. Background radiation was automatically subtracted and blank tubes provided a verification that it remained nil. Each sample was counted for 10 minutes. The five wells of the gamma counter had been calibrated with the same radioactive source at the beginning of the set of experiments.

Method of potassium uptake measurement

$^{86}\text{Rb}^+$ is commonly used as a substitute for potassium (eg. Van Dyke and Scharschmidt, 1983; Haber et al., 1987; Lijnen et al., 1990; Petit et al., 1991; Montero et al., 1991; Llewelyn et al., 1991) with no large artefactual bias since the sodium pumps have a similar affinity for both cations: the affinity of the pumps on the extracellular side for monovalent cations is in the order $\text{K}^+ > \text{Rb}^+ > \text{NH}_4^+ > \text{Cs}^+ > \text{Li}^+ > \text{Na}^+$ (Skou, 1992). Haber et al. (1988) experimentally determined the $\text{K}^+$ to $\text{Rb}^+$ ratios for active and passive uptake in clone 9 liver cells from rat. They were 1.05 and 0.83, respectively. In the current experiments I have assumed a value of 1.0.

After addition of 50 µCi of $^{86}\text{Rb}^+$ to a well containing carbogen-saturated saline at 37°C, slices were added in pairs and incubated for either one or five minutes, in triplicate, after which time they were retrieved with a paintbrush. In a preliminary experiment, the rate of $^{86}\text{Rb}^+$ accumulation was linear and
maximal for up to 5 minutes (Figure 3.2). The uptake for sheep kidney in this preliminary experiment was not as linear as in the final experiments, as can be seen in Figure 3.16.

![Figure 3.2](image)

**Figure 3.2**

Accumulation of potassium (using $^{86}$Rubidium) in mouse and sheep liver and kidney cortex slices, as a function of incubation time. Each point represents the mean and range of two means, obtained from separate experiments (n=2 for each point in a given experiment).
To maximise the measurement of intracellular \(^{86}\text{Rb}^+\), after the incubation in the radioactive saline, slices were transferred to 20 ml of non-radioactive saline and a second, non contaminated paintbrush was used to gently stir the slice in that saline for 5 seconds. Given the potassium efflux rates measured in the experiments (maximum 6.2% loss/min. in the presence of ouabain), this process does not allow for a significant loss of accumulated \(^{86}\text{Rb}^+\). It is however sufficient to remove most of the extracellular \(^{86}\text{Rb}^+\) and to eliminate brush contamination. Following this short wash the slice was transferred to a tube and processed as in Section 3.2.9.2. At the end of these manipulations the saline was sampled in duplicate, for the calculation of CPM/\(\mu\)l (used below).

**Calculations**

After the slices were weighed and homogenized, half of the slice homogenate was used for the determination of \(^{86}\text{Rb}^+\) CPM. The CPM from the 500 \(\mu\)l of sample used for counting was multiplied by 2 to obtain the total \(^{86}\text{Rb}^+\) CPM for that slice. To minimize the contribution of extracellular \(^{86}\text{Rb}^+\), the rate of potassium uptake was calculated as the difference in \(^{86}\text{Rb}^+\) accumulation between the averages of the triplicates for \(t=1\) and \(t=5\) minutes, assuming linear accumulation.

To calculate the accumulation at each time point, the following formula was used:

\[
\text{Potassium accumulation (\(\mu\)mol K+/g wet)} = \frac{(\text{Total }^{86}\text{Rb}^+ \text{ CPM in slice } \cdot 5.93\text{nmol K}^+/\mu\text{l})}{(\text{Saline CPM/\(\mu\)l } \cdot \text{mg wet})}
\]
3.2.7 Potassium efflux

Equipment and measurement of radioactivity

As described for potassium uptake, Section 3.2.6.

Method

Two slices of each tissue were preloaded with $^{86}\text{Rb}^+$ in a saline containing 50 $\mu$Ci (initially in a vial containing 5 ml saline and 30 $\mu$Ci $^{86}\text{Rb}^+$ for the animal tissues obtained at the abattoir, as described in Section 3.2.3) and incubated for either 110 (sheep and cattle slices) or 130 minutes (mouse, rat, rabbit).

After the incubation period, each slice was transferred to a well containing non-radioactive saline and 5 mM ouabain to block the recycling of $^{86}\text{Rb}^+$ by the sodium pumps. Every 4 minutes for 28 minutes, 200 $\mu$l of saline were sampled in alternation from each of the four wells and placed in gamma counter tubes. After 32 minutes, the slice was removed from the well with a paintbrush and placed in a tube. The saline was then sampled for a last time. The $^{86}\text{Rb}^+$ CPM in each tube was then determined as in Section 3.2.6.

Calculations

The rate of potassium efflux was expressed in "% potassium loss/minute". It was calculated as follows: for each time point, the amount of radioactivity remaining in the slice was back-calculated as the sum of the losses of radioactivity to the medium at that time added to the radioactivity retained
by the slice at the end of the efflux measurement. The % K+ remaining in the slice relative to t=0 was then plotted semilogarithmically to transform the absolute potassium contents into a linear relationship with time. The slope of the semilogarithmic relationship was then converted back to "% K+ loss·min⁻¹":

\[
% \text{K}^+ \text{ loss·minute}^{-1} = 100 - e(\ln 100 + \text{slope}^*)
\]

\[\text{(*in ln}% \cdot \text{minutes}^{-1})\]

It is possible to estimate "µmol K⁺·g wet⁻¹·min⁻¹" from "% K+ loss·minute⁻¹" by using the [K⁺]₀ of the control slices as an initial value (t=0). This method was not applied for the final experiments as the significance of the interpretation of the results would be jeopardised by the variability of [K⁺]₀. These calculations were however performed for theoretical estimates of the rates of potassium efflux using physiological [K⁺]₀ and for comparison with the rates of potassium uptake (Section 3.3.2.6).

3.2.8 Determination of intracellular water volume

Equipment and measurement of radioactivity

The 500 µl slice homogenates (processed as in Section 3.2.9.2 after the manipulations in this section) were added to 5 ml of scintillant in plastic scintillation vials. The incubation medium samples (100 µl in duplicate) were quenched with 500 µl of whole tissue homogenate. This procedure, yielding very similar quenching in all vials, eliminated the need for quenching
correction. CPM could then be used directly in the calculations. Background radiation was obtained by adding 500 μl of whole tissue homogenate to the scintillator, in duplicate.

Each sample was counted 5 minutes for CPM determination using a LKB Wallac 1219 Rackbeta Liquid Scintillation Counter. The channel used was set on automatic window selection for the discrimination of counts due to $^3$H or $^{14}$C.

Method

To measure the intracellular volume, 1 μCi of $^{14}$C-dextran and 5 μCi of $^3$H$_2$O were added to a well containing 10 ml of saline. Three liver slices were then incubated in this medium for 5 minutes. Figure 3.3 shows that the estimation of intracellular volume was independent of incubation time after 5 minutes of incubation. After removal of the slices from the incubation medium, they were processed as described in section 3.2.9.2.

Calculations

The intracellular volume was calculated as the difference between the volume of the extracellular compartment and the total water space. The volume of each compartment was determined by relating the amount of radioactivity of the appropriate marker in the slice homogenate to the radioactivity of that marker per volume of saline. The background radioactivity was subtracted from all measured CPM prior to calculations.
Figure 3.3

Effect of incubation time in the presence of $^3$H$_2$O and $^{14}$C-Dextran as water space and extracellular markers, respectively, on the estimation of intracellular volume. Data are presented as mean ± range of two experiments, each done in duplicate, except for sheep liver (one experiment).
3.2.9 Processing of slices and whole tissue samples

3.2.9.1 Determination of wet weight

Equipment

A four decimal place balance (Chyo JH-200, HD Scientific) was used for all weight measurements. The wet weight measurements were performed during the time-course of the experiment, as soon as the slices were available. Before a slice was removed from the saline, a tube of the type used for a gamma counter was tared on the balance. After dabbing\(^1\) the slice to absorb the excess saline as described below, wet weight was recorded (to a tenth of a milligram). The accuracy of this measurement, as estimated by weighing the same slice three times was ±0.1 mg or 0.5 to 5% of the measured weights.

To dab a slice, a wick of absorbent tissue was inserted in the tube containing the slice. The excess saline was absorbed by touching gently the edges of the slice with the absorbent paper. The reproducibility and the similarity with whole tissue values of the water content of slices weighed wet using that method were assessed and are reported in the preliminary experiments, Section 3.3.1.4.1.

3.2.9.2 Preparation of slices and whole tissue samples for enzyme activity, radioactivity and cation measurements

Immediately after the determination of wet weight of slices, 1000 µl of ice-cold homogenization buffer (refer to Appendix III) was added to the tube.

---

\(^1\) The term "dabbing" is used in this chapter to describe the action of removing the extraneous saline with a wick of absorbent paper. The wick was obtained by folding a laboratory absorbent tissue in two to obtain a 10x10 cm square and rolling the tissue square to a diameter only slightly inferior to the inside diameter of the tube.
For whole tissue samples, triplicate 3x3x3mm sections of tissue were cut with a scalpel or razor blade. These whole tissue samples were prepared at the beginning of the manipulations. They were accurately weighed and 100 times the weight of the slice of ice-cold homogenization buffer was added to the tube containing the tissue sample.

From the moment that the ice-cold homogenization buffer was added, the tubes were kept on ice. Homogenization was achieved using a T25 Ultra-Turrax (Janke and Kunkel, IKA labortechnik) with a 8 mm diameter probe. The samples were homogenized for 20 seconds at maximal speed, while ensuring that the sample tube remained immersed in ice-cold water during the procedure. Homogenising longer did not increase enzyme activity.

500 µl aliquots of the slice homogenate were sampled and transferred to other tubes for the measurements of radioisotope activity or cation concentration:

- for volume measurements, 500 µl of sample homogenate was added to 5 ml scintillant in a plastic scintillation vial.

- for the potassium uptake determinations, 500 µl of sample homogenate is transferred to a gamma counter tube.

- for the measurement of potassium and sodium levels on whole tissues, control slices and on the slices for which oxygen consumption was measured, 500 µl of sample was transferred to a gamma counter tube containing 1 ml of 0.3 M trichloroacetic acid (TCA).
These fractions were then processed as described in their respective sections. The remainder of the samples were kept at 0-4°C until the enzyme assays were performed.

3.2.9.3 Enzyme assays

Equipment

A Varian Cary 210 spectrophotometer, equipped with a chart recorder and a cuvette holder with a capacity of 5 cuvettes, changing position automatically every 5 seconds, was used. The wavelength was adjusted to 340 nm, as required when measuring the disappearance of NADH. The enzymes were assayed using a range of 1 (1 absorbance unit for the width of the chart paper) and a chart speed of 50 sec/cm. The cuvettes used were made of plastic, transparent at 340 nm and with a total capacity of 2 ml.

Method

The media used for the enzyme assays are of common use. The lactate dehydrogenase (LDH, EC 1.1.1.27) medium has been modified from my master’s thesis (Couture, 1989) while the glutamate dehydrogenase (GDH, 1.4.1.3) medium was modified from Schmidt (1974). The concentration of the media components and the pH chosen are the results of optimizations on mouse and cattle tissues. The composition of the media used for the measurement of LDH and GDH activity are described in Appendix IV. They were prepared fresh daily, as many of their components, such as NADH and substrates, are rapidly degraded in solution.

Enzyme assays were performed at room temperature within 24 hours.
after homogenization. Over that period, enzyme activity in homogenates did not decrease significantly and, rather the opposite, it tended to stabilise and to become more repeatable. In any case, as all samples for a given experiment are assayed together, the conclusions concerning the % LDH loss or % GDH loss, relative to whole tissue values, are not expected to be affected by this procedure.

The reaction was initiated by adding 5 to 50 µl of slice or whole tissue homogenate to 1000 to 1500 µl of LDH or GDH medium, according to the wet weight of the slice and to the activity of the enzyme in that tissue. The amount of sample and of medium were adjusted in order to give a linear reaction with a slope of between 15 and 75° (equivalent to 0.015 to 0.155 IU² in cuvette). The samples, deposited above the surface of the medium on the side of the cuvette, were mixed by sealing the cuvette with a parafilm and mixing.

Four cuvettes were measured at once and samples were always assayed in duplicate. As the duplicates were recorded side by side, a disagreement between them could be noted by non-parallel lines. A third replicate was performed on the fifth channel with the next four samples if the slope of the initial 2 replicates disagreed by more than 5%. The reaction was recorded until at least 3 minutes of linear trace was obtained. No controls were performed as it was initially assessed that without substrate the rate of oxidation of NADH was negligible when a range of 1 absorbance unit is used and the slope of the reaction is more than 10°.

2 International Units. 1 IU = 1µmole of substrate converted to product per minute
Calculations

The slope of the traces was calculated from the chart paper in ΔO.D. (optical density, or absorbance unit)·Δt⁻¹. Activity in cuvette (IU) = ΔO.D.·volume (in litres)·Δt⁻¹·EmM⁻¹, where EmM, the extinction coefficient of NADH, is 6.22 mM⁻¹·cm⁻¹ and the cuvette light path is 1 cm. The total activity in the slice, or per gram of whole tissue, was then calculated from the amount of tissue in the cuvette.

3.2.9.4 Determination of potassium and sodium contents in tissue slices and whole tissues

Equipment

A model 410 flame photometer (Corning) and a TC2 Econospin bench centrifuge (Sorvall Instruments, Dupont) were used.

Method

At the end of a week (24 hours after the last samples were added to TCA as described in Section 3.2.9.2), 2.5 ml water were added to each tube. After mixing, the tubes were centrifuged at 4000 RPM for 10 minutes. Potassium and sodium were measured with the flame photometer in the supernatant, against a standard curve consisting of 0 and 500 nmol K⁺ or Na⁺ in tubes containing also 500 μl of homogenization buffer, 1 ml TCA to a total of 4 ml. This standard curve corresponded directly to 0 and 1000 nanomoles K⁺ or Na⁺ in the original 1 ml homogenate. Within this range, the response of the flame photometer was
shown to be linear.

The amount of potassium or sodium calculated to be in the slice or whole tissue sample (in nanomoles) was divided by the wet weight of the slice or sample to obtain \( \mu \text{mol/g wet} \). It was then possible to calculate the \% potassium loss in slices, relative to whole tissues.

For the estimations of the intracellular concentrations of sodium or potassium ([K]_i or [Na]_i, in mM) the volumes in \( \mu l \cdot g \text{ wet}^{-1} \) determined in other slices of the same preparation were used. Using this method directly would yield an overestimation, important in the case of sodium or for slices with low intracellular potassium, due to the presence of extracellular fluid. This extracellular contribution was estimated then subtracted in the following calculations:

\[
[K]_i (\text{mM}) = \\
((\mu \text{mol K}^+ / \text{g wet slice} - \text{extracellular } K^+ (\mu \text{mol/g wet})) \cdot 1000 \\
\text{intracellular volume (}\mu l/\text{g wet})
\]

where

\[
\text{Extracellular } K^+ (\mu \text{mol/g wet}) = \\
\text{extracellular volume (}\mu l/\text{g wet}) \cdot \text{saline } [K^+] (\mu \text{mol}/\mu l)
\]

and

\[
\text{Extracellular volume (}\mu l/\text{g wet}) = \\
(\mu l \text{ water/g wet whole tissue}^*) \cdot \text{intracellular volume (}\mu l/\text{g wet})
\]

* given as \( \mu g/g \text{ wet} \) in Table 3.2.

[Na]_i was calculated the same way.
The assumptions on these calculations were:

a) that the extracellular volume of the slice for which potassium or sodium levels were measured was the same as calculated for whole tissue from its water content and from the intracellular volume measured on slices. The possible over- or underestimation of the intracellular volumes using wet weight, discussed in Section 3.3.2.1, could have affected the accuracy of the estimates of intracellular cation concentrations, but only to a small extent.

b) that the extracellular fluid has the same sodium and potassium concentration as that of the saline. The high diffusion rates in the system used (Section 3.3.1.3) suggest that this assumption is true enough not to significantly affect the results.

3.2.9.5 Determination of water content in whole tissues and slices

Dry weights for Figure 3.9 and Table 3.2 were obtained by using pre­weighed tubes and determining wet weights as described above (Section 3.2.9.1). The tubes containing slices or whole tissue samples were then transferred to a vacuum jar containing drierite, left to dry to a constant weight (at least 48 hours) and reweighed for the determination of dry weight.

3.2.9.6 Protein determination in whole tissues

Equipment

A LKB (Biochrom Ultrospec II) spectrophotometer was used for the determination of absorbance with the cuvettes used for enzyme assays.
Method

For the determination of proteins for the whole tissues, reported in Table 3.2, the method of Lowry et al., (1951) was used.

Calculations

The regression line from the standard curve was used to calculate the amount of proteins in the 10 μl sample. As whole tissue samples were diluted 1/100, each 10 μl contained 100 μg tissue. The amount of proteins in the assay was then multiplied by 10 to give mg protein/g wet tissue.

3.2.10 Statistical analyses

StatView 512+ was used to calculate the statistical values for the determination of the level of significance of the interspecific comparisons. The methods and tables of critical values are from Porkess (1988).

The small number of values in each group (4) and of groups (5) requires non-parametric tests, as parametric tests require a normal distribution around the mean, a requirement that cannot be met or tested with small samples. The tests were performed as follows.

For a given parameter and tissue, for which there are 5 groups (species) of 4 data (mean values in each experiment) each, a Kruskall-Wallis one-way analysis of variance tested the null hypothesis that there was no difference between the groups, at the 5% significance level. If this hypothesis was rejected, then a two-tailed Mann Whitney U-test was performed for each pair of groups to determine which groups differed significantly from each other, at the 5%
level. With n=4 for each group, one single tie between groups (i.e. the lowest value of the group with the highest mean is lower or equal to the highest value of the group with the lowest mean) renders the comparison non significant. Thus, the significant differences between a pair of groups reported in the tables and figures throughout this chapter imply that there is no overlap between the values of these two groups.

The linear regressions were tested for significance using critical values of r, the Pearson product moment correlation coefficient, with n-2 degrees of freedom.
3.3 Results

3.3.1 Early experiments

Much of the research conducted during this investigation has been directed at developing reliable methods for the measurement of physiological parameters on liver and kidney slices of different-sized mammals and for the assessment of slice quality. The methods used in this thesis are the end result of this process. Two early unsuccessful series of experiments are only summarised here but have given invaluable information on the problems involved in the work with tissue slices and on ways to circumvent them.

My initial study of tissue slices in mammals of different size was performed with mouse and cattle liver. In this set of experiments, a system of wells in which mixing was ensured by bubbling was used. Slices of 225 μm thickness (slicing method otherwise as in Section 3.2.3 for the manual slicer) were made using a Sorvall TC2 automatic slicer. The experiments with cattle liver were performed in a mobile laboratory stationed at the abattoir to minimize the time between the time after death and the initiation of the measurements. The results showed that cattle liver slices were damaged beyond repair during the manipulations as they did not have the ability to retain $^{86}$Rb$^+$ and as $^{22}$Na$^+$ influx was so rapid that it could not be measured. Oxygen electrodes were not available at that time to measure respiration rates. These results could not be used in this thesis but they lead to the development of methods for the assessment of slice quality and of the stirring well system.

In a second set of experiments, 225 μm slices were used for a comparison of mouse and sheep liver and kidney cortex slices, using an early version of the
stirring well system. Assessing slice quality revealed that sheep liver slices suffered much greater damage than mouse liver slices during the manipulations, as sheep liver slices displayed around 80% LDH loss vs. around 40% for mouse. (refer to Section 3.3.1.1 for the assessment of slice quality). As well, bacterial contamination rendered these early oxygen consumption measurements useless. These experiments, however, clearly indicated the need for equipment sterilisation and to improve the slicing technique.

A summary of the methodological investigations, that also serves as a justification of the final methods chosen, is given below. Four particular aspects have received most of the attention in the methodological investigations and are detailed: the methods of assessment of slice quality, the slicing technique, the system in which the slices were maintained and the basis of expression of the rates measured.

3.3.1.1 Use of % LDH loss, % GDH loss and % potassium loss for the characterization of tissue slice preparations

A common way to assess the integrity of membranes in hepatocytes is to monitor losses of LDH, a cytoplasmic enzyme that leaks out when plasma membranes are damaged (Berry et al., 1983; Marsh et al., 1991). In tissue slices, an informative way to characterize cell damage is to compare the levels of LDH in slices with whole tissues. The index "% LDH loss" can serve to estimate the damage caused by slicing and to monitor the integrity of the membranes during the following manipulations, as well as to compare various slice preparations.

