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Studies in the evolution of endothermy: mammals from reptiles

Paul Lewis Else

University of Wollongong

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STUDIES IN THE EVOLUTION OF ENDOHERMY:
MAMMALS FROM REPTILES

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by PAUL LEWIS ELSE, B.Sc (Hons.I)
This thesis is submitted in accordance with the regulations of the University of Wollongong in partial fulfilment of the requirements for the degree of Doctor of Philosophy. The work described in this thesis has not been submitted at any university or similar institution except where specifically indicated.
Abstract

In a comparison of a single mammalian (*R. norvegicus*) and reptilian species (*A. vitticeps*) of the same body weight and "preferred" body temperature, the metabolic capacity for energy production was assessed in liver, heart, brain, kidney, lung and skeletal muscle tissues by two methods: measurement of mitochondrial enzyme activity (cytochrome oxidase) and measurement of mitochondrial volume and membrane surface area densities. Both methods showed the mammal to possess an average 2-3 fold greater metabolic capacity per gram of tissue. When the larger relative tissue sizes of the mammal were included into the comparison the differences were increased to approximately 5 fold.

When the mitochondrial enzyme activities of the six mammalian and reptilian tissues were expressed per gram of tissue protein the differences were reduced. This was because all the reptilian tissues measured had significantly less protein per gram of tissue than the mammalian tissues. Isolated mitochondria from the same six tissues showed the mammalian mitochondria in general to have more protein and less lipid than the reptilian mitochondria and similar oxygen consumptions (measured using cytochrome oxidase) per mg of protein for liver, brain and kidney mitochondria. Isolated mammalian lung, heart and skeletal muscle mitochondria however, consumed twice the amount of oxygen consumed by the same reptilian mitochondria per gram of mitochondrial protein.

To assess the use of energy by the same mammalian and reptilian species the "in vitro" oxygen consumption of liver, kidney and brain
tissues were measured. "Sodium dependent" metabolism (i.e., the amount of energy used on pumping Na\(^+\) out and K\(^+\) into the cells of tissues) was also measured in these tissues. The mammal had "in vitro" tissue metabolisms generally 4 times greater and spent 4 times more energy on Na\(^+\) pumping than did the same reptilian tissues. In the liver particularly this difference was 5 fold.

To examine the reasons for this difference in "sodium dependent" metabolism between the mammal and reptile, the liver cell membrane Na\(^+\) permeabilities of the mammal and reptile plus an amphibian, _B. marinus_ were examined using primary monolayer liver cell culture. The mammalian liver cells were found to be 5 times more permeable to Na\(^+\) than either the reptilian or amphibian liver cells. This increased permeability of the endothermic liver cells probably accounts for the large differences in the amounts of energy used by the mammal and ectotherms on Na\(^+\) and K\(^+\) pumping. This inefficiency in terms of relative energy use of the mammalian cells compared to the ectothermic cells is postulated as having evolved as a means of increasing heat production.

The effects of body size and phylogeny on metabolic capacities were examined by comparing the mitochondrial capacities of 6 mammalian and 4 reptilian species representing 100 fold body weight ranges. The mammals examined included 3 eutherian, 2 marsupial and a monotreme and the reptiles 2 saurian, 1 crocodilian and 1 testudine species. The tissues examined were the same six tissues previously mentioned. Allometric equations were derived for tissue weights, mitochondrial
volume densities, internal mitochondrial membrane surface area densities, tissue mitochondrial membrane surface areas both per gram and per total tissue and summated tissue mitochondrial membrane surface areas.

For the mammals and reptiles studied a 100% increase in body size results in average increases of 68% in internal organ size and 107% in skeletal muscle mass. Similarly, total organ mitochondrial membrane surface areas increase in mammals and reptiles by an average 54% and for skeletal muscle by an average 96%. These values are similar to increases in standard (54 and 71%) and maximum (73 and 77%) organismal metabolism values found by other authors for mammals and reptiles respectively.

Although the allometric exponents or rates of change with increasing body size of the mitochondrial parameters in mammals and reptiles are statistically the same in general the total amount of mitochondrial membrane surface area in the mammalian tissues are four times greater than found in the reptilian tissues. These differences were not the result of any single "quantum" factor but are the result of the mammals having relatively larger tissues with a greater proportion of their volume occupied by mitochondria. Mitochondrial volume density from this present study would appear to be the major factor involved in changing weight specific metabolism of tissues both as a result of changes in body size and in the evolution of endothermy in mammals from reptiles.
Acknowledgements

I would like to thank many people for helping me with various aspects of this thesis but I will thank them individually in the various publications. In this acknowledgement I would just like to thank separately the woman I love, Claudia Teresa and my supervisor Tony Hulbert for his complete aid and both for their support. As a group I would like to thank the Biology Department of Wollongong University.
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CHAPTER 1

LITERATURE REVIEW:

THE EVOLUTION OF MAMMALS FROM REPTILES
1.1 The Metabolic Differences Between Mammals and Reptiles

1.1.1 Energy metabolism

Two sources of heat were associated very early with biological systems. One was the sun, the second was that produced within living organisms (Fernel, 1637). Internal heat is produced by the "combustion" of oxygen with organic substances (Lavoisier and de La Place, 1780), and this is a product of metabolism. Metabolism is the overall use of chemical energy by biological organisms and the production of heat is called thermogenesis (Hochachka, 1974, and Himms-Hagen, 1976). Oxygen consumption can therefore be used to measure metabolism and heat production (Kleiber, 1961). As animals become larger the amount of oxygen they consume is greater but this increase in oxygen consumption is not in direct proportion to their increased size (i.e., is not proportional to Weight\(^{1.0}\); Hemmingsen, 1960).

1.1.2 Allometry

Allometry is the study of size and its consequences (Gould, 1966 and Calder, 1981). A consequence of increasing body size on metabolism is to decrease the amount of oxygen consumed per unit of body weight (Brody, 1945 and Hemmingsen, 1960). The empirical relationship is generally expressed as:

\[ M = aW^{0.75} \]

where M is the metabolic rate or the rate at which oxygen is consumed, W is the body weight and "a" is a constant the value of which is
different for resting (standard) and active animals and for different phylogenetic groups (and depends on the units of M and W). This equation can alternatively be expressed as weight specific metabolism where:

\[ \frac{M}{W} = aW^{-0.25} \]

The reason for the 0.75 slope value or body mass exponent is unclear (Kleiber, 1961). The surface law (Sarrus and Rameaux, 1839) states that heat loss, and thus heat production or oxygen consumption should be proportional to the 0.66 mass exponent (surface area \( \text{L}^2/\text{volume L}^3 \)). Intraspecies relationships between metabolism and body weight (Thonney et al., 1976 and Heusner, 1982) show mass exponents may vary widely (0.4-1.00), although this may reflect other factors (i.e., developmental metabolic rates and fat stores).

1.1.3 Mammalian and reptilian metabolism

The standard (resting animals in a postabsorptive state in a thermoneutral zone) and maximum oxygen consumption of mammals is 5-15 fold greater than the corresponding values for reptiles, even when measured at the same body temperatures (Dawson and Hulbert, 1970; Bennett and Dawson, 1976; Bennett, 1978; and Taylor et al., 1980). This greater oxygen consumption of the mammals is the source of their endothermy. Endotherms maintain high body temperatures by internal heat production, ectotherms depend on external heat sources, primarily solar radiation (Schmidt-Nielsen, 1979). Mammals are endotherms and reptiles are ectotherms, the consequences of which are reflected at many different levels of biological organization.
1.1.4 Ecological and organismal levels

The aerobic scope (Fry, 1947), the difference between maximal and oxygen standard consumption, of reptiles is far lower than for mammals. In reptiles aerobic scope is temperature dependent and normally maximal at or near preferred body temperatures (Bartholomew and Tucker, 1963; Templeton, 1970; Bennett and Dawson, 1976 and Bennett, 1978). The reptiles therefore have to survive on less energy derived from aerobic metabolism than mammals. This must place different relative costs on some physiological processes.