The loss of GDH gives a different account of slice damage, since this
enzyme is situated in the mitochondrial compartment. Although all slices lost some of their LDH, in many cases they retained most of their GDH. As a consequence of the variability of wet weight measurement illustrated in Figure 3.9 and discussed in Section 3.3.1.4.1, the index % GDH loss could occasionally take slightly negative values. These indexes are calculated as follows:

\[
\% \text{ LDH loss} = \left( 1 - \left( \frac{\text{IU LDH/g wet slice}}{\text{IU LDH/g wet whole tissue}} \right) \right) \times 100\%.
\]

\[
\% \text{ GDH loss} \quad \text{was calculated the same way.}
\]

Another useful indicator of slice condition is the potassium content, expressed in \( \mu \text{mol K}^+ \cdot \text{g wet}^{-1} \) or in \( \% \text{ potassium loss} \) compared to whole tissues. In each experiment, the potassium content of non-radioactive slices (radioactivity was incompatible with the use of the flame photometer) was measured. As for % LDH loss and % GDH loss, this indicator was used for a comparison of slice preparations and to monitor membrane integrity at the end of the manipulations. In addition, since intracellular volume was also measured in other slices of the same preparation, it was possible to estimate the intracellular potassium concentration as another indicator of slice quality, with the assumptions mentioned in Section 3.2.9.4.

Sodium levels were also monitored in the same slices for which potassium was measured. The reliability of this measurement was, however, less than that of potassium since the contribution of the extracellular sodium, calculated with the assumptions mentioned in the above paragraph, is important. A more detailed discussion of the significance of the assessors of slice quality and viability is provided in the discussion of the final experiments.
3.3.1.2 The slicing technique

Using 225 μm liver and kidney cortex slices made with an automatic Sorvall tissue slicer in the initial experiments yielded satisfactory slices for mice and rats, as these slices retained much of their enzymes and potassium, displayed steady rates of oxygen consumption and accumulated $^{86}\text{Rb}^+$ rapidly. This slicing technique however did not produce usable slices for the liver of larger mammals.

An experiment using tissues from rabbits excised as rapidly as for mice or rats provided evidence that using thicker slices could help to retain cell viability for the tissues of larger mammals. In this experiment, the results of which are reproduced in Figure 3.4, slices of 225 and 500 μm (Sorvall automatic slicer and manual slicer, respectively) were compared for their enzyme and potassium content both immediately after slicing and after 60 minutes on ice followed by 60 minutes in saline at 37°C (ice-cold saline was used at that time for short term storage of slices). The thicker slices consistently showed higher enzyme and potassium levels. These slices also retained a greater ability to recover their potassium content than the 225 μm slices.

The increase in enzyme levels that was observed between after slicing and after recovery (except LDH in 225 μm slices, Figure 3.4) may be an artefact due to damaged cells being eliminated during the recovery process, yielding slices richer in healthy cells, instead of being a reflection of some recovery of the enzyme levels themselves. Figure 3.4 indicate that, after slicing, the thinner slices had lost more of their mitochondrial enzyme GDH. Once the recovery process eliminated highly damaged cells, the levels of GDH were more similar between slice preparations and with whole tissues. This increase in GDH in 500
Figure 3.4
Enzyme and potassium content in whole tissues compared to 225 μm or 500 μm thick rabbit liver slices after slicing and after recovery (60 minutes on ice +60 minutes at 37°C). Results are mean ± s.e. (n=3).
\(\mu m\) slices was paralleled by an increase in LDH levels. The thinner slices (225 \(\mu m\) thick), however, had continued to lose LDH during the storage and recovery process.

It was then obvious that using a manual slicer and 500 \(\mu m\) slices yielded a higher slice quality. They have therefore been used in the final experiments. Thicker slices were not tried as diffusion could have become a problem. The diffusion rate in 500 \(\mu m\) slices is reported in the following section.

### 3.3.1.3 The stirring well system

A system in which diffusion is enhanced by stirring the saline with a magnetic stirrer, under the slice which was located on a grid was developed (Figures 3.5 and 3.6). This system was designed with the intention of eliminating the dependence on bubbling for mixing, as it was thought that the slices stirred this way could become damaged more rapidly. As well, in a bubbling well system the slices spend most of the time resting on the bottom of the wells, limiting diffusion.

In the system designed, saline oxygenation was ensured by bubbling the solution above the slice. With 12 wells, this system allowed to install all the slices to be used in an experiment at the beginning of the manipulations. 10 ml of saline were added to each well and gas bubbles trapped under the grid were removed with a Pasteur pipette before laying the tissue slice(s). The slices were transferred with a fine paintbrush and placed on the grid, ensuring that the whole of the slice rested on it and not on the annulus. The slices were maintained this in position by the gentle downward convection created by the magnetic stirrer, under the grid.
The main concerns about such a system are to ensure that the stirring rate is appropriate for diffusion in the slice without causing any significant mechanical stress. Using a 5mmx11mm stirring bar, it was observed that below 300 RPM the slices were not stabilized on the grid, whereas above 1200 RPM the more fragile slices, such as mouse liver slices, could be damaged as they are slowly forced through it. An intermediate stirring rate of 700 RPM was therefore used throughout this chapter. In an early experiment, the effect of stirring rate on potassium content of 225 μm mouse liver slices was assessed (Figure 3.7) and the results agreed with the choice of 700 RPM as stirring rate.

Figure 3.5 The 12 stirring wells system
A- Grid

23 mm

Piece of rubber tube to hold grid in position in the vial

The annulus is made of Perspex.

The grid is made out of nylon and has a 500 μm mesh

B- Stirring well

Flow observed using ink droplets injected with a fine needle. Total mixing occurs in less than 10 seconds, except above the annulus

Diffusion through slice

Grid with slice

5x11mm stirring bar at 700 RPM

Electromagnetic stirrer

Figure 3.6 The Stirring Wells System
Figure 3.7

Effect of stirring rate on potassium content of mouse liver and kidney cortex slices. Slices were 225 μm thick. Results are mean of duplicates.

Mixing in wells could be physically observed when a slice was placed on the grid and a drop of ink added to different areas using a fine needle. The observations made using this technique are illustrated in Figure 3.6 and show that mixing occurs everywhere except on top of the annulus.

In such a system, the time required to saturate the extracellular space as well as the intracellular space could be estimated by the use of radioactive markers. The diffusion characteristics of our system, reported in Figure 3.8, show that the extracellular space was replaced in 1 minute or less, as by that time no further increase in dextran space (the extracellular marker) was observed. The maximal oxygen consumption rate recorded in these experiments (refer to the final results section) is 2.3 μmol O₂·g wet⁻¹·min⁻¹, for mouse kidney slices. The extracellular space can be estimated at 322 μl/g wet
Figure 3.8

Effect of incubation time on the penetration of $^3$H$_2$O and $^{14}$C-dextran in 500 μm slices of mouse and sheep liver and kidney cortex. Data are the mean and range of 2 experiments, each done in duplicate, except for sheep liver.
for this tissue (by subtracting the intracellular volume in Table 3.3 from the tissue water contents, reported in Table 3.2). The saline being constantly saturated with a gas mixture containing 95% O₂ contains around 0.8 μmol O₂·ml⁻¹. The oxygen content of the freshly replaced extracellular space can therefore be estimated at 0.26 μmol O₂/g wet. In consequence, without extracellular saline replacement, the total extracellular oxygen supply would be depleted within 7 seconds in mouse kidney slices, making the measurement of such a high oxygen consumption rate impossible. Obviously then, in our system, diffusion in the extracellular space occurred very fast.

My observations have confirmed the findings reported in early literature (Elliot 1955) that oxygen consumption rate of tissue slices is independent of saline oxygen concentration in the upper half of the saturation range. This is the range in which the measurements were always performed.

Similarly, using the oxygen consumption rates measured and assuming a ratio of 6 oxygen molecules consumed for each glucose molecule metabolized, the most active slices used in these experiments (mouse kidney slices) would use 0.4 μmol glucose/g wet·min if they derived all of their energy from this substrate. As the saline contains 5 mM glucose, the extracellular volume of those slices would, if saturated with fresh saline, contain an estimated 1.6 μmol glucose/g wet. It would therefore take around four minutes for those slices to deplete their extracellular environment of glucose, were it not replaced.
3.3.1.4 Basis of expression of rates measured on tissue slices

The most common methods for expressing the measured parameters in tissue slices involve the use of wet or dry weight or protein content. Wet weight is influenced by the capacity of the experimenter to remove a constant and appropriate amount of saline from the slice once transferred to a pre-weighed tube, in order to leave the same proportion of fluid as is present in the whole tissue. For this reason, many investigators have used dry weight or protein content.

The use of wet weight has an advantage over dry weight in that a homogenate can be prepared for each slice and aliquots drawn for various analyses, such as enzyme activity, sodium and potassium contents, radioactivity or even protein content. Protein content cannot be thought, in view of the findings reported in this chapter, to reliably represent the amount of cells, since cytoplasmic proteins (such as LDH) and even mitochondrial proteins (e.g. GDH) leak out of the slice’s cells to a varying extent depending on the species and tissue. On the other hand, destroyed cells have protein-laden membranes which remained attached to the slice as well as some extracellular proteins (such as collagen) will also have remained in the slice. These three methods have in common that they do not fully account for the active cell mass of a slice, that could vary depending on the species, tissue and slicing technique. Cell membrane debris present in tissue slices will constitute a variable and difficult to measure proportion of slice mass.

An alternative basis of expression was sought and the activity of glutamate dehydrogenase (GDH) was chosen. This enzyme is in the part of the urea cycle that is situated in the mitochondrial matrix. GDH links the urea acid
cycle to the citric acid cycle. Because of its mitochondrial location, GDH represented a sensible choice. Other enzymes entrapped in membranes, such as those from the nuclear envelope or the endoplasmic reticulum, may have been equally appropriate. Most of these enzymes, however, display a low cellular activity, making their accurate measurement difficult. GDH activity is high in mammalian liver and kidney cortex cells, thus facilitating reliable routine measurements.

Indeed, Elliott and Pogson (1977) had already independently used GDH to evaluate LDH loss in their guinea pig hepatocyte preparations, by measuring LDH/GDH ratios in hepatocytes and whole tissues. As they expressed their results using dry weight, the activities of both enzymes were higher in hepatocytes than in whole liver, presumably because the weight of hepatocytes does not include any connective tissue or blood vessels, present in whole tissue. From a calculation using their data, there was in their hepatocytes a 15% LDH loss relative to GDH.

Some liver and kidney cortex slices from mice and sheep were fixed and examined using electron microscopy. From an examination of these electron micrographs it appeared that cells either had normal mitochondria or all mitochondria in the cell were swollen and had no mitochondrial matrix (this was especially true for cells on the edge of the tissue slice). I have therefore assumed that cells either retain their GDH or lose it all and that those cells that lost their GDH are no longer active metabolically. Thus GDH content of a tissue slice (relative to that in whole tissue) can be used to measure the active cell mass of a slice.

If the assumption above is incorrect and loss of GDH occurred from
functional cells, the rates expressed using GDH will be overestimated. Conversely, using wet weight will yield an underestimation due to some cell mass not being active. Thus, the rates of oxygen consumption and of potassium uptake for the final experiments have been expressed using wet weight as well as GDH. This will provide a range within which the rates per unit of active cell mass should be found. The rates obtained using wet weight will be emphasised since they were less variable than those same rates expressed using GDH and since the data available in the literature for comparative purposes is commonly expressed using wet weight. These rates (using wet weight as a basis of expression) will be used for all calculations except when specified.

3.3.1.4.1 Accuracy of wet weight measurements on tissue slices

The transfer of slices from the incubation saline to pre-weighed tubes with the help of a paintbrush cannot be done without transferring a variable but significant (in terms of weight) amount of saline to the tube. This excess fluid was removed by dabbing as described in Section 3.2.9.1. To test the variability of water content in slices by comparison to whole tissues, 32 slices of each of mouse and sheep liver and kidney cortex were dabbed, their wet weight determined and then left to dry under vacuum for 48 hours. The water content (%) of these slices was then calculated. The distribution of the slices for their water content is illustrated in Figure 3.9. The values in this figure compare the mean water content of these 32 slices with that of the 4 whole tissues used in the final experiments. The whole tissues from the final experiments were used for this comparison since the 32 slices of each tissue were from only 2 experiments, not enough for errors on mean whole tissue water contents to be calculated. Since water content from whole tissues was highly repeatable between experiments and consistent with early experiments, the values
Figure 3.9

Distribution of the water content in liver and kidney cortex slices of two mice and two sheep, after dabbing. These values are from the potassium uptake preliminary (Section 3.2.6). The values for whole tissues are extracted from the data used for Table 3.2. The results are expressed as mean ± s.e. (n).
presented in Figure 3.9 are expected to be representative of in vivo values. An exception to this is for sheep liver. Sheep livers often appeared pale in colour, instead of the usual dark red found for other species and were probably bled efficiently at the abattoir. Their water content was more variable than for other species (Table 3.2) and one of them (63.2% water) was too low to be included in the dataset.

The comparison of the water contents of slices and whole tissues (Figure 3.9) suggest that this method is more accurate for some species and tissues than others. After dabbing, mouse liver and sheep kidney slices had on average the same water content as in the whole tissues.

The mouse kidney slices were the smallest of all due to the size of the tissue sample. Probably because of the increased surface area of these slices, more water was absorbed than in the whole tissue. The kidney cortex slices from other species were larger and of comparable size. Consequently, they are expected to have been as representative of whole tissue water content as for sheep kidney slices. Therefore, only the error on the weight of mouse kidney cortex slices was considered in this chapter.

A small difference in water content leads to more important errors in the wet weight. With the values reported in Figure 3.9 for mouse kidney cortex slices, the average wet weight of these slices was underestimated by 28%. Systematic corrections of this potential error were not conducted in the tables of this chapter, since dabbing efficiency was not monitored for all species and since, given the high variability for mouse kidney slices (Figure 3.9), the 28% error did not apply to all slices. In fact, since the results from this experiment were available before the final experiments, additional care was taken not to
"overdab" very small slices. Thus, in each of the following sections dealing with tissue slices the 28% correction was only applied as a mean to estimate the maximal impact that this potential error could have had on the allometric coefficients.

Since liver slices from all species were of similar size, systematic errors on wet weight due to improper dabbing were unlikely for these slices. In sheep liver the differences in water content of whole tissues and slices were probably due to errors in whole tissue water content. No corrections were performed for errors on the wet weight of liver slices of any species since it is likely that all had very similar water content to those of in vivo whole tissues.
3.3.2 Final experiments

All the results presented in this section were obtained under the same conditions, (Section 3.2), designed to test the hypotheses 2 and 3 of this thesis. To this end, intracellular water volume, oxygen consumption rates and potassium uptake and efflux rates were determined in separate slices of liver and kidney cortex in each of five mammalian species. Slices and whole tissues were also tested for potassium and sodium content and LDH and GDH activity.

The body weight of the animals used ranged from 30g in the mouse to more than 300 000g for cattle with the weight of the other three species being fairly evenly spread along the logarithmic scale (Table 3.2). Mammals of all body size had very similar water and protein content in their liver and kidney cortex. Liver differed in composition from kidney cortex in all five species examined, with kidney cortex containing more water and less protein per unit mass than liver.
Table 3.2

Total body weight of the animals used and water and protein content of liver and kidney cortex. Results are presented as mean±s.e. (n=4 for each mean, except for mouse tissues protein content and sheep liver water content where n=3)

<table>
<thead>
<tr>
<th>Animal</th>
<th>Body weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>30 ± 4</td>
</tr>
<tr>
<td>Rat</td>
<td>457 ± 6</td>
</tr>
<tr>
<td>Rabbit</td>
<td>3248 ± 268</td>
</tr>
<tr>
<td>Sheep</td>
<td>32900 ± 787</td>
</tr>
<tr>
<td>Cattle</td>
<td>369000 ± 34315</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Water content (mg/g wet)</th>
<th>Protein content (mg/g wet)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>Kidney</td>
</tr>
<tr>
<td>Liver</td>
<td>Kidney</td>
</tr>
<tr>
<td>Mouse 730 ± 6</td>
<td>835 ± 7</td>
</tr>
<tr>
<td>175 ± 29</td>
<td>156 ± 9</td>
</tr>
<tr>
<td>Rat 702 ± 4</td>
<td>818 ± 0</td>
</tr>
<tr>
<td>181 ± 16</td>
<td>157 ± 5</td>
</tr>
<tr>
<td>Rabbit 715 ± 3</td>
<td>799 ± 6</td>
</tr>
<tr>
<td>189 ± 8</td>
<td>170 ± 14</td>
</tr>
<tr>
<td>Sheep 707 ± 16</td>
<td>806 ± 5</td>
</tr>
<tr>
<td>191 ± 4</td>
<td>152 ± 9</td>
</tr>
<tr>
<td>Cattle 710 ± 7</td>
<td>791 ± 3</td>
</tr>
<tr>
<td>192 ± 5</td>
<td>153 ± 9</td>
</tr>
</tbody>
</table>

3.3.2.1 Intracellular water space in liver and kidney cortex slices and estimates of the proportion of the total water space that is intracellular in whole tissues

Although there was no significant difference in the volume of the intracellular compartment per unit weight for the kidney cortex of the 5 species investigated, cattle liver had a significantly (p<0.5) higher intracellular volume than mouse or rabbit (16.5 and 14.2% higher, respectively) (Table 3.3). The relationship, reproduced in Figure 3.10, was significant (p < 0.05). The
allometric variations were not of major importance however, with a doubling in body size corresponding to a 1.1% increase in liver intracellular volume.

Table 3.3

Intracellular volume (μl/g wet) of liver and kidney slices in five mammalian species. Results are presented as mean ± s.e. (n=4). Values in a column with different letters differ significantly (p≤0.05).

<table>
<thead>
<tr>
<th></th>
<th>Liver</th>
<th>Kidney</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>432 ± 22 a</td>
<td>513 ± 36 a</td>
</tr>
<tr>
<td>Rat</td>
<td>457 ± 19 ab</td>
<td>491 ± 22 a</td>
</tr>
<tr>
<td>Rabbit</td>
<td>440 ± 3 a</td>
<td>453 ± 20 a</td>
</tr>
<tr>
<td>Sheep</td>
<td>482 ± 26 ab</td>
<td>542 ± 35 a</td>
</tr>
<tr>
<td>Cattle</td>
<td>503 ± 9 b</td>
<td>523 ± 7 a</td>
</tr>
</tbody>
</table>

Using wet weight as a basis of expression could have led to an overestimation for the very small mouse kidney slices (Section 3.3.1.4.1). Correcting for the possible 28% underestimation of wet weight for mouse kidney cortex slices would give 401 μl intracellular volume·g wet⁻¹ for this tissue. The trend could then be as in liver, with cattle displaying more intracellular volume per gram of tissue than mouse. Using the average values from Table 3.3 with the corrected value above for mouse kidney cortex slices, the allometric increase in intracellular volume in this tissue was not statistically different (0.05 < p < 0.10). However, the trend would have been statistically significant using the individual values (p < 0.01). The allometric exponent (-
0.028) would suggest that a doubling in body size corresponded to a 2% increase in the mass-specific intracellular volume or kidney cortex.

![Liver](image)

**Figure 3.10**

Allometric relationship of intracellular volume in mammalian liver. The allometric coefficient is given. Values are mean ± s.e. (n=4).

As the infraspecific variability of % LDH loss and % GDH loss was high in the slices used for the measurement of intracellular volumes, there were no significant differences with respect to the % GDH loss in any tissue, nor in the % LDH loss of kidney cortex slices. Cattle liver slices used in these measurements however had lost significantly more LDH than the slices of other animals except sheep, while sheep had lost more LDH than rat or rabbit liver slices (p≤0.05). Nonetheless, Figures 3.11a and 3.11b illustrate that the measurement of intracellular volumes in liver and kidney cortex slices was independent of % LDH loss or % GDH loss. Would this not be the case, the intracellular volume per gram of wet tissue would decrease in slices displaying high % GDH loss or % LDH loss, indicating that $^{14}$C-dextran had penetrated
the plasma membranes of more damaged cells. Consequently, intracellular volumes could not be expressed using GDH as a basis of expression.

Figure 3.11a
Relationship between intracellular volume and % LDH loss in liver and kidney cortex slices. Each data point is the average of triplicate measurements.
**Figure 3.11b**

Relationship between intracellular volume and % GDH loss in liver and kidney cortex slices. Each data point is the average of triplicate measurements.

It is likely that the estimates of intracellular volumes using wet weight represented a slight underestimation compared to whole tissues since some of the cells would have been lost but their membranes and surrounding connective tissue could have still accounted for some weight. Nevertheless, the
reliability of the intracellular volume measurements was further confirmed by the estimates of \([K^+]_i\) for whole tissues. Applying the measurements of intracellular volume performed on tissue slices to whole tissues yielded estimates of \([K^+]_i\) comparable to those reported in the literature (Section 3.4.2). Intracellular volume could not be used systematically as a basis of expression of other parameters as it was not measured on all slices.

The method used for the determination of the intracellular volume measured the difference between the total water space and the space that is not accessible to dextran, which is assumed not to penetrate plasma membranes. From these measurements it is possible to estimate the proportion of intracellular water in whole tissues in relation to their total water content (Table 3.2). For liver, the proportion (%) of total water space that is intracellular is 59, 65, 62, 68 and 71% for mouse, rat, rabbit, sheep and cattle, respectively. For the same species in kidney cortex the values are 61, 60, 57, 67 and 66%, respectively.

### 3.3.2.2 Potassium and sodium levels and LDH and GDH activity in whole tissues

Tables 3.4 and 3.5 describes these parameters for the whole tissues used in this study. The potassium content in the liver of all the species investigated is similar, at around 60 to 70 \(\mu\)mol K\(^+\)/g wet. Statistical analysis revealed that mouse and sheep liver contain significantly \((p \leq 0.05)\) less potassium than rat liver, with intermediate, but not statistically different values for rabbit and cattle.

The levels of potassium were lower in kidney cortex than in liver and
showed a highly significant (p < 0.01) allometric increase with body size (Figure 3.12). This could reflect intracellular concentration, or be due to an increase in intracellular volume with body size in this tissue. Although the results reported in Table 3.3 do not support the latter hypothesis, as mentioned in Section 3.4.1 we cannot discard the possibility that a small increase in intracellular volume with body size in kidney cortex occurred.

Table 3.4
Sodium and potassium content (μmol/g wet) of whole liver and kidney in five mammalian species. Results are presented as mean ± s.e. (n=4 in each group, except mouse and cattle for sodium content, where n=3). Values in a column with different letters differ significantly (p<0.05).

<table>
<thead>
<tr>
<th></th>
<th>Liver</th>
<th>Kidney</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Potassium</td>
<td>Sodium</td>
</tr>
<tr>
<td>Mouse</td>
<td>60.0 ± 2.5 a</td>
<td>41.1 ± 4.9 a</td>
</tr>
<tr>
<td>Rat</td>
<td>70.0 ± 2.5 b</td>
<td>36.0 ± 2.5 a</td>
</tr>
<tr>
<td>Rabbit</td>
<td>61.5 ± 2.8 ab</td>
<td>42.0 ± 1.2 a</td>
</tr>
<tr>
<td>Sheep</td>
<td>55.6 ± 4.5 a</td>
<td>38.6 ± 5.0 a</td>
</tr>
<tr>
<td>Cattle</td>
<td>65.6 ± 2.0 ab</td>
<td>38.2 ± 1.2 a</td>
</tr>
</tbody>
</table>

Although no significant differences in the sodium content of whole liver were observed, the kidney cortex of sheep and cattle contained significantly (p<0.05) less sodium than that of the smaller mammals. Since sodium is mostly extracellular, this would reinforce the case of larger mammals having slightly higher intracellular volumes in their kidney cortex: the sodium contents
recorded relate, inversely, to the potassium levels in this tissue. In liver however, larger intracellular volumes did not directly correspond to higher potassium and lower sodium content. Cattle liver, which displayed significantly higher intracellular volume in that tissue, did not show higher potassium levels or lower sodium levels, although it ranked second on both, after rat liver.

Provided that no important loss of fluid from the extracellular space of whole tissue samples occurred before transfer of the samples to their tubes for processing, the amounts of sodium and potassium should be reflective of in vivo tissue contents even if some of the sodium leaked in and potassium out of the cells before sampling.

It is possible to estimate the in vivo intracellular concentrations of potassium and sodium in whole tissues, assuming that no significant loss of extracellular fluid occurred, that the extracellular fluid contained 5.93 mM potassium and 145 mM sodium in vivo and that the intracellular volumes in Table 3.3 calculated with tissue slices were applicable to whole tissues. The intracellular potassium concentrations ([K]i) can be estimated using the formula in Section 3.2.9.4.

The estimated [K]i, in mM, are 135, 150, 136, 113 and 128 for mouse, rat, rabbit, sheep and cattle liver and 79, 89, 100, 93 and 112 for kidney cortex, respectively. The average values of [K]i in liver including all mammals was 132±6 mM (mean±s.e.), with no allometric trend. In kidney cortex, the average [K]i of 95±6 mM could be slightly higher (99 mM) if mouse kidney values were corrected for a possible overestimation of intracellular volume due to too efficient dabbing (Section 3.3.1.4.1). This artefact could also lead to the
disappearance of the allometric trend of increase in potassium content observed in kidney cortex (Table 3.4 and Figure 3.12).

The same estimations can be made for sodium levels. The [Na]i (mM) in liver obtained from those estimations is 0, 0, 5, 18 and 16 (mean ± s.e.: 8±4 mM) and in kidney cortex, 65, 58, 74, 46 and 48 (mean ± s.e.: 58±5 mM) for mouse, rat, rabbit, sheep and cattle respectively. The estimations of [Na]i can be expected to be less reliable than [K]i because of the impact of small errors on the estimation of the sodium-rich extracellular volumes. For example, in mouse kidney cortex if an overestimation of only 10% on intracellular volumes had occurred, the [Na]i would be 25 mM, a 61% decrease over the value reported above and a normal value for proximal tubule cells (Lote, 1987). The same error for this tissue would decrease the estimate of [K]i by only 11%.

![Figure 3.12](image)

**Figure 3.12**

Allometric relationship of potassium content in mammalian kidney cortex. The allometric coefficient is given. Values are mean ± s.e. (n=4).
The maximal activities of LDH and GDH in whole liver and kidney cortex reported in Table 3.5 did not show any significant allometric trends. Rabbits had typically no more than half the GDH activity found in the other mammals in both tissues. In the liver, rats showed more than twice as much LDH activity as any of the other species, while the larger mammals had less activity of this enzyme than smaller mammals. The differences in LDH activity observed in liver were not reflected in kidney cortex, where the lowest activities were found for rats and rabbits.

Table 3.5
GDH and LDH activity (IU/g wet) of whole liver and kidney in five mammalian species. Results are presented as mean ± s.e. (n=4). Values in a column with different letters differ significantly (p≤0.05).

<table>
<thead>
<tr>
<th></th>
<th>Liver</th>
<th></th>
<th>Kidney</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GDH</td>
<td>LDH</td>
<td>GDH</td>
<td>LDH</td>
</tr>
<tr>
<td>Mouse</td>
<td>138 ± 28 ab</td>
<td>227 ± 22 b</td>
<td>42 ± 7 ab</td>
<td>169 ± 13 c</td>
</tr>
<tr>
<td>Rat</td>
<td>181 ± 29 ab</td>
<td>590 ± 36 c</td>
<td>96 ± 20 bc</td>
<td>87 ± 8 ab</td>
</tr>
<tr>
<td>Rabbit</td>
<td>94 ± 10 a</td>
<td>161 ± 35 ab</td>
<td>26 ± 4 a</td>
<td>55 ± 5 a</td>
</tr>
<tr>
<td>Sheep</td>
<td>248 ± 50 b</td>
<td>56 ± 13 a</td>
<td>79 ± 11 c</td>
<td>121 ± 14 bc</td>
</tr>
<tr>
<td>Cattle</td>
<td>172 ± 28 b</td>
<td>89 ± 15 a</td>
<td>81 ± 21 bc</td>
<td>143 ± 22 c</td>
</tr>
</tbody>
</table>
3.3.2.3 Characterization of tissue slices and viability

The slices used as controls, which were all collected at the same time after death and around the time of the measurements of the various rates reported further in this chapter (Table 3.1), were used to compare the variation of slice damage between species and tissues. This comparison was performed with the help of %LDH loss, %GDH loss and %K⁺ loss, reported in Table 3.6.

Each slice used for the measurements of the rates reported further in this chapter, except those for potassium efflux, was also monitored for these parameters and compared to those control values. This would have allowed for the elimination of values from slices clearly more damaged than the controls of this preparation. This selection was not performed as the slices used for the measurements of rates displayed indicators of quality comparable to those presented in this section for control slices.

The three indexes in Table 3.6 indicated that in liver, the slices that suffered the least damage were those from rats and rabbits. The sheep and cattle liver slices consistently suffered more damage than those from the other species. The mouse liver slices occupied an intermediate position.

Kidney cortex slices were much more comparable in terms of damage as estimated with % LDH loss and % GDH loss. However, the % K⁺ loss in this tissue showed a clear allometric increase (Table 3.6). Although there was no significant difference in the % LDH loss for the slices of this tissue, the significance of the differences in % GDH loss (Table 3.6) suggest that unlike liver slices kidney cortex slices had suffered less damage in the sheep and more damage in the rat. Rabbits again had the least damaged slices using enzyme
indicators. These differences, however, were not reflected in the capacity to recover potassium levels.

Table 3.6
% LDH loss, % GDH loss and % potassium loss in liver and kidney slices of five mammalian species. The slices used for this Table are the controls. Results are presented as mean ± s.e. (n=4). Values in a column with different letters differ significantly (p≤0.05).

<table>
<thead>
<tr>
<th></th>
<th>LIVER</th>
<th>% LDH loss</th>
<th>% GDH loss</th>
<th>% K+ loss</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>49.5 ± 10.7 ab</td>
<td>33.0 ± 15.0 ab</td>
<td>52.9 ± 9.9 bc</td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td>24.1 ± 4.8 a</td>
<td>16.1 ± 3.5 a</td>
<td>20.5 ± 3.8 a</td>
<td></td>
</tr>
<tr>
<td>Rabbit</td>
<td>11.6 ± 13.3 a</td>
<td>10.5 ± 6.6 a</td>
<td>28.2 ± 14.5 ab</td>
<td></td>
</tr>
<tr>
<td>Sheep</td>
<td>69.1 ± 6.2 b</td>
<td>45.3 ± 4.6 b</td>
<td>80.1 ± 6.5 bc</td>
<td></td>
</tr>
<tr>
<td>Cattle</td>
<td>71.9 ± 0.8 b</td>
<td>35.3 ± 5.6 b</td>
<td>81.3 ± 3.6 c</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>KIDNEY</th>
<th>% LDH loss</th>
<th>% GDH loss</th>
<th>% K+ loss</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>40.8 ± 4.4 a</td>
<td>18.6 ± 7.6 ab</td>
<td>16.9 ± 9.0 a</td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td>46.1 ± 2.4 a</td>
<td>40.2 ± 4.5 b</td>
<td>37.7 ± 2.7 a</td>
<td></td>
</tr>
<tr>
<td>Rabbit</td>
<td>29.5 ± 10.9 a</td>
<td>4.9 ± 7.2 a</td>
<td>38.6 ± 3.8 a</td>
<td></td>
</tr>
<tr>
<td>Sheep</td>
<td>34.2 ± 2.9 a</td>
<td>18.3 ± 6.2 a</td>
<td>69.9 ± 3.9 b</td>
<td></td>
</tr>
<tr>
<td>Cattle</td>
<td>56.8 ± 8.2 a</td>
<td>33.6 ± 8.5 ab</td>
<td>74.2 ± 5.0 b</td>
<td></td>
</tr>
</tbody>
</table>

While the % LDH loss and % GDH loss give an account of the damage that occurred mostly during the slicing, the % K+ loss in Table 3.6 also include the ability of slices to recover their transmembrane cation gradients. At the time
of sampling the control slices, the slices' cells, except for mouse and rat kidney slices, had not managed to recover their potassium content above what they could be estimated to have initially lost, if % LDH loss reflected the loss of cytoplasm and thus of cytoplasmic K⁺. If % K⁺ loss reflects recovery capacity, in liver slices rats and rabbits showed significantly higher recovery ability and the larger mammals showed a definite inability for restoring their potassium levels. In kidney cortex slices, sheep and cattle also showed the lowest ability to recover their potassium levels. These findings will be recalled later in relation to the activity of the sodium pumps (Section 3.3.2.5).

Although the variability of % K⁺ loss in the triplicates for a given experiment tended to be low, it was often important between experiments. The least variable slice preparations were those from rat in both tissues (Table 3.6).

The intracellular concentrations of potassium and sodium have been estimated for the control slices (as described in Section 3.2.9.4) and are reported in Table 3.7. The trends observed for intracellular potassium concentrations reflect those reported in Table 3.6 for % potassium loss. Since the total of [K⁺]ᵢ and [Na⁺]ᵢ was relatively constant, potassium loss was accompanied by a gain in sodium. While all slices did maintain potassium levels above and sodium levels below saline concentration, again in liver the rats and rabbits did better than sheep or cattle with mouse being intermediate. None of these slices however seemed to be able to maintain the concentration gradients associated with physiological conditions, like those calculated in Section 3.3.2.2 for whole tissues. The [Na]ᵢ tended to increase with body size.
In kidney cortex slices, sheep and cattle were poorer in maintaining their cation gradients. The data in Table 3.7 reflect those in Table 3.4 for whole tissues: kidney cells contain more sodium. Contrary to whole tissue estimations (Section 3.3.2.2), once the measured intracellular volumes were considered there was a clear tendency for the kidney cortex slices of larger animals to have higher \([\text{Na}]_i\).

---

**Table 3.7**

Intracellular potassium and sodium concentrations (mM) in liver and kidney control slices, corrected for the presence of extracellular saline. Results are mean±s.e. \(n=4\) except for mouse and cattle \([\text{Na}]_i\), for which \(n=3\): no statistics could therefore be computed for this parameter. Values in a column with a different letter differ significantly \((p<0.05)\).

<table>
<thead>
<tr>
<th>LIVER</th>
<th>([\text{K}]_i)</th>
<th>([\text{Na}]_i)</th>
<th>([\text{K}]+[\text{Na}]_i)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>60 ± 10 b</td>
<td>86 ± 10</td>
<td>142 ± 6</td>
</tr>
<tr>
<td>Rat</td>
<td>119 ± 5 a</td>
<td>50 ± 7</td>
<td>169 ± 8</td>
</tr>
<tr>
<td>Rabbit</td>
<td>96 ± 19 ab</td>
<td>47 ± 22</td>
<td>143 ± 5</td>
</tr>
<tr>
<td>Sheep</td>
<td>20 ± 7 bc</td>
<td>137 ± 12</td>
<td>157 ± 5</td>
</tr>
<tr>
<td>Cattle</td>
<td>22 ± 4 c</td>
<td>143 ± 2</td>
<td>165 ± 5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>KIDNEY</th>
<th>([\text{K}]_i)</th>
<th>([\text{Na}]_i)</th>
<th>([\text{K}]+[\text{Na}]_i)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>64 ± 3 a</td>
<td>79 ± 8</td>
<td>142 ± 11</td>
</tr>
<tr>
<td>Rat</td>
<td>54 ± 4 a</td>
<td>87 ± 8</td>
<td>141 ± 7</td>
</tr>
<tr>
<td>Rabbit</td>
<td>60 ± 5 a</td>
<td>98 ± 11</td>
<td>158 ± 7</td>
</tr>
<tr>
<td>Sheep</td>
<td>26 ± 4 b</td>
<td>110 ± 5</td>
<td>137 ± 4</td>
</tr>
<tr>
<td>Cattle</td>
<td>26 ± 4 b</td>
<td>120 ± 8</td>
<td>147 ± 3</td>
</tr>
</tbody>
</table>
Since the early experiments revealed that the slices could survive for only a limited time, it was necessary to monitor the decrease in slice quality with time (termed "slice viability") in these experiments. As the experimental plan did not leave room for more measurements, slice viability could not be systematically assessed in the same experiments. Nevertheless, it was possible to use the slices for which oxygen consumption was monitored at the beginning and at the end of each experiment to this end, as the time period between these measurements had been noted and indicators of slice quality monitored.

While the data reported in Table 3.6 are direct measurements of parameters relating to slice damage or recovery capacity, the results in Table 3.8 concerning slice viability are estimations calculated as the difference in slice quality between the first and second measurements of oxygen consumption, divided by the time period separating them. The interpretation of Table 3.8 relies on an assumption of linearity.

Table 3.8 suggest that there was no significant difference in the rate at which the slices deteriorate, except in liver where sheep and cattle showed a faster rate of LDH and GDH loss than rat or rabbit, with intermediate values for mouse. On average, in one hour the liver slices lost 6, 3 and 5% of their LDH, GDH or potassium, respectively while the losses for kidney cortex slices were 6, 5 and 4%, respectively. Oxygen consumption rates decreased faster in larger mammals, although not significantly.

The results in Table 3.8 should be treated in comparison with those from Tables 3.6 and 3.7 for control slices: although the results in Table 3.8 do not suggest important differences in the rate of decrease of slice quality, those from larger mammals displayed poorer initial conditions.
Table 3.8

Decrease of slice quality with time, as estimated by % LDH loss/hr., % GDH loss/hr., % potassium loss/hr. and % decrease of oxygen consumption/hr. in liver and kidney slices of five mammalian species. The slices used for this Table are those for which oxygen consumption was measured at the end of the experiment compared to those used at the beginning of that same experiment* (1 measurement per time point). Results are presented as mean ± s.e. (n=4). Values in a column with different letters differ significantly (p≤0.05).

<table>
<thead>
<tr>
<th>% decrease/hour</th>
<th>LIVER</th>
<th></th>
<th></th>
<th>KIDNEY</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LDH</td>
<td>GDH</td>
<td>Potassium</td>
<td>O₂ Cons.</td>
<td>LDH</td>
<td>GDH</td>
</tr>
<tr>
<td><strong>Mouse</strong></td>
<td>7.8 ± 3.1&lt;sup&gt;ac&lt;/sup&gt;</td>
<td>1.4 ± 4.1&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>4.9 ± 3.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14.5 ± 9.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>13.5 ± 3.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14.5 ± 12.7&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Rat</strong></td>
<td>-0.5 ± 3.3&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>-5.4 ± 3.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.3 ± 3.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-5.6 ± 4.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.8 ± 2.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.3 ± 1.9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Rabbit</strong></td>
<td>-1.1 ± 1.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-2.0 ± 1.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.2 ± 5.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.1 ± 5.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.8 ± 6.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.3 ± 2.6&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Sheep</strong></td>
<td>11.5 ± 2.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>11.1 ± 2.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.3 ± 2.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>42.3 ± 4.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.1 ± 4.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.4 ± 3.1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Cattle</strong></td>
<td>11.2 ± 3.0&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>7.0 ± 3.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.5 ± 1.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>36.7 ±18.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Calculated as the difference between the second and the first time points adjusted for one hour. For the expression of the results as % decrease/hr., there is an underlying assumption of linearity.
In summary, the tables in this section indicate that rat and rabbit liver slices suffered less damage and maintained an overall higher viability than sheep or cattle, with intermediate quality for mouse. We can also observe that kidney slices from the species investigated are more comparable in their condition and viability although they also differ in their recovery capacity.

3.3.2.4 Allometric comparison of oxygen consumption rate in mammalian liver and kidney cortex slices

This section reports on the tissue-specific rate of aerobic metabolism in an allometric comparison of mammalian liver and kidney cortex slices. First, the oxygen consumption rates measured 2 hours after death were compared. Then, corrections have been made in an attempt to estimate the importance of some artefacts on the rates of oxygen consumption.

Tissue slices from larger mammals displayed significantly lower rates of oxygen consumption than those from smaller mammals although statistically significant differences sometimes only appeared for large differences in body size (Table 3.9). The allometric relationship of oxygen consumption rates was highly significant for both tissues ($p < 0.01$) (Figure 3.13). When using only the main values, a more rigorous test for statistical significance, both relationships were still significant ($p < 0.01$ for liver and $p < 0.02$ for kidney cortex slices).

From the allometric exponents in Figure 3.13, a doubling in body size calculated to result in a 14% decrease in oxygen consumption of liver slices. In kidney cortex slices, the allometric relationship was not as pronounced as for liver, with a doubling in body size being associated with a 8% decrease in tissue slice oxygen consumption.
Table 3.9

Oxygen consumption (\(\mu\text{mol/g wet-min.}\)) in liver and kidney slices of five mammalian species. All measurements were performed between 107 and 148 minutes after death. Results are presented as mean\(\pm\)s.e. (n=4). Values in a column with different letters differ significantly (p≤0.05).

<table>
<thead>
<tr>
<th></th>
<th>LIVER</th>
<th>KIDNEY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>1.48 ± 0.18 (^a)</td>
<td>2.27 ± 0.28 (^a)</td>
</tr>
<tr>
<td>Rat</td>
<td>1.37 ± 0.11 (^a)</td>
<td>1.51 ± 0.16 (^ab)</td>
</tr>
<tr>
<td>Rabbit</td>
<td>0.78 ± 0.17 (^a)</td>
<td>1.22 ± 0.11 (^b)</td>
</tr>
<tr>
<td>Sheep</td>
<td>0.37 ± 0.03 (^b)</td>
<td>1.23 ± 0.16 (^b)</td>
</tr>
<tr>
<td>Cattle</td>
<td>0.25 ± 0.04 (^c)</td>
<td>0.66 ± 0.05 (^c)</td>
</tr>
</tbody>
</table>

The accuracy of the rates of oxygen consumption reported in Table 3.9 is dependent on dabbing efficiency (Section 3.3.1.4.1). The values for mouse kidney could represent an overestimation of up to 28%. If this is the case, the corrected rate would be 1.77 \(\mu\text{mol O}_2\cdot\text{g wet}^{-1}\cdot\text{min}^{-1}\). Using this corrected value, a doubling in body weight would have been associated with a 6% decrease in mass specific rate of oxygen consumption in kidney cortex (allometric exponent = -0.087). Errors in the determination of wet weight will not have influenced the other values to such an extent (Section 3.3.1.4.1).