The maximal observed growth rates of mammals are at least an order of magnitude greater than the maximum growth rates of reptiles (Case, 1978(a)). The growth rates of reptiles may also be temperature dependent (Hulbert and Else, 1981). In contrast to the maximal growth rates of mammals and reptiles the efficiency of energy conversion into new biomass is as much as an order of magnitude less in mammals compared to reptiles (Golley, 1968 and Gans and Pough, 1982). The field metabolic rates of reptiles are 20-25 fold lower than for similar sized mammals (Bennett and Nagy, 1977 and Nagy, 1982). These differences are further increased to 25-50 fold when annual field metabolic rates are considered due to the inactive months experienced by some reptilian species (Nagy, 1982).

1.1.5 Organ sizes, tissue metabolisms and physiological rates

Organismal metabolism is presumably a product of the metabolisms of the tissues. In a comparison of a single mammalian and reptilian species of the same body size the major internal organs, liver,
kidney and brain, of the mammal were all larger (Else and Hulbert, 1981) and consumed more oxygen per gram (Hulbert and Else, 1981). The heart weight of mammals is greater than in reptiles (Holt et al., 1968; Prothero, 1979 and Else and Hulbert, 1983) and other ectotherms (Poupa et al., 1981). The cardiac output (Altman and Dittmer, 1974), systolic pressure (Altman and Dittmer, 1974 and White, 1976) and oxygen capacity of the blood (Brody, 1945 and Pough, 1980) are all higher in mammals compared to reptiles of the same body size.

The lower oxygen delivery capacities of the reptiles are further lowered by decreased capillary densities but are offset to some extent by the low oxygen affinity of the reptilian blood and a tolerance to very low venous oxygen content allowing increased oxygen delivery to the tissues (Pough, 1980). The pulmonary surface area and lung weight in both mammals and reptiles are proportional to $W^{0.75}$ and $W^{1.00}$ respectively (Stahl, 1967 and Tenney and Tenney, 1970) but the pulmonary oxygen diffusing capacity in a reptile is only 20% that in a similar sized mammal (Glass and Johansen, 1981).

Similar sized mammals and reptiles show 2-13 fold differences in the "in vitro" oxygen consumption of their tissues even when measured at similar body temperatures (32°-37°C, Martin and Fuhrman, 1955; Wilhoft, 1966; Ismail-Beigi and Edelman, 1971; Turner and Tipton, 1972; Wong et al., 1975 and Hulbert and Else, 1981). The order of this difference is similar to that found at the organismal level.

The rate limiting metabolic factor within reptiles and mammals has been sought at the suborganismal level. The pulmonary oxygen
diffusing capacity ($D_{\text{L}O_2}$), the conductance of $O_2$ flow from air to blood in the lung has been closely associated with maximal oxygen consumption in mammals and reptiles (Gehr et al., 1981 and Mitchell et al., 1981). However, it does not appear to be limiting in lizards (Mitchell et al., 1981) while surface area and capillary length (related to $D_{\text{L}O_2}$) are not limiting in anurans (Hillman and Withers, 1979). In mammals since $D_{\text{L}O_2}$ scales with an allometric exponent of 0.99 and maximum oxygen consumption with an exponent of 0.79 (Gehr et al., 1981). If $D_{\text{L}O_2}$ is limiting as suggested (Lechner, 1978), larger mammals require a larger pulmonary diffusing capacity. Other limiting factors may involve cardiac output (Hillman, 1976), rate of blood flow (Coulson and Herbert, 1981), blood energy substrate levels (Dupre and Farrar, 1982) and alveolar-blood oxygen gradients (Taylor, 1983).

1.1.6 Mitochondrial and enzyme capacities

Tissue oxygen consuming capacities are presumably related to mitochondrial capacities. In a comparison of a single mammalian and reptilian species of the same body size and preferred body temperature (Else and Hulbert, 1981) the percentage of cell volume occupied by mitochondria, the cristae and inner mitochondrial membrane densities were all higher in the mammalian tissues (liver, heart, kidney and brain). Differences in brain mitochondrial numbers, estimated from microscope counts after separation by differential centrifugation, between different vertebrate groups had previously been noted by Wahbe et al. (1961). Homeotherms (rat and chicken) were found to have 50% more mitochondria per gram of brain than poikilotherms (turtle, frog
and catfish). Smith (1955) using similar techniques for mammalian livers has shown that the number of mitochondria increases with the 0.77 exponent of body weight. More recently Mathieu et al. (1981) analyzed the volume densities of mitochondria from four different mammalian skeletal muscles and found individual muscle mitochondrial volume densities scale with weight exponents of 0.87 to 1.03 and with weight-specific exponents between -0.06 and -0.16.

At the molecular (or enzyme) level total body mitochondrial cytochrome c content of mammalian species has been shown to give a body weight exponent of 0.7 (Drabkin, 1950). Weight specific, mitochondrial enzyme activities of mammalian skeletal muscle have shown exponents ranging from -0.07 to -0.21 (Emmett and Hochachka, 1981). An intraspecies comparison of citrate synthase activities of fish skeletal muscle has shown similar weight specific exponents ranging from -0.05 to -0.23 (Somero and Childress, 1980). These exponents are similar to the exponent for organismal weight specific (M/W) metabolism -0.25.

Isolated liver mitochondria from homeotherms and poikilotherms have been shown to consume similar amounts of oxygen at the same temperature, per mg of mitochondrial protein (Cassuto, 1971). The mammals and ectotherms reflect the differences in their organismal oxygen consumptions in the relative levels of their mitochondrial enzyme activities. Bennett (1972) has shown mitochondrial enzyme activities in the liver and skeletal muscle of a single mammalian species to be five fold greater than activities measured for several
similar sized reptilian species, at the same temperature. Else and Hulbert (1981), have shown similar differences to those found by Bennett in the activity of the mitochondrial enzyme, cytochrome oxidase, in liver, kidney, heart and brain tissues between a single mammalian and reptilian species of the same body weight and measured at the same temperature.

1.1.7 Thyroid hormone effects

Most of the work available on "in vitro" tissue metabolism in mammals (Ismail-Beigi and Edelman, 1971 and Asano et al., 1976), reptiles (Wong et al., 1975) and anurans (Packard and Packard, 1973 and Packard, 1976) has been concerned with the effects of thyroid hormones. In mammals hyperthyroid conditions produce an increase in metabolic rate, a calorigenic effect, whereas hypothyroid conditions reduce metabolism below the normal resting level of the euthyroid condition (Ismail-Beigi and Edelman, 1971). The augmented heat production elicited by thyroid hormones has been used as a possible reason to partially explain the increase in heat production from the ectothermic to endothermic state (Edelman, 1976). The reduced thyroid levels in reptiles compared to mammals (Hulbert, 1984) and the elevated metabolism of reptilian tissues in mild hyperthyroidism (Thapliyal, 1980) has produced the associations between thyroid effects, thermogenesis and endothermy. In mammals thyroid thermogenesis is associated to varying degrees, depending upon the method and tissue used (Edelman and Ismail-Beigi, 1974; Asano et al., 1976; Folke and Sestoft, 1977 and Ismail-Beigi et al., 1979) with increased Na⁺ pump activity (Lo et al., 1976 and Phillipson and
Edelman, 1977). The increased pump activity slightly increases the Na⁺ and K⁺ gradients across the cell membranes (Ismail-Beigi and Edelman, 1973 and Ismail-Beigi, 1977) against an increased Na⁺ and K⁺ membrane permeability found in skeletal muscle (Asano, 1977). In mammals thyroid hormones have been shown to increase mitochondrial numbers and cristae surface area densities (Reith, 1973; Reith et al., 1973 (a), (b); Jakovcic et al., 1978 and Wooten and Cascarano, 1980), mitochondrial phospholipid synthesis (Nelson and Cornatzer, 1965), mitochondrial ADP uptake and presumably ATP synthesis (Babior et al., 1973) and mitochondrial protein, RNA and DNA levels all increase. In reptiles very little is known of the effects of thyroid hormones apart from evidence to suggest the presence of an increased and decreased calorigenesis with hyperthyroid and hypothyroid states respectively (Wilhoft, 1966 and Turner and Tipton, 1972). However there is some evidence contrary to this in one snake species (Wong et al., 1975). Also the thyroid gland can be turned "on" and "off" by controlling the environmental temperature of reptiles (Maher, 1965).