The impact of experimental artefacts on the rates of oxygen consumption reported in Table 3.9 can be assessed to some extent. The first and perhaps most relevant parameter to consider is the “active respiring cell mass”. This parameter is calculated using the GDH content of the respiring slices (refer to
Figure 3.13

Allometric relationship of oxygen consumption in mammalian liver and kidney cortex slices expressed using slice wet weight. Each point represents an individual measurement, 107 to 148 minutes after the death of the animal. The allometric equations are given with each graph.
Section 3.3.1.4). Loss of GDH either reflects the loss of whole mitochondria or of mitochondrial enzymes, thus of those cell parameters most related to the consumption of oxygen: mitochondria are responsible for 85-90\% of the total cellular oxygen consumption in rat liver (Brand, 1990). In this correction, the oxygen consumption rates of the individual slices for which the means are reported in Table 3.9 were used, in conjunction with the GDH content of the same slices, to estimate the oxygen consumption rates at 100 \% active respiring cell mass (100\% GDH content relative to the whole tissue). The extent of the corrections did not always closely reflect the \% GDH loss reported for control slices in Table 3.6 since the \% GDH loss from the slices used for oxygen consumption may have individually differed from the average values of the control slices.

In this calculation, it was assumed that 100\% of the oxygen consumption is mitochondrial. The weight specific rates of oxygen consumption corrected for this parameter are reported in Table 3.10. This correction usually lead to more intraspecific variability than when using wet weight.

The rates of oxygen consumption corrected for active respiring cell mass yielded higher values than when using wet weight as a basis of expression (14 to 56\% higher in rabbit and cattle liver slices respectively and 7 to 84\% higher in (mouse & rabbit) and rat kidney slices, respectively) but had little effect on the allometric comparison (Figure 3.14). After correction for active respiring cell mass, a doubling in body size corresponded to a 13\% and 6\% decrease in tissue oxygen consumption for the same tissues. The allometric relationship was highly significant for both tissues (p < 0.01). When the mean values presented in Table 3.10 were used for the allometric plots, the relationship was still significant for liver (p < 0.02) but not for kidney (0.05 < p < 0.10).
Table 3.10

Oxygen consumption rate (µmol O₂/g wet-min.) of liver and kidney tissue slices corrected for active respiring cell mass (using GDH activity) (mean±s.e.).

<table>
<thead>
<tr>
<th></th>
<th>LIVER</th>
<th>KIDNEY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>1.84 ± 0.21</td>
<td>2.43 ± 0.23</td>
</tr>
<tr>
<td>Rat</td>
<td>2.09 ± 0.34</td>
<td>2.78 ± 0.53</td>
</tr>
<tr>
<td>Rabbit</td>
<td>0.89 ± 0.17</td>
<td>1.32 ± 0.20</td>
</tr>
<tr>
<td>Sheep</td>
<td>0.56 ± 0.10</td>
<td>1.57 ± 0.27</td>
</tr>
<tr>
<td>Cattle</td>
<td>0.39 ± 0.07</td>
<td>1.10 ± 0.14</td>
</tr>
</tbody>
</table>

For the calculation of oxygen consumption rates using GDH, the first rate measured for each slice preparation was divided by the IU GDH in that slice instead of wet weight and multiplied by the IU GDH/g wet of the whole tissue from which the slice comes.

Since the rates of oxygen consumption in tissue slices decreased with time (except, for the time-course used, for rat tissues, as reported in Table 3.8), it was also possible to extrapolate the oxygen consumption at t=0 after death, assuming a linear relationship between these parameters. For this correction, the 8 measurements for a given tissue and species (1 at the beginning and 1 at the end of each experiment for each animal) were plotted against the time after death of each measurement and the y-intercept of these relationships taken as “rate of oxygen consumption at t=0”. The values of oxygen consumption corrected for time after death are reported in Table 3.11.

Since the relative decrease in oxygen consumption rate after death was greater in tissue slices from large mammals (Table 3.8), correcting for time after
death had a greater effect for these animals (Table 3.11). The decrease in oxygen consumption is not likely to have been linear (at least in the case of some liver slice preparations from large mammals in which the second slice had ceased respiring). Thus the high estimates that are given by correcting for time after death may not be realistic. Their significance should not be considered as informative as the values obtained by correcting for active respiring cell mass. Nevertheless, in many instances both corrections gave similar values. This was the case for mouse, rabbit and sheep liver and for rabbit and sheep kidney cortex.

![Graph showing allometric relationship of oxygen consumption in mammalian liver and kidney cortex slices estimated using GDH or time after death](image)

**Figure 3.14**

Allometric relationship of oxygen consumption in mammalian liver and kidney cortex slices estimated using GDH or time after death (the data are calculated as described in Tables 3.10 and 3.11, respectively). For the estimates using GDH, each point represents an individual measurement, 107 to 148 minutes after the death of the animal. For the estimates using time after death, only one estimate per species and tissue could be obtained.
Table 3.11

Oxygen consumption rate (μmol O₂/g wet-min.) of liver and kidney tissue slices corrected for time after death (one estimate per species and tissue).

<table>
<thead>
<tr>
<th></th>
<th>LIVER</th>
<th></th>
<th>KIDNEY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>2.14</td>
<td></td>
<td>3.21</td>
</tr>
<tr>
<td>Rat</td>
<td>1.20</td>
<td></td>
<td>1.58</td>
</tr>
<tr>
<td>Rabbit</td>
<td>0.84</td>
<td></td>
<td>1.59</td>
</tr>
<tr>
<td>Sheep</td>
<td>0.67</td>
<td></td>
<td>2.29</td>
</tr>
<tr>
<td>Cattle</td>
<td>0.50</td>
<td></td>
<td>1.17</td>
</tr>
</tbody>
</table>

The correction using time after death is an extrapolation to t = 0 after death of a plot of oxygen consumption vs. time after death, using the first and second measurement on each preparation and grouping all the points for a species in a given tissue (n=8).

The allometric relationship of oxygen consumption rate corrected for time after death (Figure 3.14) was highly significant for liver (p < 0.01), but was not for kidney cortex mainly because of the high corrected values for sheep. Using these values, a doubling in body size corresponded to a decrease in oxygen consumption of 10% for liver and 5% for kidney. As the estimations using this method yielded only one value per species or tissue, the significance of the allometric relationship for kidney could not be assessed further by using individual data.
I did not consider combining both corrections in a final estimate of oxygen consumption rates of tissue slices since % GDH loss was probably not independent from time after death (Table 3.8).

The transmembrane potassium and sodium gradients reported in Table 3.7 also almost certainly affected slice respiration rates to some extent. Figure 3.15 shows that the % K⁺ loss of the slices used for the measurements of oxygen consumption did not present a linear relationship with oxygen consumption. That is, at lower % K⁺ loss oxygen consumption rate was unrelated to [K⁺]. The values presented in Tables 3.9 and 3.10 are the averages of the first measurements in each experiment, those points associated with lower % K⁺ loss in Figure 3.15.

It is important when viewing Figure 3.15 to bear in mind that the most K⁺-depleted slices in each species and tissue were also used for oxygen consumption 2 hours after their counterparts. It is obvious for liver slices that those with the lowest potassium levels also displayed lower oxygen consumption rates. There was very little overlap of % K⁺ loss in this figure between the values for larger and smaller mammals, making any conclusion concerning a relationship between % K⁺ loss and liver slice respiration rates only tentative.

The case of kidney in Figure 3.15 is more informative: the points for rat show two clusters, with a very strong slope, the highest cluster representing the first slices used in an experiment. In rat kidney cortex slices, the relationship was clearly more influenced by time than by % K⁺ loss, as other slices, such as
rabbit kidney slices, displayed a much wider range of % K⁺ loss but no clear relationship with oxygen consumption rates.

Figure 3.15

Relationship between potassium loss and oxygen consumption rate for liver and kidney slices. Each point represents an individual measurement performed either 106 to 148 or 203 to 270 minutes after death (first or second slice of an experiment).
Summary

The mass-specific rate of aerobic metabolism showed a clear and statistically significant allometric decline in both liver and kidney cortex slices. A doubling in body size was accompanied by a 10 to 14% decrease in mass specific oxygen consumption in liver and 5 to 8% decrease in kidney cortex. The loss of potassium in some slice preparations did not clearly affect oxygen consumption, at least when the most depleted slices (those measured last of two in each experiment) were not considered. The poorer condition of liver slices from large mammals may have lead to some underestimation of their metabolic rate but the corrections performed showed that the allometric trend would be most unlikely to disappear had all slices shown similar quality.

3.3.2.5 Potassium uptake rate and its relationship with tissue metabolic rate in liver and kidney cortex slices of mammals

Allometric comparison of the rates of potassium uptake in mammalian tissue slices

The rates of potassium uptake were calculated as the difference between the accumulation of potassium (calculated from the accumulation of $^{86}$Rubidium) in slices after 1 and 5 minutes of incubation in radioactive saline, as described in Section 3.2.6. Figure 3.16 shows representative examples of four single experiments. Using wet weight as a basis of expression typically yielded little variability in the triplicates for each incubation time.

The results reported in Table 3.12 reflect the individual experiments in Figure 3.16 in that larger mammals show lower rates of potassium uptake. It is
also apparent from this data that kidney cortex slices displayed higher rates of potassium uptake than liver slices. This was true for all species examined.

Figure 3.16

Representative examples of accumulation of potassium (as $^{86}$Rubidium accumulation) calculated using wet weight in mouse and sheep liver and kidney cortex slices as a function of incubation time. The rates of potassium uptake are calculated from the slopes.
Table 3.12

Rate of potassium uptake (µmol K⁺/g wet-min.) using wet weight, as measured using ⁸⁶Rubidium in liver and kidney cortex slices of five mammalian species. The results are presented as mean±s.e. (n=4). Values in a column with different letters differ significantly (p≤0.05).

<table>
<thead>
<tr>
<th></th>
<th>Liver</th>
<th>Kidney</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>0.74 ± 0.12  ab</td>
<td>1.90 ± 0.19  a</td>
</tr>
<tr>
<td>Rat</td>
<td>0.79 ± 0.12  a</td>
<td>1.89 ± 0.39  ab</td>
</tr>
<tr>
<td>Rabbit</td>
<td>0.38 ± 0.03  b</td>
<td>1.11 ± 0.15  b</td>
</tr>
<tr>
<td>Sheep</td>
<td>0.23 ± 0.01  c</td>
<td>1.27 ± 0.20  ab</td>
</tr>
<tr>
<td>Cattle</td>
<td>0.26 ± 0.02  bc</td>
<td>0.56 ± 0.13  c</td>
</tr>
</tbody>
</table>

In liver slices, significant differences in potassium uptake rate were found between rat, rabbit and sheep (Table 3.12). Mouse displayed similar rates as rat and was only statistically different from sheep. Similarly, cattle displayed comparable rates of potassium uptake as for sheep liver slices and the rates measured in this larger mammal were only statistically different from rat. In kidney cortex slices as in liver slices, mouse and rats showed similar rates of potassium uptake. For kidney cortex slices, mouse, rabbit and cattle differed significantly in their rates of potassium uptake.

Figure 3.17 show that the rate of potassium uptake varied inversely with body size in mammals. From the highly significant (p < 0.01) allometric equations reported in Figure 3.17, it can be concluded that the rate of potassium uptake show a similar decrease with body size in liver and kidney cortex slices: a doubling in body size corresponded to around 9% decrease in potassium uptake in both liver and kidney cortex slices. The cattle slices accumulated
Figure 3.17
Allometric relationship of potassium uptake in mammalian liver and kidney cortex slices expressed using wet weight. Each point represents a measurement on a different animal. The allometric equations are given with each graph.
potassium at about a third of the rate of those from mouse.

There are three assumptions to consider in the interpretation of these results. The first is that the rate of $^{86}\text{Rb}^+$ uptake is representative of that of potassium. Haber et al. (1988) reported that the ratios of $^{42}\text{K}^+$ to $^{86}\text{Rb}^+$ uptake in cultured rat liver cells was 1.05. Thus, this assumption is probably correct at least for rat liver cells. I have not found published values for other species or cell types.

The second assumption is that during the time-course chosen for the uptake measurements (between 1 and 5 minutes incubation) no substantial loss of $^{86}\text{Rb}^+$ occurred. In the following section, tissue slices were loaded with $^{86}\text{Rb}^+$ to estimate the rates of potassium efflux (Section 3.3.2.6). The rates of potassium uptake were calculated by using the ratio of $\text{K}^+$ to $^{86}\text{Rb}^+$ in the saline. As long as this ratio was not achieved in the intracellular space (it usually took at least 15 minutes for the equilibrium to be reached, see Figure 3.2), $\text{K}^+$ loss would have been more important than $^{86}\text{Rb}^+$ loss, for which the maximal efflux rates were around 3.5 and 6.2% loss per minute in liver and in kidney respectively, in the presence of ouabain (Table 3.15). Thus the underestimation of potassium uptake rates due to loss of $^{86}\text{Rb}^+$ would have been less than 5%.

The third assumption concerns the accuracy of the wet weight measurements and probably only affected mouse kidney cortex slices (Section 3.3.1.4.1). If the possible underestimation of the wet weights by 28% is accounted for, the value of potassium uptake for mouse kidney cortex slices in
Table 3.12 is 1.48 μmol K⁺/g wet⁻¹. The allometric exponent in Figure 3.17 becomes -0.101, suggesting that a doubling in body size corresponded to a 7% decrease in rate of potassium uptake instead of the 9% reported above using uncorrected values.

The rates of potassium uptake were also corrected for active cell mass (Table 3.13). For this correction, GDH activity was assumed to be proportional to the amount of active cells (refer to Section 3.3.1.4). Although the values of potassium uptake rates in tissue slices corrected for active cell mass were higher than those using wet weight, the interspecific differences and the allometric decrease in potassium uptake rates remained. From the exponents of the statistically significant (p < 0.01 for both tissues) allometric relationships (Figure 3.18), a doubling in body size corresponded to a 8% decrease in potassium uptake rate for both of liver and kidney cortex slices.

<table>
<thead>
<tr>
<th>Table 3.13</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rate of potassium uptake (μmol K⁺/g wet·min.) corrected for active cell mass, as measured using ⁸⁶Rubidium in liver and kidney cortex slices of five mammalian species. The results are presented as mean±s.e. (n=4).</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Liver</th>
<th>Kidney</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>1.19 ± 0.20</td>
<td>2.55 ± 0.25</td>
</tr>
<tr>
<td>Rat</td>
<td>1.02 ± 0.14</td>
<td>3.78 ± 1.01</td>
</tr>
<tr>
<td>Rabbit</td>
<td>0.42 ± 0.06</td>
<td>1.31 ± 0.28</td>
</tr>
<tr>
<td>Sheep</td>
<td>0.45 ± 0.06</td>
<td>1.72 ± 0.21</td>
</tr>
<tr>
<td>Cattle</td>
<td>0.43 ± 0.05</td>
<td>0.94 ± 0.19</td>
</tr>
</tbody>
</table>

For the calculation of potassium uptake rates corrected for active slice mass, the potassium accumulated in each slice (calculated as described in Section 3.2.6) was divided by the IU GDH in that slice instead of wet weight and multiplied by the IU GDH/g wet of the whole tissue from which the slice comes.
Figure 3.18

Allometric relationship of potassium uptake in mammalian liver and kidney cortex slices, using GDH for the calculations. Each point represents a measurement on a different animal. The allometric equations are given with each graph.

To summarize this section, there was an allometric relationship of potassium uptake rates in tissue slices similar in liver and kidney cortex. A doubling of body size was associated with a 8 to 9% decrease in potassium uptake rate in liver slices and 7 to 9% in kidney cortex slices.
Relationship between the rates of oxygen consumption and of potassium uptake

This section does not directly involve body size as it is devoted to testing the third hypothesis of this thesis, that sodium pump activity is proportional to metabolic activity. To this end, the rates of oxygen consumption were compared to the rates of potassium uptake of the same slice preparations. The relationship between these parameters is illustrated in Figure 3.19. Both linear relationships were statistically significant ($p < 0.01$ for liver slices and $p < 0.05$ for kidney slices) using the mean values for the linear regression.

![Figure 3.19](image)

**Figure 3.19**

Relationship between potassium uptake and oxygen consumption in liver and kidney slices of mammals in an allometric comparison. The results are mean ± s.e. of the values for each species and tissue.
Estimation of the contribution of the sodium pumps to aerobic metabolism

Since the slices had been incubated in a saline containing only glucose as substrate, it was possible to estimate the contribution of the sodium pumps to oxygen consumption in this system. This calculation involved the stoichiometric assumptions that the sodium pumps carry 2 potassium ions across the plasma membrane for 1 ATP used and that the complete oxidation of glucose consumes 6 oxygen molecules and yields 38 ATP molecules. In fact, the yield is between 36 and 38 ATP, depending on the shuttle used, phosphate or malate-aspartate, to bring electrons from the cytosolic NADH to the respiratory chain (Stryer, 1988). These estimates are reported in Table 3.14.

Table 3.14

Estimation of the contribution of the sodium pumps to cell oxygen consumption for liver and kidney slices of mammals of various body size. The results of these calculations are in % of the oxygen consumption devoted to the production of ATP that is used by the sodium pumps. Values in a column with a different letter differ significantly (p≤0.05).

<table>
<thead>
<tr>
<th></th>
<th>Liver</th>
<th>Kidney</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>3.9 ± 0.5 b</td>
<td>6.8 ± 0.7 a</td>
</tr>
<tr>
<td>Rat</td>
<td>4.8 ± 1.3 ab</td>
<td>10.5 ± 2.7 a</td>
</tr>
<tr>
<td>Rabbit</td>
<td>4.4 ± 1.0 b</td>
<td>7.2 ± 0.8 a</td>
</tr>
<tr>
<td>Sheep</td>
<td>5.1 ± 0.7 b</td>
<td>9.1 ± 2.8 a</td>
</tr>
<tr>
<td>Cattle</td>
<td>10.2 ± 2.8 a</td>
<td>7.0 ± 2.0 a</td>
</tr>
</tbody>
</table>
In liver slices, the oxygen consumption calculated to be due to potassium pumping was around 4 to 5% of the total oxygen consumption of the tissue slices for all species except in cattle, where it was 10% (significantly higher than mouse, rabbit and sheep). There was a slight tendency for larger mammals to spend relatively more energy on their sodium pumps in liver slices. This allometric relationship was not statistically significant ($0.10 < p < 0.05$) and was not reproduced here. Kidney cortex slices of all species spent more energy on their sodium pumps than liver, except for cattle. More interspecific variations were observed for kidney cortex slices than for liver slices and no allometric trend could be found.

In estimating the use of cellular ATP by the sodium pumps, it was assumed that the cells respired exclusively on glucose and aerobically. If some of the cells were deriving part of their energy from anaerobic metabolism, correcting the estimates in Table 3.14 for this factor would yield lower values, as more ATP than calculated would have been produced. An overestimation of the rate of ATP consumption by the sodium pumps would also occur if the cells were producing ATP through the catabolism of more energy-producing substrates, such as endogenous lipid stores. The complete oxidation of palmitate, for example, produces 27% more ATP per mole of oxygen than the oxidation of glucose does (from calculations based on ATP yield of different pathways taken in Stryer, 1988). Indeed, according to Weidemann and Krebs (1968), in kidney cortex slices glucose does not suppress the oxidation of endogenous substrates. They also proposed that "...the endogenous fuel must have been triglyceride...". As in the liver also, glucose is not the favourite fuel (Stryer, 1988), a good proportion of endogenous substrates could have been oxidised.
The validity of the estimates of the contribution of the sodium pumps to tissue slice metabolism also relies on the assumption that the \(^{86}\text{Rb}^+\) uptake was due solely to the work of the sodium pumps. From literature evidence, this assumption is likely to be wrong. Van Dyke and Scharschmidt (1983) reported that in their cultured rat hepatocytes only 67% of the \(^{86}\text{Rb}^+\) uptake could be inhibited by ouabain. In kidney cortex, the proportion of the active (ouabain-sensitive) potassium uptake is hard to evaluate in slices since many different types of cells are present. Harris \textit{et al.} (1986) reported that, in rat proximal tubule cells, ouabain inhibited 87% of net potassium influx. The mechanisms of potassium influx not associated to the sodium pumps are still unclear. Little \textit{et al.} (1986), working with rat vascular smooth muscle, took the evidence of the 91% inhibition of the ouabain-insensitive \(^{86}\text{Rb}^+\) uptake by furosemide as suggesting that this influx is mostly mediated by a Na, K, Cl cotransport system.

The last assumption is that 100% of the oxygen consumed went for the production of ATP. This assumption is also likely to be wrong since it has been shown that in liver cells 10-15% of the oxygen is consumed extramitochondrially (Brand, 1990) and that a 20-25% of the oxygen consumed by the resting mitochondria in liver cells is involved in non-ATP producing processes to combat the proton leak (Nobes \textit{et al.}, 1990; Brown \textit{et al.}, 1990). Thus, in liver cells 30-40% of the oxygen consumption does not lead to ATP formation. For this tissue, the error that this assumption leads to will be compensated by the error on the previous assumption (33% of the potassium uptake is not due to the sodium pumps, see above). For kidney cortex cells, the error that both these assumptions lead to cannot be ascertained to the same extent because of the lack of data on the cost of the proton leak.
Allometric comparison of the outward permeability of plasma membranes to potassium and estimates of the net potassium fluxes in slices under steady conditions

In this comparison, outward membrane permeability to potassium was estimated as the efflux rate of $^{86}$Rubidium, often used as a substitute for potassium to this end (Montero et al., 1991; Harris et al. 1991 for example). The rates of potassium efflux are not expected to be as indicative of sodium pump activity as the rates of potassium uptake: sodium pump activity is controlled by $[\text{Na}^+]_i$ (Van Dyke and Scharschmidt, 1983) much more than by $[\text{K}^+]_i$ (Rossier, 1987). The measurement of $\% \text{K}^+ \text{loss \cdot min}^{-1}$ is useful in discussing the capacity of tissue slices to retain their $[\text{K}^+]_i$ since $\% \text{K}^+ \text{loss \cdot min}^{-1}$ should be equal to the rates of potassium uptake if homeostasis is to be maintained. The results from this section will also be considered in relation to membrane composition (Hypothesis 6).

Allometric comparison of potassium efflux rates

The results of this experiment are presented in Table 3.15 and illustrated in Figure 3.20. Each point in this figure represents the average of the 4 values obtained in individual experiments. The relationships were linear on this semilogarithmic scale from 4 to 28 minutes. The values at $t=0$ (100%) and at $t=0.5$ minute were also plotted for comparative purposes but were not included in the calculations as they would have included the efflux from the extracellular compartment. It was assumed that from 4 to 28 minutes the rate of efflux from the tissue slices was limited by the efflux rate across the plasma membrane.
The rates of potassium efflux in liver ranged from 1.2 to 3.5% per minute (Table 3.15). There was no significant allometric relationship of potassium efflux rate from liver slices, as illustrated in Figure 3.21. The mouse to rabbit relationship, however, was statistically significant (p < 0.01), with an allometric exponent of -0.224. In this limited comparison for small mammals, a doubling in body size was associated to a 14% decrease in potassium efflux rate. The variability of potassium efflux rates in liver slices was much greater than for kidney slices, as the spread of the data indicated in Figure 3.21.

Table 3.15
Rates of potassium efflux calculated between t = 4 and 28 minutes (using \(^{86}\text{Rb}^+\)) in mammalian liver and kidney cortex slices, expressed as % K\(^+\) loss-min\(^{-1}\) (mean ± s.e.). Values in a column with a different letter differ significantly (p≤0.05).

<table>
<thead>
<tr>
<th></th>
<th>Liver</th>
<th>Kidney</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mouse</strong></td>
<td>3.43 ± 0.56 (^a)</td>
<td>6.20 ± 0.18 (^a)</td>
</tr>
<tr>
<td><strong>Rat</strong></td>
<td>2.01 ± 0.22 (^a)</td>
<td>4.94 ± 0.14 (^b)</td>
</tr>
<tr>
<td><strong>Rabbit</strong></td>
<td>1.23 ± 0.28 (^a)</td>
<td>5.56 ± 0.40 (^{ab})</td>
</tr>
<tr>
<td><strong>Sheep</strong></td>
<td>3.47 ± 0.55 (^a)</td>
<td>4.43 ± 0.23 (^{bc})</td>
</tr>
<tr>
<td><strong>Cattle</strong></td>
<td>2.36 ± 0.16 (^a)</td>
<td>3.51 ± 0.19 (^c)</td>
</tr>
</tbody>
</table>
Figure 3.20
Permeability of plasma membranes to potassium in mammalian liver and kidney cortex slices, as estimated by the rate of $^{86}$Rubidium loss. Each point corresponds to the mean of % potassium loss at a given time after transfer to non-radioactive saline (n=4).
Figure 3.21

Allometric relationship between the rate of potassium loss and body weight in liver and kidney cortex slices. Each point is the mean of duplicate measurements on a particular animal and tissue. The allometric equation for kidney cortex is given while no relationship was found for liver.
The potassium efflux rates from kidney cortex slices were substantially greater than those from liver slices, ranging from 3.5 to 6.2% per minute (Table 3.15). The decrease of potassium efflux rate in kidney cortex slices from mammals of increasing size resulted in a statistically highly significant (p < 0.01) allometric relationship (Figure 3.21). In the comparison of all species, a doubling in body size could be associated with a 4% decrease in potassium efflux rate.

The first assumption involved in the interpretation of the results in terms of potassium loss is that the outward permeability of potassium and of $^8$Rb$^+$ through potassium channels and through the phospholipid bilayers was essentially the same. Haber et al. (1988) calculated for cultured rat liver cells that the ratio of $^{42}$K$^+$ efflux to $^8$Rb$^+$ efflux was 1.07. For cultured rat proximal tubules, Harris et al. (1986) found that K$^+$ efflux from K$^+$-loaded tubule cells exposed to ouabain was not significantly different from Rb$^+$ efflux from Rb$^+$-loaded cells in which K$^+$ was substituted for external Rb$^+$. Thus, this assumption is likely to be correct at least for rats. Only if the relative permeability of Rb$^+$ to K$^+$ varied interspecifically would the allometric comparison be affected.

The second assumption is that after 4 minutes of incubation in non-radioactive saline the $^8$Rb$^+$ efflux rate was limited by plasma membrane permeability. Given the diffusion rates in the extracellular compartment (Section 3.3.1.3 and Figure 3.8), this assumption was considered reliable.
To summarize this section, the rates of potassium efflux measured for liver slices and reported in Figure 3.20 did not show a consistent allometric decrease. Only the mouse to rabbit comparison was significant, with a doubling in body size in this range being associated with a 14% decrease in potassium efflux rate. The mouse to cattle decrease in potassium efflux rate was however statistically significant in kidney cortex slices, with a doubling in body size being associated with a 4% decrease in potassium efflux. The reliability of these rates will be discussed below.

Comparison of potassium efflux rates with potassium uptake rates

Under ideal physiological conditions, the rate of potassium uptake is matched to the rate of potassium loss so that the potassium gradient across the plasma membranes is maintained relatively constant. Under the experimental conditions imposed on tissue slices in the experiments of this chapter, this condition was not fully fulfilled since slices of all species and tissue examined showed gradual losses of potassium over extended periods of time (Table 3.8). One way to evaluate the capacity of the sodium pumps and other mechanisms of potassium uptake to compensate for potassium losses is to compare the rate at which tissue slices lost their potassium under steady experimental conditions with the net potassium efflux rates measured using $^{86}$Rb$^+$. This comparison shows that potassium uptake mechanisms compensated for nearly all of potassium losses. The rate of net potassium loss under steady conditions reported in Table 3.8 is only 0.1 to 7% of the rate of outward $^{86}$Rb$^+$ permeability reported in Table 3.15. The mechanisms of potassium uptake compensated for 93 to 98% of the losses in liver slices and for 97 to 100% of the losses in kidney cortex slices.
Another way to assess the capacity of tissue slices to maintain their $[K^+]_i$ is to compare the rates of potassium efflux with those of potassium uptake. To this end, the rates of potassium efflux from Table 3.15, expressed in $\% \text{ K}^+ \text{ loss} \cdot \text{min}^{-1}$, were multiplied by the $\mu\text{mol K}^+ \cdot \text{g wet}^{-1}$ of the control slices to obtain rates of potassium efflux in $\mu\text{mol K}^+ \text{ loss} \cdot \text{g wet}^{-1} \cdot \text{min}^{-1}$. These rates were then subtracted from the rates of potassium uptake from Table 3.12, in the same units. These estimates, expressed as "net potassium flux", are reported in Table 3.16 and represent the residual potassium flux that would have to be compensated by altering either potassium uptake rate or potassium efflux rate in order for $[K^+]_i$ to be maintained at the level estimated in control slices.

### Table 3.16

**Estimation of the net potassium flux ($\mu\text{mol K}^+ \text{ loss} \cdot \text{g wet}^{-1} \cdot \text{min}^{-1}$) in mammalian liver and kidney cortex slices under experimental $[K]_i$, calculated as the difference between potassium efflux and potassium uptake.** The results are mean $\pm$ s.e. ($n=4$). Negative values indicate net potassium loss.

<table>
<thead>
<tr>
<th></th>
<th>Liver</th>
<th>Kidney</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>-0.3 $\pm$ 0.2</td>
<td>-0.3 $\pm$ 0.4</td>
</tr>
<tr>
<td>Rat</td>
<td>-0.3 $\pm$ 0.2</td>
<td>0.5 $\pm$ 0.4</td>
</tr>
<tr>
<td>Rabbit</td>
<td>-0.1 $\pm$ 0.0</td>
<td>-0.5 $\pm$ 0.3</td>
</tr>
<tr>
<td>Sheep</td>
<td>-0.1 $\pm$ 0.1</td>
<td>0.6 $\pm$ 0.2</td>
</tr>
<tr>
<td>Cattle</td>
<td>-0.0 $\pm$ 0.0</td>
<td>0.0 $\pm$ 0.2</td>
</tr>
</tbody>
</table>
The values reported in Table 3.16 confirm that the slices were almost capable of maintaining their potassium levels and thus suggest that under experimental conditions the rates of potassium uptake closely matched the rates of potassium efflux: the net fluxes of potassium presented in Table 3.16 were close to 0. The small net gains of potassium suggested for rat and sheep kidney cortex slices using this method were not observed over a 2 hour period (Table 3.8).

The lower $[K^+]_i$ of tissue slices under experimental conditions (Table 3.7) than expected for healthy tissues (eg. values calculated for whole tissues, Section 3.3.2.2 and values from the literature in Section 3.4.2) would have been a reflection of either a greater plasma membrane permeability to potassium than for in vivo conditions or suboptimal rates of potassium uptake. The rates of potassium uptake and efflux will be compared with values from the literature in the discussion, Sections 3.4.3 and 3.4.4, respectively. Following slicing, the $[K^+]_i$ would decrease until it reaches a level at which the rate of uptake matches the rate of efflux. Thus the near 0 values in Table 3.16 only indicate that during the manipulations this equilibrium had been reached.

Rat and rabbit liver slices had the highest $[K^+]_i$ (119 and 96 mM respectively, Table 3.7). In those slices, the rates of potassium efflux were much lower than in any other liver slice preparations, although the variability in the replicates rendered the comparisons non statistically significant (Table 3.15). The potassium efflux rates reported for rat and rabbit liver slices, 2.01 and 1.23 % $K^+$ loss-min$^{-1}$ respectively, would be closest to the rates of potassium efflux that would be required in intact cells for the maintenance of physiological $[K^+]_i$. 

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3.4 Discussion

3.4.1 Allometric comparison of mass specific intracellular water volumes in liver and kidney cortex

There was a small but statistically significant increase in intracellular volume in liver slices with increasing body size. While no statistically significant trend in intracellular volume was noted for kidney cortex slices, because of a possible underestimation of the wet weight of mouse kidney cortex slices the possibility of an increase in intracellular volume in mammals of increasing size twice that of liver (2% for a doubling in body size vs. 1% in liver, Section 3.3.2.1) could not be dismissed. In any case, the allometric variations in intracellular water space will remain small. These findings eliminate the possibility that the decrease in mass-specific tissue metabolism reported observed in this study could be due to some extent to larger mammals having proportionally less cell volume per unit mass in their tissues. In fact, the mass-specific differences in rates of oxygen consumption and potassium uptake reported in this chapter were accentuated by this phenomenon.

At least for rat liver slices, the values of intracellular water space reported here appear reliable. Claret et al. (1973) calculated the intracellular water space in perfused rat liver to be 463 μl/g wet, compared to 457 μl/g wet (Table 3.3). On average, mammalian liver was estimated to have 65% of its water space intracellular compared to 62% in kidney cortex (Section 3.3.2.1). For liver these values agree with those from Claret et al. (1973) (since they also reported similar total water content in their rat livers). In human tissues, approximately 71% of the total tissue fluid volume, excluding plasma, is intracellular (from Lote, 1987).
3.4.2 Allometric comparison of whole liver and kidney cortex composition

Protein and water contents

Mammals of all body size had around 71% water per unit weight in their livers and 81% water in kidney cortex. The interspecific differences in water content did not exceed 5% in any tissue. The protein content of whole tissues did not vary much in either tissue among species, but there was an allometric trend of increase of protein content in liver, ranging from 175 mg·g wet⁻¹ to 192 mg·g wet⁻¹ in the mouse to cattle comparison. In the kidney there was no allometric trend in protein content. There was less proteins in kidney cortex than in liver for all species. Hulbert and Else (1989) reported slightly lower protein levels for rat kidney than measured here and their value for rat liver was similar to those in Table 3.2.

Then, at least the two major constituents of tissues, water and protein, show similar proportions in homologous tissues of all mammalian species investigated. The larger mammals have a slower rate of protein synthesis (protein synthesis rate scales according to \( W^{0.75} \) and is thus proportional to basal metabolic rate. Blaxter, 1989). The results presented here allow to conclude that although protein turnover is proportional to metabolic rate, protein content does not vary with metabolic rate.

Potassium and sodium levels

There was no allometric trends in sodium and potassium content of whole liver. In whole kidney cortex, the trends of increase of potassium content
and of decrease of sodium levels (Table 3.4) in mammals of increasing size were
greater than could be accounted for by increases in intracellular volume. Larger
mammals maintained higher intracellular concentrations of potassium and
lower levels of sodium in their kidney cortex (Section 3.3.2.2).

The average calculated values of \([K^+]_i\) reported in Section 3.3.2.2 for liver
of all 5 species (132 ± 6 mM K⁺) are intermediate between the values calculated
by Claret and Mazet (1972) in their perfused rat livers (113 mM) and those from
Lote (1987) for human skeletal muscle (150 mM). The estimates of \([Na^+]_i\) from
Section 3.3.2.2 (0 to 18 mM) overlap published values of 16.4 mM for rat
perfused liver (Claret and Mazet, 1972) and 12 mM in human skeletal muscle
cells (Lote, 1987). The relatively close match of the estimates of \([K^+]_i\) and \([Na^+]_i\)
with values from the literature suggest that the method used in this chapter for
the determination of intracellular volume and the calculations of \([K^+]_i\) were
reasonably accurate for liver slices.

The report in this chapter that larger mammals maintain higher
potassium levels and lower sodium levels in their kidney cortex cells than do
small mammals could not be confirmed from the literature. Because of the
labile nature of intracellular sodium and potassium gradients in kidney cortex
tubule cells when used for its main \textit{in vivo} kidney function, namely
reabsorption, little data is available. Jørgensen (1980) did not find any report of
cytoplasmic sodium and potassium concentrations in tubule cells, except the
value from Edelmann \textit{et al.} (1978) for rat kidney cells where 82 mM \([K^+]_i\) was
calculated. If the measurements of intracellular volume were as equally
accurate for this tissue as they were with liver cells, we can conclude that the
transmembrane cation gradients in kidney cortex cells were not as great as in liver. The significance of the allometric trends discussed above is unclear but could be associated with differences in reabsorption rates since in tubule cells the gradients are used for reabsorptive functions.

**LDH and GDH activities**

As well as GDH providing a basis for the estimation of oxygen consumption when considering active respiring cell mass, the aim for measuring LDH and GDH activities in whole tissues was to provide for comparative values to assess slice damage. Some interspecific differences in the scope of given metabolic pathways can also be inferred from the activity of these enzymes in whole tissues. Maximal enzyme activity, which is what was measured in these experiments, is nearly always higher than *in vivo* activity, but as Diamond and Hammond (1992) discussed, “natural selection tends to eliminate unutilised capacities because of their costs”. This concept, termed “symmorphosis” (the postulated matching of capacities to each other and to loads, Taylor and Weibel, 1981), suggests that maximal enzyme activity relates to the maximal requirements of a pathway. Nevertheless, although LDH is the only enzyme involved in the anaerobic step of glycolysis, the conclusions reached from the activities of enzymes such as LDH in gluconeogenesis and GDH in amino acid metabolism have their limitations since these enzymes are not known as limiting in their metabolic pathways.

For each species, the activity of GDH was greater in liver than in kidney cortex. This is presumably related to the role of this enzyme in urea production, which takes place predominantly in the liver (Stryer, 1988). In the liver, rabbits showed significantly (*p*≤0.05) lower GDH activity than sheep or cattle. This
suggests an overall lower level of protein metabolism in this animal. That conclusion is supported by the rates of protein synthesis of whole animals, relatively lower in rabbits than in young adult rats, sheep of ox (12.6 g/Kg \( W^{0.75} \)·day vs. 16.9, 15.7 and 14.8, respectively. See Blaxter, 1989).

With the notable exception of LDH activity in the liver, GDH and LDH activity in the tissues examined appeared to be a species specific characteristic unrelated to body weight. However, the higher activity of LDH in the liver of rat and mouse in comparison to larger mammals (Table 3.5) strongly suggest that for those smaller mammals the total organism anaerobic capacity is higher than in larger mammals. LDH in muscle cells and erythrocytes is the anaerobic step for the production of lactate from glucose. In liver, this enzyme is involved in gluconeogenesis using the lactate produced mostly by muscle cells and erythrocytes (Stryer, 1988). At times where oxygen demand is increased, for the most metabolically active mammals a capacity to temporarily perform part of the metabolic work anaerobically could be advantageous, as it would reduce the pressures for the maintenance of even higher aerobic capacities. This finding suggests that the allometric relationship of total metabolic capacity may be stronger than that of maximal aerobic metabolism, with smaller mammals partly increasing their total metabolic capacity by an increased anaerobic capacity. This is consistent with the findings of Weibel and Taylor (1981) who observed that the aerobic scope for activity of large mammals is greater than for small mammals.

In the kidney cortex, LDH is responsible for anaerobic glycolysis as well as some gluconeogenesis (Weidemann and Krebs, 1969). The interspecific variations of LDH activity are not related to size and given the dual function of this enzyme in kidney cells the significance of these variations remains unclear.
3.4.3 Allometric comparison of oxygen consumption rates and of sodium pump activity in mammalian liver and kidney cortex slices

Oxygen consumption rate

The hypothesis that differences in mass-specific basal metabolic rate in whole mammals are in part a reflection of differences in cellular metabolism is supported by the measurements of oxygen consumption of tissue slices from different-sized mammals. From the rates of aerobic metabolism measured in tissue slices it is possible to conclude that a substantial part (approximately 50%) of the allometric decrease in basal metabolism in whole organisms is due to less metabolically active tissues in larger mammals. This was found to be true for both tissues investigated.

Estimates drawn from various sources (see Chapter 1) have shown that in mammals a doubling in body size is accompanied by a 15% decrease in mass-specific basal metabolic rate and a 9% decrease in relative liver and kidney size. In the present study a doubling in body size was accompanied by a 10-14% decrease in mass-specific oxygen consumption rate of liver slices and 5-8% for kidney cortex slices depending on the method used for the calculations (wet weight, active cell mass or time after death) (Section 3.3.2.4). A doubling in body size was thus accompanied by a 18-21% decrease in liver metabolic rate per unit body weight and by a 15-17% decrease for kidney\(^3\) (if the allometric decrease in kidney cortex size is proportional to that of whole kidney). From these estimates, kidney contributed to a constant proportion of basal metabolic rate in mammals of all size while liver contributed less to basal metabolic rate in larger mammals.

\(^3\) To calculate the contribution of a combination of factors, the mass-specific allometric exponents involved are added.
The corrections of oxygen consumption rates for active cell mass (using GDH) or time after death reinforce the conclusions that the scaling of tissue metabolic rate is not an artefact of slice preparation. Although after correction the rates of oxygen consumption are typically higher and the allometric relationships are slightly lessened, the allometric trends and their statistical significance persist.

The relative contribution of liver and kidney metabolic rate to basal metabolic rate of whole animals can be estimated by multiplying the mass-specific rate of oxygen consumption of slices by the organ size and dividing this product by the rate of basal metabolism reported for this animal in Table 1.1. Since in this calculation the oxygen consumption rates measured in vitro with tissue slices were compared to basal metabolism in vivo (Table 1.1), these are only rough estimates. Nevertheless, such estimates agree with the above conclusions. The relative contribution of liver to basal metabolism decreased in larger mammals as this tissue was estimated to produce 6.9, 8.1, 7.3, 4.0 and 3.7% of basal heat in mouse, rat, rabbit, sheep and cattle, respectively. Kidney would have contributed equally to basal metabolism in all species as this tissue would have been responsible for 2.6, 2.0, 2.5, 2.7 or 2.2% of basal metabolism for the same respective species. When the active respiring cell mass is used for these estimates, the total contribution of these tissues to basal metabolism would have been 8.7, 12.4, 8.3, 6.0 or 6.6% of the total basal heat for liver in mouse, rat, rabbit, sheep and cattle respectively and 2.8, 3.7, 2.7, 3.4 or 3.6% for kidney in the same respective species.