1.1.8 Anaerobic metabolism

Anaerobiosis is the primary energy producing pathway in skeletal muscle during the first few minutes of maximal activity in mammals and reptiles and unlike aerobic capacities, the anaerobic capacities in mammals and reptiles are similar (Bennett, 1978 and Ruben and Battalia, 1979). For example, in a mammalian and a fish species the glycolytic enzyme content of skeletal muscles are similar per gram of tissue protein (Avelar et al., 1978). The advantage of anaerobic metabolism is it can be mobilized rapidly, possibly due to the
simplicity of the reaction and its localization in the cytosol, for this reason anaerobiosis is used in burst and maximal activities (Moberly, 1968; Bennett and Dawson, 1971; Gatten, 1974; Ruben, 1976; Ruben and Battalia, 1979 and Bennett et al., 1981). The disadvantages of anaerobic metabolism are its inefficiency, producing only 2 ATP compared to 36 ATP produced in aerobic metabolism per molecule of glucose (Lehninger, 1975), and the detrimental effect of increasing concentrations of the major byproduct, lactate, on the tissues (Bennett, 1978 and Ruben and Bennett, 1981). Possibly for these reasons in mammals lactate is either resynthesised to glucose or glycogen or consumed as substrate by the heart (Banks et al., 1976).

Anaerobic enzymes unlike aerobic enzymes from mammals and fish per unit of body weight scale positively with increasing body size (Somero and Childress, 1980 and Emmett and Hochachka, 1981). The larger the animal the greater the anaerobic potential of its skeletal muscle. Anaerobic metabolism also differs from aerobic metabolism in mammals and reptiles by being virtually temperature independent (Bennett, 1978). This temperature insensitivity of anaerobic metabolism may be of paramount importance in some reptilian species to evade predation when they are not at their preferred temperature levels (Pough, 1983).

1.1.9 The effects of body temperature

In reptiles anaerobic metabolism is virtually temperature independent with Q10's of ≤ 1.3 (Bennett, 1978) and aerobic metabolism is temperature dependent with Q10's normally between 2-3 (Bennett and
Dawson, 1976). Q_{10} is the increase in a rate caused by a 10°C increase in temperature (Schmidt-Nielsen, 1979). Burst speed and distance running capacities in lizards show some thermal independence with Q_{10} ≤ 1.5 (Bennett, 1980(a)). The minimum cost of transport, attained at a maximal sustainable speed for a lizard species has also been shown to be temperature independent (John-Alder and Bennett, 1981) while lactate removal after activity has been shown to exhibit a temperature dependence (Gleeson, 1980).

Reptiles will increase their oxygen consumption with increasing body temperature up to preferred levels (exceptions noted are varanid lizards, Bartholomew and Tucker, 1964 and some turtle species, Gatten, 1974). Beyond this point Q_{10}'s are normally reduced, plateauing and suppression of oxygen consumption may occur (Dawson, 1960 and Dawson and Templeton, 1966). Heart rates (Dawson, 1967; Templeton, 1970; Greenwald, 1971 and Wilson, 1974), blood oxygen affinities (Pough, 1969), assimilatory efficiencies (Ruppert, 1980 and Huey, 1982) and thyroid function (Maher, 1965) all show increases with increasing temperature. Some further effects of temperature on reptile physiology are reported by Dawson (1975).

In mammals the effects of temperature on physiological processes are sometimes masked by the maintenance of homeothermy. Birds and mammals are both homeotherms and maintain high and fairly constant body temperatures, independent of the environmental temperatures. Lower vertebrates are poikilotherms, poikilotherms have body temperatures dependent on the environmental temperatures (Gunn,
In the "primitive" mammals the effects of changing body temperature become slightly more visible with a $Q_{10}$ for metabolism of 2.1 in echidnas (Schmidt-Nielsen et al., 1966). Metabolism during mammalian hibernation is reduced but is proportional to body temperature with a $Q_{10}$ of about 2.5 (Bligh, 1973).

1.1.10 Thermoregulation

Thermoregulation in reptiles is mainly behavioural and in mammals physiological but there are also many similarities. One of the responses to cold in reptiles and mammals is to reduce heat loss by controlling the blood flow to the peripheral tissues (Bartholomew and Tucker, 1963 and Bligh, 1973). The effects of heat stress in reptiles are normally behaviourally avoided or they can lose heat by panting (Bogert, 1950). One of the major mechanisms for heat loss in mammals is also panting; other heat loss mechanisms used by mammals but not found in reptiles include wallowing, sweating and licking (Bligh, 1973). This additional repertoire of the mammals has probably evolved because unlike reptiles mammals also have to lose the large amounts of heat generated from their own metabolism.

Mammals also respond to cold by increasing thermogenesis using microtremors or shivering (Jansky, 1965) and nonshivering thermogenesis (N.S.T.) in some mammals (Hochachka, 1974 and Cannon et al., 1981). Different combinations of responses to cold and heat are used by the different mammalian groups (Hulbert, 1980). Shivering evokes thermogenesis through mechanical activity from myosin ATPase and ion pumping (Himms-Hagen, 1976). In mammals shivering can increase heat
production several fold above resting levels (Jansky, 1965). Brooding pythons have also been shown to utilize muscular contractions to raise the temperature of body and clutch 7°C above ambient (Hutchinson et al., 1966). Nonshivering thermogenesis in mammals is generally found in hibernators, small mammals and cold acclimated mammals. The thermogenic mechanism for brown fat nonshivering thermogenesis is uncertain but possibilities include mitochondrial uncoupling (Hochachka, 1974) such as a short circuiting of the proton current by a protein (Thermogenin) in brown adipose tissue mitochondria (Cannon et al., 1981). A further mechanism for augmented heat production associated with N.S.T. is increased sodium pump activity (Guernsey and Stevens, 1977).

Thermoregulation in reptiles was first recognized by Sergeyev (1939) and Cowles and Bogert (1944). Since which time numerous subtleties have been found concerning reptilian thermoregulation (for reviews see Templeton, 1970 and Huey, 1982). Examples include differential heating and cooling rates (Claussen and Art, 1981), panting in response to heat stress, capable of threshold shift with acclimation (Heatwole et al., 1975) and behavioural thermoregulation as a circadian rhythm (Cowgell and Underwood, 1979). For a review of reptilian thermoregulation and its effects on reptilian physiology and ecology, see Huey (1982).

The role of the nervous system in temperature adaptation and regulation in mammals and reptiles is reviewed by Bligh (1966 and 1973), Satinoff (1978) and Prosser and Nelson (1981) and the evolution of thermoregulation by Bligh (1973) and Wittow (1973).
1.1.11 The advantages and disadvantages of being either a mammal or a reptile

One disadvantage of being a mammal compared to being a reptile is that mammals are limited in their minimum size. In reptiles 8% of lizard and 2-4% of snake and amphisbaenians species are smaller than 1g as adults. Nearly 80% of lizard species are less than 20g and in amphibians 20% of salamander and 17% of anuran species are less than 1g, an extreme not approached by any mammalian group (Pough, 1983). Mammals are limited in their minimum size by their metabolism and thermal environment (Tracy, 1977). A further disadvantage of endothermy is the substrate (or food) requirement compared to ectothermy. Mammals require far more food than similar sized reptiles and their secondary productivity is reduced compared to reptiles (Pough, 1983). However as a result of endothermy, mammals have the advantage of being more capable of procuring their increased food needs. A further demand of homeothermy is increased autonomic temperature regulating mechanisms and an insulative coat (Bligh, 1973). The obvious advantage offered by endothermy is the ability to stay active independent of environmental temperatures (within certain extremes). A further advantage is that endotherms can grow an order of magnitude faster than ectotherms (Case, 1978(a)) possibly as a result of their independence of environmental temperature and their greater energy intake. A further advantage of endothermy is that by increasing the aerobic scope over ectothermy by an order of magnitude the sustainable activity range of mammals is increased (Bennett and Ruben, 1979).
What is the advantage of maintaining high body temperatures? One possibility is that enzymes work better at 37°C than at lower temperatures. For example in fish mitochondrial enzymes from liver and heart continue to increase in activity, past organismal preferred levels, up to 35-45°C (Irving and Watson, 1976) and the Na⁺K⁺-ATPase activity of *Bufo marinus* skin is maximally active at 37°C, several degrees above the preferred organismal maximal temperature (Park and Hong, 1976). This may also explain the concomitant increase in oxygen consumption with body temperature above the preferred body temperatures in most lizard and snake species.
1.2 Hypotheses Concerning the Evolution of Mammals from Reptiles

The evolution of endothermy and the evolution of homeothermy in mammals are the subject of several hypotheses. The major differences being the selective advantages offered by both endothermy and homeothermy. There is further disagreement as to whether endothermy preceeded homeothermy or vice versa or if they occurred concurrently in mammals.