To calculate tissue weight, the body weights in Table 1.1 are used in Brody’s allometric equations: Y=0.0333M^{0.867} for liver and Y=0.00732M^{0.846} for kidney (Brody, 1945). The rates of oxygen consumption of tissue slices, in \( \mu \text{mol O}_2/\text{g wet min.} \) (Table 3.9) are converted to Kcal/day by assuming that resting cells had a respiratory quotient of 0.7 as is typical of fasting, resting mammals (Blaxter, 1989). Palmitate, which yields this quotient, gives 435.5KJ/mol O\(_2\) (1 cal = 4.184 joules) (Blaxter, 1989).
The estimates above should only be considered in the allometric comparison since the absolute contribution of these tissues to metabolism are likely to have been grossly underestimated by the method used. While the rate of metabolism of the tissue slices reported in this chapter may be normal for tissue slices (see the comparisons with literature values, later in this discussion), they may be very different in vivo. For rat, the liver has been estimated to be responsible for 20% of basal metabolic rat (Jansky. 1965). In man, splanchnic organs and kidneys have been estimated to be responsible for 34% and 8% of basal heat production, respectively (Aschoff et al., 1971).

Since indicators of slice quality are available for each preparation, some information can be extracted relating to the likely metabolic condition of the slice’s cells. Given the differences in cation gradients reported in Table 3.7, the cells from slices of different species were probably not operating at the same membrane potential and this membrane potential could have been lower than normal for all tissues examined. Collapsed membrane potential and cation gradients would undoubtedly eventually affect slice viability. However, it is apparent that slices that display high intracellular sodium levels sometimes spend more energy for sodium pumping (Van Dyke and Scharschmidt, 1983; Clausen et al., 1991) and display higher oxygen consumption rates (Clausen et al., 1991). Potassium loss in tissue slices was compensated by gains in sodium (Table 3.7). In the experiments described in this chapter, oxygen consumption rates were not clearly affected by potassium loss (and thus by sodium gain) except for those slices that were extremely depleted in potassium at the end of some experiments (Section 3.3.2.4). Thus, the cation gradients present in the tissue slices used for the measurements of oxygen consumption may not have negatively affected oxygen consumption rates and it could even be that there was an artificial rise of metabolism from resting levels for some slices.
Nevertheless, the possibility that high intracellular sodium and low potassium concentrations may have negatively affected the metabolic rate of some slice preparations in the present experiments cannot be dismissed. The possible negative long-term effects of collapsed transmembrane sodium and potassium gradients on tissue metabolism can be inferred from early experiments with slices and cell preparations incubated in ouabain for extended periods (see Chapter 1). Early experiments with ouabain suggested that the disappearance of transmembrane cation gradients could lead to an inhibition of 30-50% of cell metabolic rate. The intracellular concentrations of sodium or potassium necessary for such an effect on cell metabolism are not known and thus no systematic correction could be attempted. In addition, these experiments are not directly comparable to those from this chapter where no use of ouabain was made. In the latter experiments, the cells presumably still had an opportunity to restore transmembrane gradients. Increasing the values of oxygen consumption for tissue slices from large mammals (the most potassium-depleted) by 30-50% would not abolish the allometric decline in tissue metabolism. These estimates corrected for transmembrane potassium and sodium gradients would be most similar to those in Table 3.11 where a correction for time after death was performed. This reinforces the suggestion made in Chapter 1 that a likely negative effect of collapsed transmembrane cation gradients is to reduce slice viability, possibly through inhibition of metabolic processes dependent on the sodium gradients. Considering a correction for time after death in the allometric decrease of tissue metabolic rate, a doubling in body size corresponded to a 10% decrease in liver slice aerobic metabolism and 5% in kidney cortex slices.

In the following paragraphs the rates of oxygen consumption reported in this chapter will be compared with literature values. While this exercise is
informative since a new system for maintaining slices (the stirring well system, Section 3.3.1.3) was developed in this thesis, the reader should bear in mind that oxygen consumption rates of tissue slices vary according to experimental conditions and are almost certainly different to in vivo rates, where the cells perform specific metabolic functions for other tissues that they are not performing when isolated in vitro. The experiments in this chapter were not designed to determine precisely the rates of in vivo tissue metabolism but rather for allometric comparisons under constant external conditions. Finally, glucose, the only exogenous substrate available to slices in the experiments of Section 3.3.2.4, is not a favourite substrate for liver and kidney cortex. While kidney cortex slices prefer such substrates as acetoacetate, butyrate or oleate (Weidemann and Krebs, 1969), liver cells prefer keto acids derived from the metabolism of amino acids (Stryer, 1988).

Krebs (1950) has published the most extensive comparison of oxygen consumption rates on tissue slices from different-sized mammals. His measurements were performed at 40°C with a Warburg manometer over a time-course of 45 minutes. I have converted his values from dry weight to wet weight\(^5\). They are 3.4, 2.9, 1.4, 1.3 and 0.7 \(\mu\)mol O\(_2\) g wet\(^{-1}\) min\(^{-1}\) in liver slices from mouse, rat, rabbit, sheep and cattle, respectively and 5.0, 4.6, 4.5, 3.5 and 3.2 \(\mu\)mol O\(_2\) g wet\(^{-1}\) min\(^{-1}\) in kidney cortex slices respectively from the same species. These values are higher than those presented in Tables 3.9 and 3.10 (see below) and are also higher than any others reported in the literature for tissue slices. It is possible that the experimental temperature that he used (40°C) was partly responsible for these high values.

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\(^5\) To convert \(\mu\)l O\(_2\)/g dry hr.: at 37°C, 1 \(\mu\)l O\(_2\) = 39.31 nmol O\(_2\) (using \(V_m = RT/p\), refer to Atkins, 1990, p. 11) and water contents reported in Table 3.2 for whole tissues were used.
With the data reported in Section 3.3.2.4, a doubling in body size was associated with a 10-14% decline in mass-specific oxygen consumption in liver slices and 5-8% in kidney cortex slices. These declines are greater than those calculated from Krebs' (1950) data, where the values are 8% for liver slices and 4% for kidney cortex slices (from the allometric exponents in Figure 1.2). The differences between Krebs' and our estimates were greater for larger mammals, explaining the differences in the allometric relationships. Krebs' estimates are 2.3, 2.1, 1.8, 3.5 and 3.1 times mine for liver slices from mouse, rat, rabbit, sheep and cattle respectively. In kidney, the relative differences are also related to body-size of the mammals (2.2, 3.0, 3.7, 2.8 and 4.9-fold respectively for the same species).

A possible explanation for Krebs' tissue slices displaying 3 times the rates of oxygen consumption measured in Section 3.3.2.4 while the difference was around 2-fold in smaller mammals is that the tissue slices from larger mammals used in this thesis could have been in poorer condition than those used by Krebs. Another avenue which cannot be ignored is potential bacterial contamination of the measuring equipment in Krebs' experiments. Even using fresh saline, when measuring oxygen consumption over 45 minutes a bacterial buildup is likely to occur in the presence of tissue slices. This was commonly observed during the preliminary experiments and only thorough sterilisation of the equipment could prevent this from occurring. In the experiments by Krebs (1950), who did not mention any other precautions for this potential problem than the preparation of fresh saline, minor contamination would have had more relative impact on the rates of oxygen consumption of metabolically slower slices. Krebs did not mention performing controls of the saline oxygen consumption once the slices were removed.
Elliott (1955), commenting on the unusually high rates of oxygen consumption reported by Krebs, published values for rat liver slices (converted as I have done for Krebs' data) of $2.5 \mu\text{mol O}_2\cdot\text{g wet}^{-1}\cdot\text{min}^{-1}$. The estimates reported here, $1.4$ to $2.1 \mu\text{mol O}_2\cdot\text{g wet}^{-1}\cdot\text{min}^{-1}$ (using wet weights or correcting for active respiring cell mass, respectively) are more in agreement with his values. More recently published values of tissue oxygen consumption better match the measured values reported in Table 3.9 for rat liver slices using wet weight. The review by Clausen et al. (1991) covered rates of oxygen consumption for rat liver slices under various experimental conditions. The values they gathered ranged between $1.7$ to $1.9 \mu\text{mol O}_2\cdot\text{g wet}^{-1}\cdot\text{min}^{-1}$ for Na⁺-loaded slices, from $0.8$ to $1.5$ for prechilled liver slices and for liver slices preequilibrated at $37^\circ\text{C}$ the rates of oxygen consumption were found to be around $0.9 \mu\text{mol O}_2\cdot\text{g wet}^{-1}\cdot\text{min}^{-1}$. Thus the values reported here (using wet weight) are similar to those from slices with high $[\text{Na}]_i$ from this review (Clausen et al., 1991). This agrees with the average 50 mM $[\text{Na}]_i$ estimated for these rat liver slices (Table 3.7).

The published values of oxygen consumption rates for isolated liver cells (hepatocytes) are much higher, ranging from $1.7$ to $7.1 \mu\text{mol O}_2\cdot\text{g wet}^{-1}\cdot\text{min}^{-1}$ (see Clausen et al., 1991). The control values for hepatocytes published by Berry et al. (1983) ranged between $3.2$ to $4.7 \mu\text{mol O}_2\cdot\text{g wet}^{-1}\cdot\text{min}^{-1}$. Van Dyke et al. (1983) reported values of $7.0$ to $8.9$ in various hepatocyte experiments. The typically higher values reported for hepatocytes when compared to liver slices are probably due in part to these weights not including any non-cell weight such as extracellular matrix that is present in tissue slices.
Data from the literature for kidney cortex slices are more scarce. Those available are higher than reported in Table 3.9. Else and Hulbert (1987) obtained values which, converted (see footnote 5) are 3.2 μmol O₂·g wet⁻¹·min⁻¹ in rats. This value is twice as high as that reported in Table 3.9 (1.5 μmol O₂·g wet⁻¹·min⁻¹). Elliott (1955), measured rates of 2.6 μmol O₂·g wet⁻¹·min⁻¹ for rat kidney cortex slices, similar to the value reported in Table 3.10 after correction for % GDH loss. Weidemann and Krebs (1969) reported rates of oxygen consumption of 3.2 μmol O₂·g wet⁻¹·min⁻¹ for rabbit kidney cortex slices in substrate-free saline, more than twice the value reported here (1.2 μmol O₂·g wet⁻¹·min⁻¹).

Sejersted et al. (1971) estimated, using thermocouples, that the oxygen consumption of in vivo kidney cortex of dogs would be between 2.7 to 3.6 μmol O₂·g wet⁻¹·min⁻¹. According to the evidence presented by Jørgensen (1980), the filtering and reabsorbing whole kidney cortex would display much higher oxygen consumption rates than non-filtering and non-reabsorbing slices. In his review, this author reported rates of 3 to 6 μmol O₂·g wet⁻¹·min⁻¹ as typical of whole kidney in rat, rabbit or dog kidney. Unlike for liver, most authors do not commonly put an emphasis on the absolute rates measured for whole kidney cortex or slices, as it is apparent that it does vary widely depending on the experimental conditions (such as the extent to which the tissue is actively involved in reabsorptive processes). Thus the rates of oxygen consumption for isolated kidney cortex slices measured here are most probably lower than that for in vivo conditions.
A fact worth mentioning from Table 3.9, where the rates of oxygen consumption on a wet weight basis are reported, is that rat liver slices did not differ to those from mouse in their rates of oxygen consumption. Correcting for active respiring cell mass (Table 3.10) did not bring about any difference. Hulbert and Else (1990) also reported that mouse and rat liver slices displayed the same rate of tissue oxygen consumption. This contrasts with Krebs' data (1950), who reported 34% higher values for mouse than rat liver. As in the present experiments mouse liver slices showed a much greater decrease in oxygen consumption rate with time than rat, using the latter factor to correct oxygen consumption in conjunction with GDH loss leads to mouse liver slices displaying rates of oxygen consumption that are 21% higher than those of rat liver slices (Table 3.11).

Rates of potassium uptake

The results reported in this chapter have shown that the liver and kidney cortex of smaller mammals display higher rates of potassium uptake. Unlike the oxygen consumption rates, the allometric decline in potassium uptake rates was similar in both liver and kidney cortex slices, with a doubling in body size corresponding to a 9% decline in the mass-specific rate of potassium uptake in liver slices and 7 to 9% for kidney slices.

Given that the estimated [Na⁺]i of tissue slices ranged between 47 and 143 mM in the control slices, sodium pump activity would most likely have been maximal (or at least very close to maximal) under the experimental conditions. The KNa of sodium pumps lies around the preferred cell sodium concentration and maximal sodium pump activity is rapidly reached when
[Na$^+$]$_i$ rises above this level (Haber et al., 1987; Rossier, 1987; Skou, 1992).

Van Dyke and Scharschmidt (1983) described the dependence of the activity of the sodium pumps on intracellular sodium concentration in rat hepatocytes. In those cells, the $K_{Na}$ was reported to be around 18 mM, meaning that near maximal activity of the sodium pumps was reached at around 36 mM [Na]$_i$, lower than the lowest of our estimated [Na]$_i$ (Table 3.7). Harris et al. (1986) determined experimentally that in cultured rat proximal tubules a rise from 12 mM to 100 mM [Na]$_i$ resulted in a 3-fold increase in sodium pump activity, although they did not report the $K_{Na}$ of the sodium pumps in those cells. Jørgensen (1980) and Soltoff and Mandel (1984) reported that at 100 mM [Na]$_i$ maximal pump activity is reached. If the estimates reported in Table 3.7 are correct (79 to 120 mM [Na]$_i$), from the available evidence, and assuming similar $K_{Na}$ for the sodium pumps in the species examined, I consider that the sodium pumps in the slices examined were functioning near their maximal rate. It is however clear from the review by Jørgensen (1980) that the rate of sodium pump activity can be greatly altered in non-filtering kidney cortex. Consequently, the results will only have their significance when interpreted in an allometric perspective and in the context of the cells of these tissue slices not performing their normal in vivo physiological work.

Little comparative data on rates of potassium uptake by liver is available in the literature. In perfused rat liver, Claret et al. (1973) calculated the rate of potassium uptake in perfused rat liver. The value they reported, 1.04 $\mu$mol K$^+$.g wet$^{-1}$.min.$^{-1}$ is not greatly dissimilar to the 0.79 $\mu$mol K$^+$.g wet$^{-1}$.min.$^{-1}$ reported in Table 3.12 for rat liver slices. The control value published by Van Dyke and
Scharschmidt (1983) for cultured rat hepatocytes, converted using the protein content of whole liver from Table 3.2 (assuming that those values are representative of hepatocyte protein content/cell volume) is 0.75 \( \mu \text{mol} \ K^+ \cdot g \text{ wet}^{-1} \cdot \text{min}^{-1} \), nearly identical to the value reported here. Most authors have reported relative potassium uptake rates, or units irrelevant for this study. For example, Wiley and Cooper (1975) express the rates per ml of erythrocytes. Many researchers have monitored the activity of the sodium pumps by the rate of release of inorganic phosphate. If the maximal activity of the sodium pumps published by Haber et al. (1987) for cultured rat liver cells are converted using 1 ATP/2 K and the method mentioned above for conversion of mg protein to wet weight, their estimate is around 1.9 \( \mu \text{mol} \ K^+ \cdot g \text{ wet}^{-1} \cdot \text{min}^{-1} \).

In kidney, the literature on the rate of sodium pump activity and sodium reabsorption is vast, although little of it was performed with tissue slices. Jørgensen (1980) estimated that the actual reabsorptive capacity of intact mammalian kidney (rat, rabbit, dog, pig) for sodium would be around 6-24 \( \mu \text{mol} \ Na^+ \cdot g \text{ wet}^{-1} \cdot \text{min}^{-1} \). With a stoichiometry of 3 Na/2 K, the values would be in the range 4-18 \( \mu \text{mol} \ K^+ \cdot g \text{ wet}^{-1} \cdot \text{min}^{-1} \). From his estimates, he also suggests that only 24-54\% of this sodium reabsorption is linked to sodium pump activity. This makes the expected values range between 1 and 10 \( \mu \text{mol} \ K^+ \cdot g \text{ wet}^{-1} \cdot \text{min}^{-1} \). Still smaller values would be expected in slices where normal \textit{in vivo} reabsorptive mechanisms have been disrupted. Thus the values presented in Table 3.12 are clearly lower than would be expected for \textit{in vivo} situations but are credible for kidney cortex slices.
Relationship between the rates of aerobic metabolism and of potassium uptake in liver and kidney cortex slices

This part of the present study was aimed at testing the third hypothesis of this thesis, that sodium pump activity is directly proportional to metabolic activity. For this purpose, the rates of oxygen consumption of liver and kidney cortex slices was taken as an indicator of tissue metabolic activity and the rates of potassium uptake were assumed to reflect sodium pump activity. The statistically significant relationship between oxygen consumption rate and potassium uptake rate for both tissues, illustrated in Figure 3.19, is in agreement with the third hypothesis. In statistical terms, since the rates of oxygen consumption and of potassium uptake were independent variables, the coefficients of determination ($r^2$) indicated either that for liver 95% of the variation of potassium uptake was explained by oxygen consumption rate or conversely that 95% of the variation in oxygen consumption of tissue slices was due to variations in potassium uptake rates. For the kidney cortex slices, 79% of the variation in one of these parameters was explained by the level of the other.

The demanding statistical analysis (only 5 values) thus allows me to conclude that oxygen consumption rate and potassium uptake rate are closely related parameters in mammalian liver and kidney cortex slices. If oxygen consumption rate is indicative of metabolic activity and if potassium uptake rate is representative of sodium pump activity, sodium pump activity is related to metabolic activity or the reverse. Which one of these variables is dependent on the other cannot be determined from the present experiments.

Investigations by others have already suggested that sodium influx was related to metabolism. Else and Hulbert (1987) showed that rat liver slices had

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2-4 times higher oxygen consumption rates than liver slices from a lizard of the same size and preferred body temperature and that the liver slices from rat were about 3-fold more permeable to potassium. With isolated liver cells from the same species they also demonstrated that rat liver cells were almost 8 times leakier to sodium. Else and Hulbert (1987) thus postulated that the higher metabolism of endotherms than ectotherms was partly explained by leakier membranes which necessitate greater ion pump activity. This causal relationship cannot be justified from these experiments since just as in the case of this study both variables had been measured independently. In such cases, a causal factor cannot be inferred. They further stated that “it is the increased leakiness of the mammalian cell membrane that is driving the system and resulting in an increased consumption of energy relative to the same system in the ectotherm”. Given the experimental methods, it could also be said from their findings that it is the increased consumption of energy that drives the increased leakiness of the mammalian cell membranes relative to the reptilian cell membranes. In this alternative view, since the sodium gradient serves as an energy store for metabolic purposes, higher metabolic activity results in a greater use of the gradients and consequently of sodium pump activity.

Further to this, Hulbert and Else (1990) asked whether membrane leakiness was related to cellular oxygen consumption and showed that plasma membrane permeability to sodium was strongly correlated to the part of metabolism that was dependent on the sodium gradients (if I can interpret the part of aerobic metabolism that is inhibited by long incubation periods in the presence of ouabain as “sodium gradient-dependent metabolism”). The correlation that they reported, with isolated liver cells from trout, lizard, toad, pigeon and rat, is indeed tighter than that reported in Figure 3.19 between potassium uptake and total mammalian tissue slice aerobic metabolism in
mammalian liver and kidney cortex slices. The liver slices of the vertebrates used by Hulbert and Else (1990) varied in the proportion of metabolism that could be inhibited by long incubation periods in the presence of ouabain. Their findings lead to the logical proposition that sodium pump activity (which is expected to match sodium influx) could be more related to the part of metabolism that is dependent on the sodium gradient than to total tissue aerobic metabolism. Since in Figure 3.19 total tissue slice aerobic metabolism was related to potassium pumping, it is possible that the tissues of different-sized mammals are similar in the proportion of their metabolism that is dependent on the sodium gradients. This question will be indirectly examined in the next section which investigates the proportion of liver and kidney cortex slices aerobic metabolism that is used by the sodium pumps.

Estimates of the proportion of cell energy turnover devoted to the sodium pumps in liver and kidney slices

The estimates of the contribution of sodium pumps to mammalian tissue slice metabolism (Table 3.14) suggest that in liver slices the small mammals spent less than 5% of their energy turnover on the sodium pumps (Section 3.3.2.5). This is in agreement with the values reported by Brand et al. (1991) and Clausen et al. (1991). Cattle liver slices clearly devoted relatively more energy to their sodium pumps, around 10%. Since pump activity was probably maximal for all slice preparations, the trend of increase in energy devoted to the sodium pumps in the liver slices of larger mammals could have been a reflection of increasingly lower rates of oxygen consumption due to experimental artefacts. An alternative explanation is that in metabolically slower mammals the passive permeability of liver plasma membranes to sodium and potassium becomes relatively more important. Sodium influx, the likely major influence on sodium
pump activity, occurs partly for metabolism-related functions and partly passively. The passive part of sodium influx could have become relatively more important in the liver of animals with lower metabolic demands.

From the estimates in Table 3.14, kidney cortex slices spent relatively more energy on sodium pumping than liver slices from small mammals and under the experimental conditions there was no allometric trend for the metabolic cost of the sodium pumps. The slopes of the linear regression lines in Figure 3.19 reflect the differences in cell energy devoted to the sodium pumps between liver and kidney cortex slices. In kidney cortex slices, the values estimated here are different to those reported by Clausen et al. (1991). While the estimates reported here ranged from 7 to 10%, Clausen et al. (1991) report that up to 70% of the oxygen consumption in kidney could be devoted to cation pumping. Lote (1987) states that kidney cortex in vivo can devote around 50% of its energy for sodium transport.

With the methods used in this chapter, the estimates are likely to be much closer to in vivo rates for liver slices than for kidney cortex slices. Kidney tubule cells use the sodium gradients for reabsorptive processes and in addition are directly involved in sodium reabsorption from the plasma ultrafiltrate produced in the glomeruli. The estimates in Table 3.14 probably represent the energy spent by the kidney cortex cells to maintain potassium and sodium gradients in non-reabsorbing conditions.

In addition, according to Lote (1987), there is some evidence for a Na-ATPase which operates independently of potassium in kidney cortex cells. In this case, more energy than was estimated by $^{86}$Rb accumulation in Table 3.14 would have been spent for sodium pumping in the kidney cortex slices.
The estimations in Table 3.14 suggest that mammals do not greatly differ in the proportion of their tissue metabolism devoted to the sodium pumps in homologous tissues. This could imply that the tissues of different-sized mammals have a similar proportion of their metabolism that is dependent on the sodium gradients. Such mechanisms involve protein synthesis since the inward transport of more than half of the amino acids is directly sodium-dependent (see Guidotti et al., 1978). The suggestion above is supported by experimental evidence since Blaxter (1989) have shown from data collected by Reeds and Harris that body protein synthesis is related to body weight with an exponent of approximately 0.75, the same exponent as that of basal metabolism.

In summary for this section, the estimates of the metabolic cost of sodium pumps for liver and kidney cortex slices in vitro suggest that under these conditions sodium pumps are a minor contributor to tissue metabolism. The results of this chapter in conjunction with evidence from the literature also indicate that mammals, whatever their size, spend a similar proportion of their total energy turnover for protein synthesis and for sodium pumping. Whether the sodium gradient-dependent part of metabolism can be generalized beyond protein synthesis to be a constant in mammals can only be suggested by the similar proportion of the tissue slice energy turnover devoted to the sodium pumps and remains to be thoroughly investigated.
3.4.4 Further analysis of the condition of the mammalian liver and kidney cortex slices used in this thesis and general comments on the work with tissue slices

Discussion of the rates of potassium efflux measured on tissue slices

Potassium efflux rate can be used as an indicator of plasma membrane integrity in conjunction with estimates of intracellular concentrations of sodium and potassium. The \([K^+]_i\) of liver slices reported in Table 3.7 was normal for rats (Claret and Mazet, 1972 reported 113 mM for perfused rat liver) but lower than expected under physiological conditions for all species by comparing values for slices to values for whole tissues (Section 3.3.2.2). The \([Na^+]_i\) was higher in all liver slice preparations than expected for normal conditions such as those estimated for whole tissues. In rat liver, \([Na^+]_i\) was 50 mM, similar to that in rabbit slices, lower than for the other species and three times that estimated by Claret and Mazet (1972) for perfused rat liver. Kidney cortex slices also showed lower transmembrane cation gradients than expected \textit{in vivo}. For both tissues, larger mammals had a lower capacity to maintain cation gradients. For liver slices however, the superior slices in this respect were those from rat and rabbit. This low ability of some tissue slices to maintain transmembrane cation gradients was possibly due to increased passive permeability of the plasma membranes to potassium. Given the lower sodium pump activity in the tissues of larger mammals a rate of potassium efflux slightly higher than normal could be enough to prevent the tissue slices from maintaining ion gradients.
Few values are available in the literature for rates of potassium efflux in tissue slices. For 500 µm thick rat liver slices, the rate of potassium loss published by Haber and Loeb (1984), 0.9% loss-min⁻¹, is much lower than that published by Else and Hulbert (1987) :3.0% loss-min⁻¹. The potassium efflux rate reported here for rat liver slices, 2.01% loss-min⁻¹, compare better with the control rates of 1.8% loss-min⁻¹ for cultured rat liver cells reported by Haber et al. (1988). For comparative purposes, data on other tissues are also available. Montero et al. (1991) report 7.7% loss-min⁻¹ in chicken enterocytes, whilst in rat Islets of Langerhans, Petit et al. (1991) reported control rates of 2.4% loss-min⁻¹. As mentioned above, the rat liver slices had the ability to maintain high [K⁺]. The rates of potassium efflux measured may have been normal for this tissue.

To support the hypothesis that the high potassium efflux rates measured in many tissue slice preparations were an artefact of slice preparation, it is relevant to report some results from early experiments. When 225 µm slices and bubbling wells were used (early experiments in Section 3.3.1), these techniques induced substantial slice damage and the potassium efflux rates measured in liver slices were around 9, 8 and 11% K⁺ loss/min. for mouse, sheep and cattle respectively. Improving the methods has yielded repeatedly smaller efflux rates.

Finally, in liver slices the phospholipid fatty acid composition of plasma membranes may have played a role in their passive permeability to potassium. This possibility will be investigated in Chapter 4.
To summarize, the liver slices that showed consistently superior viability and quality are those from rat and rabbit. They are followed by mouse, then sheep and cattle slices. In kidney cortex slices larger mammals showed a clearly lower ability to maintain their cation gradients, presumably mainly because of lower sodium pump activity.

Suggestions for further studies with tissue slices from different-sized mammals

To improve the viability of the slices, many avenues can be explored in the future. One could try to obtain fresher tissues from the abattoir, removed within a few minute after death or, if impossible, it could be fruitful to attempt reperfusion with serum before slicing. A more physiological saline, such as serum, could also help the cells to recover. In any case, the slices should be used as soon as possible.

Before attempts are made to measure sodium influx in an allometric comparison with mammalian tissues, methods have to be developed under which the cells display a plasma membrane permeability that allows them to maintain their ion gradients for extended periods. If this condition is not met, the results of such comparisons cannot be interpreted in a physiological perspective.
3.5 Conclusions

The findings reported in this chapter confirm that the allometric decline in whole organism metabolism is reflected in part in tissue metabolism. The differences in tissue metabolism reported by Krebs (1950) in different-sized mammals could not be attributed to variations in cell volume. Indeed the less metabolically active mammals tended to have slightly higher mass-specific intracellular volumes (at least statistically significant in their livers). Total kidney metabolic activity was estimated to be proportional to basal metabolic rate in mammals. The relative decrease of total kidney cortex metabolic rate in larger mammals was due equally to a decrease in kidney size and in tissue metabolic rate. From estimates, total liver metabolic rate relative to basal metabolic rate decreased in larger mammals, when considering the decrease in organ size and a still greater decrease in mass-specific tissue metabolic rate. Thus, from the conclusions reached with these tissues the scaling of basal metabolic rate according to $W^{0.75}$ could be fully accounted for by a decrease in organ size and in mass-specific tissue metabolic rate. The latter would be due to lower cell volume-specific metabolic rate (less metabolically active cells of similar size or larger-sized cells with a similar rate of metabolic activity in the tissues of larger mammals) and not to differences in cell volume.

There was a statistically significant correlation between tissue slice oxygen consumption rate and potassium uptake rate. This reinforces an association between metabolic functions and sodium and potassium fluxes across the plasma membranes. Under the conditions used in the experiments of this chapter sodium pumping was estimated to be of little importance in terms of metabolic cost. By comparing with literature evidence, it can be proposed that sodium and potassium transport only become major contributors for the
total cell energy turnover in conditions where the cation gradients are used for specific metabolic functions such as reabsorption of various substances.

The monitoring of slice condition during this work with tissue slices from mammals of different size has also revealed that slices from larger mammals suffered more damage and possessed lower recovery capacity than those from smaller mammals. While slice damage could be associated with the time between death and slicing, recovery capacity was clearly related to the activity of the sodium pumps.
Chapter 4

General Discussion

“Organisms may be pictured as systems of precise multiple interrelations”

E.F. Adolph (1949)
One part of this thesis aimed at investigating some of the mechanisms involved in the allometric decrease in basal metabolism for mammals. Thus in Chapter 3 the hypothesis that the scaling of mass-specific basal metabolic rate is partly due to the scaling of mass-specific liver and kidney cortex metabolic rate was tested. An allometric decrease of oxygen consumption rate of liver and kidney cortex slices in larger mammals was found (Figure 3.13, p. 169). The results allowed to conclude that tissue metabolic rate was partly responsible for about half of the allometric decrease in mammalian mass-specific basal metabolic rate. The intracellular water volumes measured also permitted the inference that the observed decrease in tissue metabolism in larger mammals could not be attributed to these mammals having less cell volume per unit mass in their liver and kidney cortex. Accounting for the decrease relative in organ size as well as for the decrease in mass-specific tissue metabolism in larger mammals could potentially provide for an explanation of the -0.25 allometric exponent for the allometric relationship of mass-specific metabolism, a hypothesis proposed by Holliday et al. (1967).

There was an allometric decrease in potassium uptake rates with increasing body size in both of liver and kidney slices (Figure 3.17, p. 179). A statistically significant linear relationship between the rates of oxygen consumption and of potassium uptake was found in both tissues (Figure 3.19, p. 183), in agreement with Hypothesis 3 that sodium pump activity is directly proportional to metabolic activity.

In the other part of this thesis, body size was used as a system (in which tissue metabolism varies) to investigate membrane-related aspects of metabolism. Parameters describing membrane composition and permeability were investigated. The hypothesis that mammalian tissues displaying higher
mass-specific metabolic rate possess more polyunsaturated cell membranes was confirmed for some tissues as the Unsaturation Index of kidney cortex, heart and skeletal muscle phospholipids showed a statistically significant inverse relationship with body size (Figure 2.4, p. 70), reflecting variations in other indexes of membrane composition.

This chapter will further investigate the relationships between body size, tissue phospholipid fatty acid composition, tissue metabolism, membrane permeability to some cations and sodium pumping. The last three hypotheses of this thesis have been formulated to this end. Specifically, they are that mammalian tissues displaying higher mass-specific metabolic rate possess more polyunsaturated cell membranes, that mammalian tissues whose cell membranes are more polyunsaturated also display higher sodium pump activity and that tissues whose cell membranes contain higher levels of highly polyunsaturated fatty acids display higher rates of potassium efflux.

The experimental system chosen imposes limitations on the interpretation of the results. The first is the choice of the species. While the body weight of these species shows an even spread on a logarithmic scale, they had a different diet and lifestyle since both smaller species were rodents and both larger species were ruminants while the intermediate-sized species, rabbit, was a lagomorph. Some of the differences observed in composition and physiological parameters could have been associated to taxonomic differences. In particular, rabbits showed a lower membrane polyunsaturation (UI) in liver and a lower GDH activity in this tissue than would have been predicted for their body weights.
The second limitation of this investigation is the number of tissues examined. While it was possible to examine five tissues in each species for phospholipid fatty acid composition, for the work with tissue slices only liver and kidney cortex were used. The main reason is that tissues with elongated cells such as striated muscle cannot be easily sliced. As well, the time required for the slicing and the manipulations limited the number of tissues that could be handled simultaneously to two. Apart from the relative ease of slicing liver and kidney cortex, using these tissues for physiological measurements provided with one tissue showing a significant allometric relationship of membrane polyunsaturation (kidney cortex) and one for which no such statistically significant relationship had been found (liver).

The interpretation of the changes in tissue phospholipid fatty acid composition was limited by not discriminating in the present study between the various membranes or the different phospholipid classes. The parameters examined further in this chapter for their relationship with the composition of a pooled membrane fraction could possibly only be influenced by the composition of a particular subcellular membrane. Similarly, it may be that most of the variation in membrane composition of homologous tissues from different-sized mammals was due to one class of phospholipids (rather than total tissue phospholipid) and that other phospholipids remain conservative in composition and proportions in homologous mammalian tissues. In addition, in liver, phospholipids other than those of cell membranes (eg. those in the envelope of the lipoproteins assembled in the liver for export to other tissues) were analysed with all phospholipids. Examining total tissue phospholipids could have hidden some trends relating membrane fatty acid composition with tissue metabolism.
Tissue slices have their limitations since their normal physiological function would be altered \textit{in vitro} and since they were likely to differ in metabolic activity to the same tissues \textit{in vivo}. The further comparison in this chapter between membrane composition of whole tissues and physiological parameters measured with tissue slices will remain confined to very general considerations. Accordingly, mainly broad parameters have been used for this comparison, namely the Unsaturation Index (UI), which relates to the extent of polyunsaturation of membranes, oxygen consumption as an indicator of tissue metabolic rate and potassium uptake, relating to sodium pump activity. As well, it must be pointed out that the analysis of membrane composition and the measurements of tissue metabolism were performed with different animals, although they were the same breeds and were obtained from the same sources.

Finally, the time available to complete this thesis has brought limitations in the number of species and of individual in each species examined. Having only four animals per species and five species lead to very demanding criteria for the statistical significance of the correlations reported throughout this thesis. Nevertheless, the repeatability of most parameters measured has lead to statistically significant trends and differences in many cases and these may be regarded as emerging from a rigorous statistical analysis.

The major relationships experimentally established between membrane polyunsaturation and physiological parameters in the previous chapters have been summarized in Figure 4.1, along with relationships between membrane polyunsaturation and physiological parameters discussed in this chapter. The Unsaturation Index was used for this figure as an indicator of overall polyunsaturation but some other indexes of membrane composition will also be considered in the following discussion. The correlation coefficients in Figure 4.1
Figure 4.1

Relationships between the extent of membrane polyunsaturation and metabolism in mammalian liver and kidney cortex. The r values were calculated using mean values (n=5). Oxygen consumption and potassium uptake measurements were performed on tissue slices. The thickness of the arrows indicate the level of significance.
were calculated using the mean values (n=5 for each comparison) to make all correlation coefficients comparable since in the comparisons between tissue phospholipid composition and physiological parameters mean values for each species had to be used, these measurements having not been performed with the same individuals. Thus in some cases the significance levels and the correlation coefficients will differ from those reported in Chapter 3, where the allometric relationships with the rates of oxygen consumption and of potassium uptake were calculated using individual data (n=20).

Before discussing in detail Figure 4.1, it must be said that for both tissues there was a consistent trend between the parameters reported. Lower membrane UI was associated with larger mammals, with lower rates of both oxygen consumption and of potassium uptake\(^1\). This was deduced by the slopes of the regression lines which were always consistent with the above statements. Presumably because the parameters measured were only approximate indicators of membrane composition, of *in vivo* tissue metabolic rate, of sodium pump activity and of plasma membrane permeability and because the sample size was small, many relationships which may be physiologically important did not become statistically significant.

**Tissue phospholipid polyunsaturation and oxygen consumption rate**

The hypothesis that mammalian tissues displaying higher rates of metabolism have more polyunsaturated membranes can be tested by observing the correlation between the UI and oxygen consumption rate (Figure 4.1). In the liver, mammals of increasing body size proportionally decreased tissue slice

\(^1\) In this chapter, the rates of oxygen consumption and of potassium uptake of tissue slices were taken from Tables 3.9 and 3.12, respectively (which used wet weight for the calculations) except where specified, since this the results obtained using this method showed less variability.
metabolism without a statistically significant decline in total tissue phospholipid Unsaturation Index (UI). As a result the UI was not statistically significantly correlated with oxygen consumption rate. In kidney cortex the total body size of the mammal was found to be correlated with both the UI and the rates of oxygen consumption and the UI was also correlated to aerobic metabolism ($p < 0.02$, Figure 4.1 and solid line, Figure 4.2). Plotting the UI of both tissues against their rates of oxygen consumption to give a more general overview also yielded a significant ($p < 0.05$) relationship (dotted line, Figure 4.2). From these figures, a general relationship between membrane polyunsaturation and cell metabolic activity can be inferred. Those tissues that clearly did not follow the general trend were livers from sheep and cattle, which displayed a high membrane polyunsaturation for their rates of aerobic metabolism (the lower points in Figure 4.2).

![Figure 4.2](image)

**Figure 4.2**

Relationship between Unsaturation Index and oxygen consumption rate of mammalian liver slices (○) and kidney cortex slices (●) (mean±s.e., n=4).
The results presented in Chapter 2 have revealed that kidney cortex phospholipid fatty acid composition of larger mammals showed a decline in Unsaturation Index and average chain length and an increase in the proportions of monounsaturates (mainly due to increases in Ω9 monounsaturates). The increase in % monounsaturates for larger mammals failed to show a statistically significant correlation with tissue oxygen consumption (r=0.7532). The relationship between %Ω9 content and kidney cortex oxygen consumption rate was not statistically significant (r=0.791) although the estimates of oxygen consumption rates corrected using tissue GDH content yielded a significant relationship (r=0.924, p < 0.05). In kidney cortex then, the aspects of membrane composition that could be correlated with body size showed a comparable association with oxygen consumption rates. Since in kidney cortex of larger mammals Ω9 fatty acids substituted for more polyunsaturated fatty acids in smaller mammals, the negative relationship of %Ω9 fatty acids with oxygen consumption rates could simply reflect the relationship already described between UI and oxygen consumption.

The data from Krebs (1950) plotted in Figure 1.1 (p. 11) also suggest a relationship between oxygen consumption rates and body size in mammalian brain. The analysis of brain phospholipid fatty acid composition in Chapter 2 unveiled no allometric relationship between membrane composition and body size. If the data from Krebs (1950) were to be relied upon they would suggest that membrane composition and tissue metabolism are not very closely associated in the brain. An examination of Figure 4.1 would be in agreement with this view. In liver, while there was a clear relationship between body size and oxygen consumption rates, the Unsaturation Index did not correlate so tightly to body size. This could be interpreted as meaning that some parameters

2 With 5 points, a correlation is significant at the 5% level if the "r" value is equal or above 0.878 and at the 10% level for a r value higher than, or equal to, 0.805 (Porkess, 1988).
related to membrane polyunsaturation, for example a particular class of phospholipids or a particular subcellular membrane, can be strongly related to parameters related to tissue slice metabolism, like the activity of some membrane enzymes, the permeability of some membranes to some molecules or ions or in vivo tissue metabolic rate. Thus, using general indicators such as UI and slice oxygen consumption rate could obscur underlying relationships present in all tissues between some aspects of membrane composition and of cell metabolism. The significance of the relationship between UI and tissue slice rate of aerobic metabolism found in the present study for both kidney cortex and liver and kidney cortex together cannot be inferred from the data available.

**Tissue phospholipid polyunsaturation and potassium uptake rate**

Since the sodium pumps are membrane-bound enzymes and that there is some evidence, presented in Chapter 1, that membrane composition can affect the activity of such enzymes, in this section the hypothesis that sodium pump activity is higher in more polyunsaturated membranes is examined. Sodium pump activity was estimated by the rates of potassium uptake (using rubidium). Sodium pumps are responsible for 67% to 87% of the total potassium uptake in rat liver and kidney tubules, respectively (Van Dyke and Scharschmidt, 1983; Harris *et al.*, 1986). In Figure 4.1, the relationships between the UI and potassium uptake rates in liver and kidney cortex are reported. For both tissues there was a tendency for those tissues with higher rates of potassium uptake to be more polyunsaturated but the differences were not statistically significant.

The increase in % monounsaturates in the kidney cortex of larger mammals could not be correlated with a significant decrease in potassium
uptake of tissue slices ($r=0.796$). The amounts of ω9 fatty acids showed a
greater correlation with potassium uptake ($r=0.903$, $p < 0.05$).

Thus, although in both tissues no statistically significant relationship
was found, 50 to 55% of the variability of potassium uptake rates could be
attributed to the Unsaturation Index or the reverse (from the coefficients of
determination). This statistical statement should be interpreted in the context of
Figure 4.1 which shows that body size, UI, oxygen consumption rate and
potassium uptake rate are all related parameters to some extent. The UI itself is
influenced by many aspects of membrane composition. Thus a safe statement
concerning the relationship between the UI and tissue potassium uptake rates is
that in homologous tissues with higher UI a higher rate of potassium uptake is
to be expected. Whether this is due to higher membrane permeability, sodium
pump activity, or tissue metabolic rate or to a combination of these factors
cannot at the present be ascertained.