The obvious consequence of "warm-bloodedness", as discussed by Rodbard (1953) is it affords the reduction of the diurnal temperature cycle. This may have allowed early "mammals" the selective advantage of occupying habitats regardless of their temporarily shifting temperature profiles and possibly give these animals some advantage over "cold-blooded" reptiles. This basic idea is incorporated in the hypothesis proposed by Crompton, Taylor and Jagger (1978) for the evolution of homeothermy in mammals. Crompton et al. propose that mammalian homeothermy was acquired in two steps, the first step involved the invasion of temporally wide vacant nocturnal niches by early mammals. These mammals could maintain constant body temperatures around 25-30°C without any concomitant increase to high resting metabolic rates typical of most extant mammals. At various times these nocturnal mammals acquired higher metabolic rates, enabling them to regulate their body temperatures at higher levels and to reinvade diurnal niches. Crompton et al. (1978) use activity patterns, behaviour and body temperatures from three extant "primitive" insectivore species, a monotreme and marsupial species to
show typically mammalian type energetics in all except the insectivores which they claim have remained in the nocturnal niches and retained reptilian-type energetics.

Dawson and Hulbert (1970) who developed the experimental relationships used by Crompton et al. (1978) to distinguish between mammalian and reptilian energetics have been critical of the experimental interpretations. Dawson et al. (1979) notes that the temperatures at which the metabolic responses to exercise were normalized were in some cases considerably above those reported under resting conditions (Dawson, 1973) while others were not. Dawson et al. (1979) suggest that the temperature variations between the mammals may reflect differences in thermoregulatory capabilities, not metabolic capacities. Dawson et al. (1979) also point to the unsupported assumption by Crompton et al. (1978) that the eutherians from which the exercise data were obtained (Taylor et al., 1970) had body temperatures during exercise of only 38°C, neglecting any increase in body temperature due to thermal storage during exercise. Hulbert (1980) also comments on the implications of Crompton et al.'s hypothesis. Hulbert points out that if the insectivores have retained reptilian-type energetics then their ancestors and the ancestors of monotremes and marsupials also had reptilian-type energetics. This means monotremes, marsupials and advanced eutherians have independently evolved high metabolic levels to the same mammalian level. Hulbert suggests that a far more logical interpretation is the regaining rather than retaining of reptilian-type energetics.

Subsequently the authors of the original paper (Crompton et al., 1978)
have also come to the same conclusion (Oron et al., 1981). That the low metabolic rate observed in these insectivores is a specialization for conserving energy rather than a retention of primitive metabolic characteristics during the course of evolution.

One of the implications of Crompton's et al. hypothesis is the need to improve alternative senses to compensate for the loss of visual, sensory information. Jerison (1971) has argued that the increase in relative brain size of the early mammals seems due chiefly to improved senses of smell and hearing, compatible with the invasion of nocturnal niches by early mammals. Nyberg (1971) commenting on Jerison's hypothesis also confronts the problem of why the brain/body weight ratio of reptiles has not increased during evolution. Nyberg speculates that the necessity of operating at different temperatures represents an informational burden which inhibited the development of larger brains in poikilotherms. It is interesting that both Jerison and Nyberg talk of capacities although Jerison's emphasis is on those of the nervous systems and Nyberg's on the genetic systems.

The informational capacity of poikilotherms that is necessary because of temperature variation is unknown. However, it is known that proteins may exhibit distinct functional temperature optima and sometimes the information needed to operate at different temperatures is preserved in multiple alleles (Nyberg, 1971). The reason mammals have evolved to regulate high stable body temperatures may well be related to enzyme specialization for the maintenance of high activity rates.
As proposed by Heinrich (1977) specialization of enzymes to operate at specific temperatures (either high or low) improves the potential to promote high rates of substrate turnover while still remaining subject to the necessary mechanisms of metabolic control. Heinrich (1977) suggests that the high mammalian set point evolved from inabilities to dissipate heat produced from high activity rates. To sustain these activities the enzymes and macromolecules needed to function at higher temperatures. Biochemical restructuring for activity at high tissue temperatures has evolved, in part, to extend the ability to be maximally active beyond the short time otherwise required to overheat. Maintenance of high body temperatures between bouts of activity allows the animal to be "instantly" ready for activity without the need for prewarming.

The selective advantage offered by high and sustainable aerobic activity is the basis for the hypothesis proposed by Bennett and Ruben (1979) for the evolution of endothermy. These advantages are sustained greater levels of pursuit or flight, gathering food or avoiding becoming food, superior territorial defence or invasion, more success in courtship or mating. For these advantages endothermic mammals maintain high and constant body temperatures increasing their energetic costs. As stated by Nyberg (1971) the trait must not require too many resources relative to its value. Bennett and Ruben consider the advantages of endothermy to outweigh the increased energetic costs, particularly since enhanced capacities give their possessor the ability to increase energy intake to meet new energy demands. A criticism used by Bennett and Ruben (1979) of alternative
hypothesis is the assumption that the endothermic condition is the end product of selection for a high and stable body temperature.

An alternative hypothesis attributes the evolution of endothermy in mammals to a decrease in the earth's surface temperature by 15°C or more (Hammel, 1976). This hypothesis assumes early "mammals" to be preadapted with insulation (present to protect early "mammals" from U.V. radiation), evaporative cooling, external testes and concentrated urine as a result of living in hot, arid environments, Hammel proposes these "mammals" retained bradymetabolism (or the reptilian rate of heat production), the increase in metabolic heat production (tachymetabolism) occurring as a result of a global decrease in temperature. These small preadapted early mammals which possessed the abilities to increase heat production with homeothermic control survived. The smaller reptiles that retained thermal lability survived this cooling trend late in the Tertiary (Hammel, 1976). One problem (other than those proposed generally by Bennett and Ruben, 1979) associated with this hypothesis is its dependence upon large changes in the temperature of the earth's surface. Also no reason is given for the large differences in the relative brain size between reptiles and mammals.

An interesting mechanism suggested by Hammel for the evolution of increased heat production in mammals is an increase in the Na⁺ permeability of the cell membranes. Sodium transport and the transition from poikilothermy to homeothermy and the possible role of thyroid hormones has also been linked by Edelman (1976). Prior to
this, Stevens (1973), has related functionally and phylogenetically
the Na+ pump, thyroxine and thermoregulatory responses to cold in an
attempt to account for the evolution of increased heat production in
mammals. These ideas are based on the large contribution of the Na+
pump to the resting metabolism of mammals (Ismail-Beigi and Edelman,
1971).