**Membrane polyunsaturation and rates of potassium efflux**

As detailed in Chapter 1, it has been suggested from experiments with
cells, mitochondria and reconstituted membranes that membrane permeability
to protons or to various molecules could be related to some aspects of
membrane phospholipid fatty acid composition (Brand et al., 1992; Stillwell et
al., 1993; Brand et al., in prep.). The extent of membrane polyunsaturation and
in particular the amount of 22:6ω3 have been proposed to be related to
membrane permeability. Thus the sixth hypothesis, aimed at testing the
relationship between potassium efflux and membrane polyunsaturation was
formulated.
No statistically significant relationship could be established between the UI of membrane fatty acids or the relative amount of 22:6Ω3 and potassium efflux rates in either liver or kidney cortex. These correlation coefficients were 0.552 and 0.856 for liver and kidney cortex UI vs. potassium efflux rates, respectively and 0.825 and 0.661 for liver and kidney cortex %22:6Ω3 content vs. potassium efflux rates, respectively. Following this analysis, the sum of relative amounts of the most abundant highly polyunsaturated fatty acids found in liver and kidney cortex (22:5Ω3 and 22:6Ω3) was considered for a comparison with potassium efflux rates. In liver, a very significant relationship between the rates of potassium efflux and the sum of the relative amounts of 22:5Ω3 and 22:6Ω3 was found (p < 0.02, Figure 4.3). In kidney cortex, no such relationship was found (r=0.503).

Figure 4.3

Relationship between the amount of the highly polyunsaturated 22:5Ω3 and 22:6Ω3 and potassium efflux rate in mammalian liver slices. Shown are mean ± s.e. (n = 4).
The relationship in liver is thus supportive of the hypothesis that membrane permeability to potassium is influenced by the amount of highly polyunsaturated fatty acids. These results also give an interesting insight that there could be physico-chemical mechanisms (see Chapter 1) regulating potassium efflux in living cells that would be altered in tissue slices. The high rates of potassium efflux found in sheep and cattle liver slices in Chapter 3 may not have been mainly caused by slice damage with the improved technique used but by some mechanisms of regulation of potassium efflux not being fully operative in liver slices of some species. Consequently, in liver slices the rates of potassium efflux measured could have reflected passive plasma membrane permeability, proportional to the amounts of highly polyunsaturated fatty acids, instead of a more metabolically controlled rate of efflux. In kidney cortex slices, which did not show such a correlation between membrane polyunsaturation and potassium efflux rates, the latter parameter could have remained more under metabolic control.

Another avenue for exploring the relationship between membrane polyunsaturation and permeability to sodium and potassium is by examining the activity of the sodium pumps in relation to the proportions of 22:5\(\omega3\) and 22:6\(\omega3\). The rates of potassium uptake could be expected to be indicators of maximal in vivo rates because of the high [Na\(^+\)]\(_i\) found in slices (Chapter 3). In order for the cells to maintain potassium gradients, the rates of potassium efflux should match the rates of potassium uptake. The rates of potassium uptake measured with tissue slices were possibly related to the maximal rates of potassium uptake and thus of potassium efflux and of sodium influx encountered in vivo in the various tissues examined.
The positive relationships between \( \%22:6\Omega3 \) and potassium uptake rates were not statistically significant (\( r=0.567 \) and \( r=0.585 \) for liver and kidney cortex, respectively). The relative content of \( 22:5\Omega3 \) and \( 22:6\Omega3 \) also failed to show relationships with potassium uptake rates (\( r=0.089 \) and \( r=0.442 \) for liver and kidney cortex, respectively). Since the rates of potassium uptake may be indicative of maximal *in vivo* permeability, these findings could lend support to the proposition above that plasma membrane permeability to potassium *in vivo* is not mainly determined by passive membrane permeability (which could be largely controlled by membrane phospholipid fatty acid composition) but by some mechanism of metabolic control.
General conclusion

The work presented in this thesis has enlarged the early findings by Krebs (1950) who had reported that the tissues of larger mammals displayed lower mass-specific metabolic rates. This study pointed out that the differences in liver and kidney cortex metabolic rate were not due to differences in cell volume but narrowed the explanation to either larger mammals having less metabolically active cells or having less, larger cells. The decrease in relative size of liver and kidney of larger mammals has been considered together with the decrease in tissue metabolism and from these estimates it appears that the scaling of basal metabolic rate according to $W^{0.75}$ could be fully accounted for by these parameters if other tissues also follow the trends observed in liver and kidney cortex.

Another significant finding in this thesis is that the rates of potassium uptake, indicators of sodium pump activity, are proportional to tissue metabolism. These results are in agreement with the mammal-reptile comparison, where it was found that although mammals display higher tissue metabolic rate than similar-sized reptiles at the same body temperature, the proportion of cell metabolism that depended on sodium pump activity was similar in liver and kidney cortex (Else and Hulbert, 1987; Hulbert and Else, 1990). It can therefore be concluded that in vertebrates sodium influx, potassium efflux and sodium pump activity are related to tissue metabolic rate.

In the mammal-reptile comparison, differences in metabolic activity, membrane permeability to sodium and potassium and sodium pump activity have been found to be paralleled by changes in membrane fatty acid composition. Animals with a higher metabolic activity had more
polyunsaturated membranes (Hulbert and Else, 1989). The present study with
different-sized mammals also found that tissues with higher metabolic activity
or coming from animals with higher basal metabolic rate were usually more
polyunsaturated. An important exception to this was for brain, which showed a
high constancy in composition in all the mammals investigated although Krebs
(1950) found a decrease in metabolic rate of brain slices from larger mammals
(the allometric decrease reported by Krebs was small, but as important as
what he had found in kidney cortex. See Figure 1.2). The striated muscles
skeletal muscle and heart showed the strongest allometric decrease in
polyunsaturation. Heart is known to vary in metabolic activity according to
body size. The pulse rate of a mouse is around 650 beats per minute, while that
of a horse is around 40 (Kleiber, 1961). It can be concluded from these findings
that the extent of membrane polyunsaturation is generally correlated to tissue
metabolic activity.

The reasons for the association between membrane polyunsaturation
and tissue metabolism remain to be elucidated and it is likely that the
explanation for this relationship is complex. Porter and Brand (1993) have
found that the proton permeability of mitochondrial inner membranes is
related to body size in that the larger mammals display lower membrane
permeability to protons. The mitochondrial membranes from larger mammals
have also been found to be less polyunsaturated (Brand and Hulbert, pers.
comm.). In the present study, there was a significant correlation between the
relative amounts of 22:5Ω3 and 22:6Ω3 and potassium efflux rates in liver.
These findings suggest that the presence and proportions of highly
polyunsaturated fatty acids in membranes may play a role in membrane
permeability.
Another potential role of membrane composition is to ensure optimal functioning of membrane enzymes. In both liver and kidney cortex, slices from tissues with more polyunsaturated membranes displayed higher rates of potassium uptake. The differences were not statistically significant but about half of the variation of potassium uptake could be associated to the Unsaturation Index. Since potassium uptake rate was correlated to oxygen consumption rate and since both these parameters were correlated to body size, it is clear from this study that tissue metabolism, sodium pump activity and membrane polyunsaturation vary together in comparative systems such as different-sized mammals where the metabolic requirements differ.
Views and Future directions

A question that remains to be addressed in the investigation of the causes of the lower metabolism of larger mammals is whether different-sized mammals have similar cell size and cell number in their tissues. The present study has left two possibilities for the decrease in tissue metabolism in larger mammals: either the tissues from larger mammals have similar-sized cells, in which case the differences in tissue metabolism are due to lower cell metabolic rate, or tissues from larger mammals are made of less, larger cells which may individually display a similar metabolic rate in all mammals. If the differences in liver cell volume between a mouse and an elephant are 8-fold, as the doubling in linear dimensions reported for these mammals by Berrill (1955) suggest, the explanation of the decrease in mass-specific tissue metabolic rate in larger mammals will be shared by larger mammals having larger and metabolically slower cells, while the total mass-specific cell volume would only increase marginally in the tissues of larger mammals.

Indeed, it is possible that cell membrane fatty acid composition correlates more with cell-specific metabolic rate than with tissue metabolic rate. It could be that only small differences were observed in liver phospholipid UI in ruminants compared to rodents, although there was a considerable allometric decrease in mass-specific metabolic rate, because individually the liver cells of these mammals display similar metabolic rates but ruminants have larger liver cells.

Concerning the reasons for larger mammals possessing less polyunsaturated and shorter fatty acids in their tissue phospholipids, it is quite possible that the elongases and desaturases vary in activity or substrate affinity.
An investigation of the activity of these enzymes would bring insights on the level of control of membrane fatty acid composition. Membrane composition can be controlled from within the membrane itself by the activity of elongases and desaturases or by external modulators. Elongase activity also has a potential for controlling polyunsaturation since it was observed that only the longest fatty acids need become highly polyunsaturated.

Detailed analysis of the composition of subcellular membranes and phospholipid species in an allometric comparison will help to understand the level at which changes in membrane composition occur and may give further insights on the role of these changes.

If changes in plasma membrane composition are found in the tissues of different-sized mammals in the study above, membrane protein function and membrane permeability could be investigated. The importance of membrane composition for sodium pump activity could be examined by measuring the activity of sodium pumps from the tissues of small mammals in reconstituted liposomes made of phospholipids from the tissues of large mammals. This simple experiment would help to determine if the changes in membrane composition that accompany changes in metabolic activity partly occur for accommodating membrane enzyme function.

The dependence of some aspects of cell metabolism on the sodium gradients remains to be quantified. In future experiments, cells or slices can be gradually deprived of their sodium gradients, by using ouabain and monitoring intracellular sodium and potassium gradients, to measure the time-course of the inhibition of metabolic processes, such as protein synthesis, RNA synthesis or membrane phospholipid turnover.
Adolph (1949) pointed out that organisms can be described as systems of precise multiple interrelations. In this thesis I have on occasions expressed my views on the question of causality in the relationships reported between metabolic rate, membrane composition and sodium transport. My investigations have lead me to adopt the view that the causal factors may be outside these three parameters. In this view, basal metabolic rate, which differs between tissues, will be dictated by body size (with its physical implications such as heat loss), lifestyle and phylogenetic position of a species. Basal metabolic rate will be to some extent modulated by the organism by means of hormones and other messengers. Membrane composition will be adjusted by compromising between metabolic needs for permeability and membrane protein function and efficiency of the membrane as a barrier. Sodium transport will be proportional to metabolic activity. While the results presented in this thesis support this view, a causal factor cannot be inferred from them. Although it may be interesting to speculate about the identity of the causal factors, I wish that the emerging principle from this work will remain that metabolic activity, sodium transport and membrane fatty acid composition are interrelated parameters in mammals.
References


Brand Martin D., Couture Patrice and Hulbert A.J. (submitted). Liposomes from mammalian liver mitochondria are both more polyunsaturated and leakier to protons than those from reptiles.


Appendix I

Preliminary experiments with silicic acid chromatography columns for the determination of optimal column height and elution volume for maximal recovery of phospholipid and minimisation of contamination by neutral lipids

The determination of the height of 325 mesh silicic acid column required to achieve maximal recovery of phospholipids and minimal contamination by neutral lipids was performed using as sample a mixture of phospholipids and neutral lipids. Both fractions were kept for an assessment of the contamination by the other fraction.

To test for the loss of phospholipids in the neutral fraction, this fraction was assayed for its inorganic phosphorus content using the method of Mrsny et al. (1986). Very small amounts of phospholipids could be detected in the neutral lipid fractions. This loss of phospholipids was quantified as follows, using two aliquots of equal volumes from a sample of tissue total lipids in chloroform. One of the aliquots was purified using silicic acid column chromatography. The amount of total phosphorus (determined as in Appendix IV) was then compared in the control (total lipid) aliquot and in the phospholipid fraction from the purified aliquot. With a 100 mg sample, a 7 cm 325 mesh silicic acid column and 10 ml of both of chloroform and methanol for the elution of neutral lipids and phospholipids, respectively, a triplicate experiment yielded 97.4 ± 6.1% recovery of the phospholipids.
Similarly, it was important when setting the method to verify that no neutral lipids contaminated the phospholipid fraction. To this end, samples of phospholipid fractions after purification on silicic acid column as described above were dotted on glass plates pre-coated with silica gel 250 μm. Mixtures of pure triglycerides or phospholipids were used as standards. The plates were then left in the chromatography tanks (the solvent level was less than the height of the sample, 1 cm from the bottom) until the solvent line reached near the top of the plate. The solvent used was the lower phase of chloroform:methanol:acetic acid:water (85:15:10:20). The plates were then air-dried. The position of all lipids was determined by staining with iodine vapour (a few crystals of iodine in a sealed tank, for five minutes). After the lipids had been circled with a pencil, the plates were stained for phosphorus by spraying with Hanes-Isherwood reagent (1 g ammonium molybdate, 8 ml water, 3 ml concentrated HCl, 3 ml 70% perchloric acid and 85 ml acetone). This allowed to discriminate between neutral lipids (negative) and phospholipids (positive, blue). This experiment was carried in triplicate and no neutral lipids could be detected in the phospholipid fractions, using a 7 cm 325 mesh silicic acid column and 10 ml of both of chloroform and methanol for the elution of neutral lipids and phospholipids, respectively.
Appendix II

The saline solution

<table>
<thead>
<tr>
<th>Chemical</th>
<th>M.W.</th>
<th>Concentration</th>
<th>grams/litre</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCl</td>
<td>74.55</td>
<td>4.74 mM</td>
<td>0.354</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>136.09</td>
<td>1.19 mM</td>
<td>0.162</td>
</tr>
<tr>
<td>NaCl</td>
<td>58.44</td>
<td>120 mM</td>
<td>7.018</td>
</tr>
<tr>
<td>NaHCO$_3$</td>
<td>84.01</td>
<td>25 mM</td>
<td>2.100</td>
</tr>
<tr>
<td>MgSO$_4$·7H$_2$O</td>
<td>246.47</td>
<td>1.2 mM</td>
<td>0.296</td>
</tr>
<tr>
<td>Glucose</td>
<td>180.16</td>
<td>5 mM</td>
<td>0.901</td>
</tr>
<tr>
<td>CaCl$_2$·2H$_2$O</td>
<td>147.02</td>
<td>1.3 mM</td>
<td>(1)</td>
</tr>
</tbody>
</table>

(1) added as a concentrated (13 mM) solution.

To prevent precipitation of calcium and magnesium, once the other components have been dissolved in 900 ml of distilled water, 100 ml of 13 mM CaCl$_2$·2H$_2$O are added gradually.

The saline is equilibrated with carbogen for 60 minutes at 37°C and the pH adjusted to 7.4.
Considering the choice of a saline

One important property of a saline solution is its buffering capacity. External (serum) pH is maintained at around 7.4 in vertebrates, higher than the cytosolic pH which is around 6.9 for endotherms (White and Somero, 1982; Hochachka and Somero, 1984). Among the variety of buffers commonly used in physiological studies, I have opted for the NaHCO₃/KH₂PO₄ equilibrated with carbogen (95% oxygen and 5% carbon dioxide). This buffer can maintain a pH between 7.3 and 7.7 at 37°C with respiring cells, provided it is saturated with carbogen. In the experiments of this chapter, the initial pH was around 7.3-7.4 and would rise slowly throughout the incubation of slices, to reach 7.5-7.6 (measured after completion of manipulations) for the saline used for liver slices of all species and for mouse and rabbit kidney slices. The kidney slices of the other three species produced a slightly higher final pH of 7.7. This slight rise in pH is thought to be at least in part an artefact of cooling, as the pH was measured 2 to 3 minutes after the glass vials were removed from the heating block (White and Somero, 1982).

Commonly used saline solutions for physiological studies with tissue slices have an osmolarity between 310 and 350 mosmol/l (Elliott, 1955; Krebs, 1950; Else and Hulbert, 1987 among others). The Krebs-Ringer saline is modified from Krebs (1950) and has an osmolarity of 340.5 mosmol/l.

The main contributor to osmolarity is NaCl (120 mM or 240 mosmol/l). Under physiological conditions, serum also contains 5 to 6 mM of potassium (Lote, 1987 for example). As well, enzymatic processes require small amounts of calcium and magnesium. All these parameters are respected in the saline used in the experiments throughout this chapter.
Finally, although slices can remain viable for several hours by using their own endogenous substrates, the addition of substrates is necessary for long-term cell metabolism. Krebs (1950) identified pyruvate, glutamate, fumarate and glucose, in physiological concentrations of around 5 mM, as the metabolites required to run the energy-producing metabolic pathways of glycolysis and of the citric acid cycle. When compared with salines containing no substrate or only glucose, such enriched salines have been reported to give unusually high oxygen consumption rates (Elliot, 1955).

In a preliminary experiment, oxygen consumption of mouse liver slices was measured in triplicate for slices kept in substrate-free saline or in a saline enriched with 5 mM of each of pyruvate, glutamate, fumarate and glucose. There was a 6% increase in oxygen consumption when the slices were incubated in the presence of substrates, using wet weight to express the rates (13.1 vs. 13.8 μmol O₂/g wet-min.). This difference raised to 28% when using GDH (from 13.8 vs. 17.6 μmol O₂/g wet-min.) for the calculations (as described in Chapter 3).

I have chosen a saline containing glucose only (5 mM) as substrate since the choice of this saline allows a comparison with published data, where glucose-enriched saline is most commonly used. It can be estimated, by assuming a ratio of 6 oxygen molecules consumed for each glucose and that the cells respire only aerobically, that a 10 mg mouse kidney slice (the most metabolically active slices used) with an oxygen consumption rate as reported in Table 3.9 would take over 200 hours to completely deplete the 10 ml of saline of its glucose.
Beyond these reasons, a saline containing only glucose provides an excellent tool to estimate the ATP yield that can be generated from a measured oxygen consumption rate. Such calculations, involving assumptions such as those mentioned above, will allow an estimation of the contribution of the sodium pumps to the metabolism, in Section 3.4.5. One must bear in mind however that although both liver and kidney cortex cells have the capacity to derive their energy from glucose, this substrate is not their favorite. While kidney cortex slices prefer such substrates as acetoacetate, butyrate or oleate (Weidemann and Krebs, 1969), liver cells prefer keto acids derived from the metabolism of amino acids (Stryer, 1988).
Appendix III

Homogenization medium

This medium is used to homogenize whole tissue samples or slices for determining enzyme activities and/or to measure sodium and potassium concentrations. It is sodium and potassium free. It was modified from Couture and Guderley (1990).

<table>
<thead>
<tr>
<th>Chemical</th>
<th>M.W.</th>
<th>Concentration</th>
<th>grams/500 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imidazole</td>
<td>68.08</td>
<td>50 mM</td>
<td>1.702</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>203.3</td>
<td>2 mM</td>
<td>0.203</td>
</tr>
<tr>
<td>EDTA¹</td>
<td>292.2</td>
<td>5 mM</td>
<td>0.731</td>
</tr>
<tr>
<td>Triton X-100</td>
<td></td>
<td>0.1% v/v</td>
<td>0.5 ml</td>
</tr>
</tbody>
</table>

pH is adjusted at 7.5 with LiOH

Solution is kept for up to 1 month at 4°C

¹ ethylenediamine tetraacetic acid. It is sodium-free.
Appendix IV

Media for enzyme assays

A- Lactate Dehydrogenase (EC 1.1.1.27, LDH) medium

<table>
<thead>
<tr>
<th>Chemical</th>
<th>M.W.</th>
<th>Concentration</th>
<th>grams/50 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>K$_2$HPO$_4$</td>
<td>174.18</td>
<td>71.2 mM</td>
<td>0.6200</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>136.09</td>
<td>38.2 mM</td>
<td>0.2600</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>110.00</td>
<td>0.80 mM</td>
<td>0.0044</td>
</tr>
<tr>
<td>NADH$^2$</td>
<td>709.4</td>
<td>0.16 mM</td>
<td>0.0057</td>
</tr>
</tbody>
</table>

Adjust to pH 7.0 with HCl.

B- Glutamate Dehydrogenase (EC 1.4.1.3, GDH) medium

<table>
<thead>
<tr>
<th>Chemical</th>
<th>M.W.</th>
<th>Concentration</th>
<th>grams/50 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>121.1</td>
<td>100 mM</td>
<td>0.6055</td>
</tr>
<tr>
<td>α-ketoglutarate</td>
<td>146.1</td>
<td>6 mM</td>
<td>0.0438</td>
</tr>
<tr>
<td>(NH$_4$)$_2$SO$_4$</td>
<td>132.1</td>
<td>30 mM</td>
<td>0.1982</td>
</tr>
<tr>
<td>ADP$^3$</td>
<td>427.2</td>
<td>250 μM</td>
<td>0.0107</td>
</tr>
<tr>
<td>NADH$^1$</td>
<td>709.4</td>
<td>0.16 mM</td>
<td>0.0057</td>
</tr>
</tbody>
</table>

Adjust to pH 8.0 with acetic acid.

---

$^1$ β-Nicotinamide adenine dinucleotide, reduced form. Sodium salt.

$^3$ Adenosine diphosphate