A further hypothesis concerned with the question of the evolution
of endothermy in the phylogeny of mammals is that of McNab (1978).
McNab suggests that reptiles ancestral to mammals developed a degree
of inertial homeothermy, a consequence of their large body mass
(30-100kg). A fur coat was added, increasing their degree of
homeothermy. Once inertial homeothermy was obtained these animals had
a "commitment" to homeothermy. Decreasing thermal conductance (a
consequence of large body size in reptiles) and increasing weight
specific rates of metabolism as body size decreases explains the
transition from reptiles to the first mammals. Why did these changes
occur? The decrease in body size is explained in a shift in food
habits and possible competition between early mammal-like-reptile
groups. The increase in weight specific metabolism is postulated as a
result of the invasion of nocturnal niches and a "commitment" to
homeothermy (McNab, 1978). But exactly the same result could occur in
the reverse, by retaining reptilian weight specific metabolism and
increasing body size eventually the animal gains mammalian metabolic
levels and decreases its thermal conductance. These animals could
then decrease in size and increase weight specific metabolism. Also a
"commitment" to homeothermy as pointed out by Hulbert (1980) is not an
explanation and teleological in its reasoning.
Hulbert (1980) suggests that there was an early development of the endothermic metabolic capacity in the history of the mammals, then came responses to nocturnal cold and later responses to heat. Hulbert considers the selective advantage of endothermy would not only be thermoregulatory but also the increase in the level of activity that such a large increase in metabolic capacity would allow.

Two further interesting views are those of Hopson (1973) and Case (1978(b)), that endothermy is directly related to a decrease in the proportion of resources that can be allocated to reproduction. That the evolution of endothermy, particularly in small endotherms, would place a selective pressure upon reproduction. This would favour the emergence of altricial (minimal energy demand) conditions and subsequently the need for parental care to ensure the survival of the immature young.

A thermodynamic interpretation for the almost universal constant of 40°C of homeotherms is the content of a paper by Calloway (1976) which provides additional reasons for endothermy other than those discussed so far. Calloway suggests that the 40°C temperature for homeothermic animals is dictated by the thermodynamic properties of water. Water constitutes 80-92% of the cellular content and dictates the nature and behaviour of all cellular substances. The fact that water is solid at 0°C and gas at 100°C suggests to Calloway that homeothermy might be expected at 50°C. This half way point is the general thermodynamic state at which the least thermodynamic stress is placed on the aqueous system. However, Calloway shows that the
relationship between the thermodynamic states of water and temperature are logarithmic. The logarithmic mean of 100°C divided by the natural base logarithm e gives 36.9°C. This explanation seems to suggest that all organisms composed of water should "prefer" to be at 37°C.
1.3 The Evolutionary History of Mammals from Reptiles

1.3.1 Introduction

Mammals evolved from a radiation of the reptiles during the Mesozoic. Fossils indicate that the ancestry of the mammals separated from the line that later gave rise to the living reptiles very soon after the origin of the class Reptilia in the Pennsylvanian or upper Carboniferous period (Kemp, 1982). This has resulted in extant reptiles having some characteristic features which may never have been present in the early reptilian ancestors of the mammals.

1.3.2 Stem reptiles

The "stem reptiles" from which the extant reptilian, mammalian and avian classes radiated constitute the suborder Captorhinomorpha (Order Cotylosauria). The extant reptiles share a common ancestry from these primitive forms of the early Pennsylvanian. Romer (1967) and Carroll (1975) consider the Squamates and Rhynchocephalians (Lepidosauria) to have arisen from the Eosuchians, descendant from the Captorhinomorpha in the early Permian. Benton (1982) also considers the Archosaurians (Dinosaurs and Crocodiles) share a common Eosuchian ancestry and not a Thecodontian one as suggested by Romer (1967). The snakes and lizards separated from one another in the middle Jurassic and the crocodiles separated from other Mesosaurs in the late Jurassic. The Sphenodon which is little advanced over the Permian reptiles became isolated in the early Triassic. The turtles and tortoises (Chelonia), the ancestry of which is subject to considerable speculation, have
been separated from the other living reptiles since at least the early Permian (Carroll, 1975; see Figure 1.1).

The adaptive radiation of the mammals (Figure 1.2) is followed within the subclass Synapsida. The Synapsids, now totally extinct, once dominated the terrestrial fauna from the late Pennsylvanian, throughout the Permian and for much of the Triassic periods (Colbert, 1971). The mammals arose from advanced synapsids in the upper Triassic, at which time the dinosaur reptiles were rapidly replacing the mammal-like-reptiles as the principal terrestrial forms (Kemp, 1980). The synapsid subclass contained many of the structural gaps existing between early reptiles and the later extremely mammal-like forms but share few diagnostic features within the subclass apart from a single lateral opening in the temporal region (Romer, 1967). The first primitive synapsids were the Pelycosaurs. The Pelycosaur radiation began early in the Pennsylvanian (for a review see Romer and Price, 1940, and appendum Romer, 1945, p.430, and Reisz, 1980). The earliest known synapsid reptile is Protoclepsydrops, a small pelycosaur from the early Pennsylvanian (Carroll, 1964; Baird and Carroll, 1967, and Kemp, 1980).

1.3.3 Order Pelycosauria

Virtually nothing is known of the early Pelycosaurs until the Late Carboniferous-Early Permian where three major subgroups became differentiated (Romer and Price, 1940 and Romer, 1945). The three Pelycosaur suborders were Ophiacodontia, Edaphosauria and Sphenacodontia. A brief survey of the pelycosaur radiation will show
Figure 11: The evolutionary history of reptiles.
Figure 1.2 The evolutionary history of mammals from reptiles.
that with the exception of the advanced sphenacodontids, the adaptations of the pelycosaurs were to an essentially "reptilian" mode of life with very few of these animals possessing any features not found in alternative reptilian groups (Romer and Price, 1940; Romer, 1967 and Romer, 1968(a); see Table 1.1).

The well know Dimetrodon of the lower Permian (Romer and Price, 1940, and Berman, 1977) can be used to show advanced sphenacodontid adaptations. The postcranial skeleton was primitive and sprawling although slender with elongated neural spines, a feature which took place in parallel fashion four or five times in Pelycosaurs, it ("dorsal sail") is suggested as having served a thermoregulatory function (Romer, 1948). In the Dimetrodon it has been shown that the ratio of the "dorsal sail" surface area to body volume remained constant (Romer and Price, 1940). The most advanced feature of the Dimetrodon and sphenacodontids in general is the skull and dentition. The teeth were well differentiated, the maxilla deep and the palate arched (Pough, 1979). The arched palate represents a preliminary solution to the problem later solved by the development of the secondary palate in the more advanced Therapsids. These advanced sphenocodontids also gained reorientation of adductor jaw musculature (Barghusen, 1968), the principal jaw closing muscles, the presence of an angled coronoid eminence and a notch or flange on the posterior part of the keeled angular bone (a large posteroventral element of the lower jaw). The original function of the notch or flange is postulated (Hopson, 1969) as aiding in the protection of dislocation of the lower jaw when prey was struck by allowing the pterygoideus
musculature to gain insertion close to the lateral surface of the jaw articulation and thereby improve the stabilizing function of this muscle (see Romer and Price, 1940, for detailed sphenocodontid structure and musculature, see also Watson, 1948; Parrington, 1955; Crompton and Parkyn, 1963, and Barghusen, 1968). This characteristic feature of the Sphenocodontids is repeated in all the primitive and many of the advanced Therapsids which were descendents of the advanced sphenacodontids.

1.3.4 Order Therapsida

The order Therapsida, best known from the richly fossiliferous Karoo beds of South Africa (Broom, 1932) link the essentially "reptilian" pelycosaurs and the primitive mammals. They represented all the carnivorous and many of the herbivorous forms of the late Permian with the rapid demise of the Pelycosaurs in the middle Permian. So varied were these forms that they are considered to have constituted a number of distinct (although closely related) orders. Watson and Romer (1956), Boonstra (1963), and Romer (1967) split the therapsids into two principal suborders: Anomodontia and Theriodontia, however, Hopson (1969) ranks three separate suborders: Anomodontia, Theriodontia and Dinocephalia, this latter group is included in Anomodontia by Romer et al.

The most sphenocodont-like of the early therapsids are the Russian eotitanosuchians (Boonstra, 1963; called phthinosuchians by Romer, 1967). From these primitive forms there is an early cleavage into the two suborders of Therapsida, the theriodontia, the "main
carnivorous therapsids which contain the ancestry of the mammals and the Anomodontia therapsids which were mostly herbivorous (for reviews of Anomodonts see Watson, 1948; Watson and Romer, 1956; Boonstra, 1963; and Romer, 1967). The eotitanosuchians (i.e., Eotitanosuchus, Phthinosuchus, Biamosuchus and related genera) closely resembled their sphenocodont ancestors with increased adductor muscle mass (indicated by increased flaring of the rear part of the skull, Hopson, 1969) and the shift in limb posture, characteristic of Therapsids (Romer, 1967).

The theriodonts of the Permian above eotitanosuchians fall into several different groups (Watson and Romer, 1956; Boonstra, 1963, and Hopson, 1969). These are the infraorders Gorgonospia, Therocephalia and Bauriamorpha (often not separated), Ictidosauria (often ignored as distinct); and the infraorder Cynodontia generally accepted as the group within which the latter mammalian radiation was initiated (Romer, 1967; Barghusen, 1968; Hopson, 1969 and Crompton and Jenkins, 1968, 1973, 1979). All these theriodonts possessed a smaller quadrate (a skull element the articular surface of which fits into the articular bone of the lower jaw to form the jaw joint in reptilian forms) loosely attached to the skull, probably by a fibrous pad, so that they were capable of a small amount of fore-aft movement (Hopson, 1969) in the jaw at its lower end (previously suggested for late cynodonts and possibly for gorgonopsids by Parrington, 1946).

The gorgonopsids had no secondary palate (although they had fused vomers, Romer, 1967), the persistence of postfrontal bones and a
modified quadrate-articular hinge (Parrington, 1955). These features (as well as many others) they shared with the primitive therocephalids. In contrast to the gorgonopsids, the therocephalids possessed elongated palates, paired vomers, development of the epiterygoid (the bone bearing a socket into which extends a projection, the basipterygoid process, from the braincase forming the basal articulation of palate and braincase) and a restructured quadrate ramus (Romer, 1968(b)). The therocephalids also retained a cleithrum (a dorsal element of the dermal girdle which tended towards rapid reduction in the early reptiles) only occasionally found in the gorgonopsids (for a paper on the stance and gait of the hindlimbs of therocephalids see Kemp, 1978). From the primitive therocephalids there developed various advanced or specialized forms (the Bauriamorphs) in the late Permian and early Triassic and in the later Permian the appearance of a series of advanced therocephalids descendents, usually of small size in which there was a trend toward various modifications and advances paralleling the cynodonts or even the later Ictidosaurs (Watson and Romer, 1956, and Romer, 1968(b)).

1.3.5 The Cynodonts

Romer (1967, 1968(b)) argues for an early gorgonopsid or theriodont ancestry for the cynodonts and therefore mammals. Brink and Hopson and Crompton (1969) believe the ancestry of the cynodonts have their origins within the therocephalid-bauriamorph radiation from the silphedestid-scalaposaurid group. All of the Permian cynodonts may be placed in the family Procynosuchidae (Watson and Romer, 1956 and Hopson and Crompton, 1969). These mammal-like-
reptiles (for a review of the origin of the idea of mammal-like-reptiles see Aulie, 1975) possessed several advanced features including multicusped cheek teeth, secondary palate, incipient doubling of the occipital condyle (Watson and Romer, 1956), a closed braincase (a cynodont hallmark) and many lower jaw advances (Barghusen, 1968), most of which can be related to improvement of the masticatory apparatus (Hopson, 1969). An increased activity would require such changes so that a greater supply of potential energy could be assimilated in a given time. Increased metabolic activity is one of three predominant adaptive features that distinguish the mammalian type from the reptilian type, the other two features being greater care of the young and an increase in intelligence (Van Valen, 1960).

1.3.6 Therapsids as endotherms

One of the results from an analysis of extinct reptiles, dinosaurs and mammal-like-reptiles (Bakker, 1975) using bone morphology, predator-prey ratio and latitudinal zonation was the conclusion that all the therapsids possessed some degree of endothermy (although not to the same extent as present day mammals). Any gradual evolution of endothermy may have brought about homeothermic responses to cold and heat, through either behavioural and or physiological mechanisms. One such possible adaptation could have been the development of an insulative coat, i.e., hair. The presence of hair in therapsids has been proposed by Brink (1957). Maderson (1972) has postulated the development of hair from complex epidermal modifications of mechanoreceptors and Cowles (1946) has suggested hair as a protection against solar radiation and heat loss. Brink (1957)
claims evidence for hair in theroccephalids from the presence of pits in the anterior parts of the maxillary, suggestive of vibrissae.

Similar observations have been made by Watson (1931) for a bauriamorph. Broom (1920) had previously interpreted Brink's pits as glands although no nutrient foramina have been detected (Van Valen, 1960). The best evidence (Van Valen, 1960) for the presence of sweat and other skin glands is provided by various superficial features of the snout. Foramina for blood vessels and nerves (analogous to the mammalian infra-orbital foramen) are present in the maxillary and sometimes also the premaxilla, lacrimal, nasal and dentary of many theroccephalids and gorgonopsids (Broom, 1920, 1936; Watson, 1931; Brink and Kitching, 1953; Brink, 1954(a), 1959; Crompton, 1955 and Attridge, 1956) and are the rule in cynodonts (Simpson, 1933 and Parrington, 1934). Brink (1957) claims direct evidence for sweat glands in Diademodon and Cynognathus (Cynodonts) from peculiar depressions situated dorsally on the maxillaries in the middle of the length of the snout (Brink, 1955(a),(b)). The presence of glands is no basis for any presumption as to their original function. It seems highly unlikely that evaporative cooling was one of their earlier tasks (Findlay, 1970). Evaporative responses to heat, except maybe salivation, were probably not acquired until after the protetherian-therian split (Hulbert, 1980). Since milk glands are modified sweat glands, Haldane (1964) has proposed lactation in hair-clad mammal-like-reptiles. Brink (1955(a)) reports evidence for viviparity in therapsids from the weak teeth of young of some cynodonts, the discovery of large and young forms of same species being found together (Brink, 1955(b)) and the lack of anything
resembling an egg ever being found in the fossil beds yielding mammal-like-reptiles (Kitching, 1968) which would mean the early separation of the monotremes (Olson, 1944 and Van Valen, 1960).

The secondary palate, developed independently by several therapsid groups, appears related to the simultaneous elaboration of the crowns of postcanine teeth. Van Valen (1960) and Parrington (1967) also speculate as to the division of the ventricles and reduction of aortic arches occurring in the synapsids as early as the lower Permian within the Pelycosaurs. The development of the secondary palate combined with a contemporaneous reduction in the number of lumbar ribs and differentiation between thoracic and lumbar ribs (Brink, 1957) in some cynodonts, a reduction in rib head size in therocephalids (Romer, 1968(a)) and the shortening and broadening of the lumbar ribs in some gorgonopsids and cynodonts (Parrington, 1967 and Romer, 1968(b)) has led to suggestions for the presence of a diaphragm in some cynodonts and therocephalids (Attridge, 1956 and Brink, 1957).

1.3.7 The mammalian lower jaw

Today the change in jaw articulation from articular-quadrate to squamosal-dentary is the "artificial" criteria used to define the class mammalia from the reptilia (Kuhne, 1956; Simpson, 1960; Van Valen, 1960, and MacIntyre, 1967). The mechanics of the jaw joint of reptiles contrast markedly to those of the mammalian jaw. The reptilian jaw hinges on the quadrate from the upper part of the skull about which articulates the articular bone of the lower jaw, so that
the lower jaw acts essentially as a third-class lever, with the jaw joint as the fulcrum. Since the jaw closing muscles of reptiles are inserted closer to the jaw joint than to the point of bite, the vertical force generated at the jaw joint is greater than that between the teeth at the point of bite (Parrington, 1955 and Crompton and Parker, 1978). Furthermore, reptiles lack a masseter muscle inserted on the outside of the jaw. In the jaw closing muscles of a reptile, the adductor muscles, insert on the inner, upper and lower surfaces of the lower jaw and do not form muscular slings. These closing muscles pull the jaw upward and inward and forward and inward. When the muscles on one side contract therefore, they tend to deflect the jaw ramus toward the midline of the skull. It then becomes the job of the flange of the pterygoid bone to limit this movement and also to serve as areas of attachment for the large ptergoideus muscle but it still remains for the jaw joint to withstand the strong medially directed forces. As would be expected in present day reptiles the jaw joint is fairly massive in relation to skull size to withstand these forces (Crompton and Parker, 1978).

The origins of the dentary-squamosal jaw joint contact of mammals can be interpreted as part of a major adaptive trend begun in the therapsids, continued within the cynodonts and culminated in the primitive mammals. Basically this process involves a reduction in the number of jaw elements, an expanding (width) dentary and the development of a retroarticular process which is later replaced by a coronoid process, eminating from the dentary with subsequent realignment and attachment of jaw musculature. For the literature
concerning these changes see Watson (1912, and 1948), Romer and Price (1940), Parrington (1955), Patterson and Olson (1961), Crompton and Parkyn (1963), Barghusen (1968, 1970), Barghusen and Hopson (1970), and Crompton and Parker (1978).

1.3.8 The mammalian middle ear

Intimately related to the formation of the mammalian masticatory apparatus was the development of the mammalian middle ear which incorporates the bones that previously formed the reptilian jaw joint. The first steps leading to this transformation probably occurred in the first reptiles (Westoll, 1945) in the transition from an aquatic to a terrestrial environment. From that point the two main groups that descended from the earliest reptiles (excluding Aves) the reptiles and mammals, solved the problem of perceiving airborne sounds separately. Parrington (1946, 1955, 1967 and 1979), Hopson (1966), Allin (1975) and Lombard and Bolt (1979) have all produced papers proposing either evidence and or means by which the mammalian middle ear could have been derived.

1.3.9 Early mammals

As previously discussed the earliest cynodonts are predominantly placed in the family Procynosuchidae. In addition to the Procynosuchidae several other families of Permian cynodonts have been recognized (Haughton and Brink, 1954 and Tatarinov, 1968), some of which have been included in the predominantly Triassic family Galesauridae (Lystrosaurus Zone, South Africa). The Galesauridae family reflects a Procynosuchian ancestry (Hopson, 1969) with a number
of progressive changes: the dentary was larger, the zygomatic arch heavier, the masseteric fossa and coronoid process essentially mammalian in shape which suggests well differentiated masseter and temporal muscles (Barghusen, 1968). The best-known galesaurid is *Thrinaxodon* (for cranial anatomy see Parrington, 1946; Estes, 1961 and Hopson, 1966 and for dental morphology, Crompton, 1963; Crompton and Jenkins, 1968 and Osborn and Crompton, 1973) the molars of which, as noted by Crompton and Jenkins (1968) and Hopson and Crompton (1969), bear a striking resemblance to the molars of *Eozostrodon* (an early mammal). Crompton and Jenkins (1968) and Hopson (1969) cite this as an important point in favour of the derivation of Eozostrodontids from thrinaxodon-like cynodonts.

The major problem with such a relationship is that the galesaurid cynodonts similar to *Thrinaxodon* possessed ribs with both overlapping extensions and fusion to the vertebrae in the lumbar region (there are three major hypothesis related to the function of these expanded ribs, see (1) Brink, 1954(b), 1955(a), 1957; (2) Gregory and Camp, 1918, and Haughton, 1924; and (3) Jenkins, 1970 which are not present in the eozostrodontids). Hopson and Crompton (1969) do not consider this problem to be irreconcilable although Crompton and Jenkins (1979) have since suggested the Chiniquodontid, *Probainognathus*, as an alternative representative to the pre-mammalian lineage since it has no precluding skeletal specializations.

It is reasonably well established that the Galesaurids served as points of radiation for several other later groups (Hopson and
Crompton, 1969; Hopson, 1969 and Barghusen and Hopson, 1970). These groups tentatively include the Chiniquodontidae, Cynognathidae (large carnivores), Diademodon ancestral to the Traversodontidae and later Tritylodontids representing the culmination of the herbivorous cynodont radiation and Diarthrognathus (Broom, 1932 and Crompton, 1963) an Ictidosaur which could also be from either a scaloposaurid-bauriamorph and cynodont ancestry (Crompton, 1958, 1963; Crompton and Parkyn, 1963; Kermack, 1967; Romer, 1967; Barghusen, 1968 and Crompton and Jenkins, 1968).

In the Late Triassic three groups possessed both functional squamosal dentary (mammalian) and quadrate-articular (reptilian) jaw articulations. These were the Ictidosaur, Diarthrognathus, the family Eozostrodontidae (= Morganucodontidae, with straight lined cusped molars) and Kuehneotheriidae (with wide angled V cusped molars). The similarities in the dentitions (Crompton and Jenkins, 1968; Hopson and Crompton, 1969 and Parrington, 1971) and jaws (Hopson, 1969 and Barghusen and Hopson, 1970) suggest a common ancestry for Eozostrodontidae and Kuehneotheriidae at or near the therapsid-mammal boundary which suggests a monophyletic origin of mammals (Reed, 1960; Parrington, 1967, 1971; Crompton and Jenkins, 1968; Hopson and Crompton, 1969; Hopson, 1970 and Kielan-Jaworowska, 1970). The differences in the teeth (Mills, 1971) and braincase (Kermack, 1967 and Kermack and Kielan-Jaworowska, 1971) in theria and non-theria mammals has been used to indicate a distant relationship between the two Triassic stocks and polyphyletic (Kermack and Mussett, 1958; Olson, 1959; Simpson, 1959, 1960 and Kermack et al., 1973)
origins for mammals from well below the therapsid-mammal boundary. The similarities are considered the result of independent parallel evolution, this would include the middle ear and therefore seems unlikely.

1.3.10 Theria and non-theria mammals

There is general agreement that the Eozostrodontidae are ancestral to the nontherian mammals and Kuehneotheriidae ancestral to all Therian mammals (see Mesozoic Mammals, edited by Lillegraven et al., 1979). The nontherian mammalian groups include the Multituberculata, Triconodonta and Docodonta, the only present day representatives of which are the monotremes, the nontherian ancestry of which is possibly multituberculate but may still be regarded as unidentified (Clemens, 1979). The theria contains the Symmetrodonta, Eutheria and Metatheria and the Eupantotheria. The history and the ideas concerning the relationships within these groupings can be followed in the literature of Simpson (1925, 1937), Kuhne (1958), Patterson (1956), Parrington (1967, 1971), Kermack (1967), Kermack, Kermack and Mussett (1968), Crompton and Jenkins (1968, 1973), Hopson and Crompton (1969), Hopson (1969), Kielan-Jaworowska (1970, 1971), Barghusen and Hopson (1970), and Lillegraven et al. (1979).

The current opinion concerning the origin of the marsupials (metatherians) and placentals (eutherians) is their origins from within the Eupantotheria which are closely related to the Symmetrodonta and probably descendants of Kuehneotheriid symmetrodonts (Kraus, 1979). The marsupial-placental dichotomy most probably
occurred during the Early Cretaceous (Kielan-Jaworowska et al., 1979). For a review on the evolution of metatherian and eutherian characters based on cladistic methods see Marshall (1979).

Since writing this current review on the palaeontology of mammals from reptiles, Kemp (1982) has published the best currently available volume on the mammal-like reptiles. The book includes summaries of all advanced mammalian like features in these early reptilian groups. Some of these features are summarized in Table 1.1.

This section of the literature review has dealt with the reptilian-mammalian phylogeny. Although the thesis does not draw directly upon the information contained within this section, it has been included to demonstrate a working knowledge of the area. This knowledge is essential as a background for this thesis. This section of the literature review discusses skull and postcranial elements to show evolutionary histories and changes associated with the development of endothermy in mammals from ectothermy in reptiles.
<table>
<thead>
<tr>
<th><strong>Pelycosaurs</strong></th>
<th><strong>Middle Carboniferous to Middle Permian</strong></th>
<th><strong>Advanced Sphenacodontids</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>- dimetrodon; well differentiated teeth, arched palate which possibly aided in breathing while holding prey in the mouth.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- jaw constructed to reduce the risk of dislocation.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- postcranial skeleton slender.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- biology essentially reptilian.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- brain case same size as contemporary reptiles.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- ectothermic temperature regulation (eg dimetrodon-dorsal sail) and bone histology (growth rings, little secondary haversian replacement).</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- possibility of impermeable skin in some Pelycosaurs.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- locomotion primitive and sprawling.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Therapsids</strong></th>
<th><strong>Middle to Late Permian</strong></th>
<th><strong>Eotitanosuchians (phthinosuchians)</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>- differentiated teeth and slender limb bones.</td>
</tr>
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<tr>
<th><strong>Late Permian</strong></th>
<th><strong>Gorgonopsids</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>- large canines, enlarged jaw musculature but still arranged as in the sphenodontid pelycosaurs and strong articulation to resist disarticulation.</td>
</tr>
<tr>
<td></td>
<td>- no secondary palate.</td>
</tr>
<tr>
<td></td>
<td>- increased olfactory epithelial surface area and also hearing was probably more acute.</td>
</tr>
<tr>
<td></td>
<td>- possibility of increased metabolic rates compared to sphenacodontids.</td>
</tr>
<tr>
<td></td>
<td>- reptilian brain size and sprawling gait.</td>
</tr>
</tbody>
</table>

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<thead>
<tr>
<th><strong>Very Early Lower Triassic</strong></th>
<th><strong>Therocephalids</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>- variable dentition, indicative of several different feeding strategies in different groups.</td>
</tr>
<tr>
<td></td>
<td>- jaw musculature advanced.</td>
</tr>
<tr>
<td></td>
<td>- some members developed secondary palate and complex postcanine teeth.</td>
</tr>
<tr>
<td></td>
<td>- possible vibrissae and higher metabolic rates (eg multicusped teeth and secondary palate).</td>
</tr>
<tr>
<td></td>
<td>- olfactory surface areas increased as in gorgonopsids.</td>
</tr>
<tr>
<td></td>
<td>- reptilian sized brains, forelimbs sprawling but hindlimbs capable of sprawling and more erect gaits.</td>
</tr>
</tbody>
</table>

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<thead>
<tr>
<th><strong>Lower Triassic</strong></th>
<th><strong>Cynodonts</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>- multicusped teeth, jaw capable of some chewing motion.</td>
</tr>
<tr>
<td></td>
<td>- secondary palate present.</td>
</tr>
<tr>
<td></td>
<td>- presence of diaphragm (shortened ribs).</td>
</tr>
<tr>
<td></td>
<td>- hair possibly present.</td>
</tr>
<tr>
<td></td>
<td>- increased metabolic rates</td>
</tr>
<tr>
<td></td>
<td>- well developed olfactory organ.</td>
</tr>
<tr>
<td></td>
<td>- brain case from reptilian up to mammalian sizes.</td>
</tr>
<tr>
<td></td>
<td>- more erect gait (increased neuromuscular control of locomotion, therefore central nervous system more developed).</td>
</tr>
</tbody>
</table>

+ Summarized from Kemp (1982)
THESIS

STUDIES IN THE EVOLUTION OF ENDOTHERMY:

MAMMALS FROM REPTILES
INTRODUCTION

Heat produced from metabolism is the result of biochemical inefficiencies and these inefficiencies are associated, as are all conversions of energy, with the first and second laws of thermodynamics. However, the heat produced in mammals and birds has become incorporated into their thermal homeostasis. Reptiles and other lower vertebrates, which do not produce the quantities of heat that mammals and birds do, have to rely upon heat from their environment to gain a level of thermal homeostasis. The mammals and birds are called homeotherms and the reptiles and other lower vertebrates poikilotherms as a consequence of their different strategies. Mammals and birds are also called endotherms and reptiles and other lower vertebrates ectotherms with reference to their abilities to produce heat. During the evolution of mammals and birds there has been a transition from the shifting body temperatures of the reptiles and its environmental dependence, to the internal production of body heat and maintenance of high and constant body temperatures independent of the environmental temperatures. The aim of this thesis is to study the development of endothermy in mammals from ectothermy in reptiles, the differences themselves, where they are, how they have occurred, and to suggest reasons for their development. Although this thesis studies mammals and reptiles the differences found are also considered to reflect similar general differences between birds and lower vertebrates.
In the attempt to examine these questions the experimental part of the thesis has been divided into four parts. The first part is a detailed comparison of a single mammalian and a single reptilian species of the same body size and body temperature, since both these parameters influence metabolism. It concentrates on where the differences in metabolic capacity are found, it compares both tissue and mitochondrial capacities and the amount of energy used to transport Na\(^+\) in both species. The second part (Chapter 3) looks at the same mammalian and reptilian species plus a similar sized amphibian species and examines why the mammal spends more energy on active Na\(^+\) transport than the ectothermic vertebrates. The importance of the Na\(^+\) pump in heat production and its relationship with the development of endothermic capacity are discussed. In Chapter 4 the effect of body size on the metabolic capacities of six tissues from a variety of mammalian species are examined. This comparison includes representatives of all three extant mammalian groups and covers a 100-fold body weight range. It looks at the metabolic capacities in the so-called "primitive" mammals and compares them to other mammals. In the fourth part of the study (Chapter 5) the mammals from Chapter 4 are compared with a similarly diverse group of reptilian species over the same body weight range. The effects of both body size and phylogeny on the metabolic capacities of the tissues are compared and the development of endothermy from ectothermy is considered. Finally there is a summary of information from all the chapters.
CHAPTER 2

A COMPARISON OF THE METABOLIC CAPACITIES OF TISSUES AND THEIR MITOCHONDRIA IN A MAMMALIAN AND REPTILIAN SPECIES
2.1 Introduction

Mammals have organismal levels of metabolism 5-15 times greater than similar sized ectothermic vertebrates when measured at the same body temperatures (Dawson and Hulbert, 1970; Hart, 1971; Bennett and Dawson, 1976; Bennett, 1978; Hillman and Withers, 1979 and Taylor et al., 1980). These large differences in organismal metabolism between mammals and ectothermic vertebrates are a reflection of similar differences at the cellular level. There is almost a complete lack of direct comparisons available in this area except for a few isolated studies. In a direct comparison Bennett (1972) has shown approximate 5-fold differences in skeletal muscle and liver mitochondrial enzyme activities between a single mammalian and three reptilian species, all of similar body weight and measured at the same temperature. Hulbert and Else (1981) have also shown similar 2-5 fold differences in the "in vitro" oxygen consumptions of liver, kidney and brain tissues between a similar sized mammalian and reptilian species measured at the same temperature. These observed differences between the mammals and ectothermic vertebrates can be supported by comparing data for individual species drawn from the literature (Morgan and Singh, 1969; Robin and Simon, 1970; Wilhoft, 1966; Ismail-Beigi and Edelman, 1971; Turner and Tipton, 1972; Lagerspetz et al., 1974; Wong et al., 1975 and Packard, 1976). Comparing this data, differences in enzyme activities and "in vitro" tissue metabolisms ranging from 3-15 times are found between similar sized mammals and ectothermic vertebrates when measured at similar temperatures.
In a direct comparison of the metabolic capacities between a small mammalian and reptilian species (Else and Hulbert, 1981) large differences were found in liver, kidney, heart and brain tissues. These differences always showed increased metabolic capacities in the mammalian tissues compared to the reptilian tissues. In the comparison two different methods were used. These methods were the determination of tissue mitochondrial membrane surface area densities and the activity of the mitochondrial enzyme cytochrome oxidase. These same two species unfortunately were too small to use in the work required for the continuation of the comparison. Their small size did not lend them to surgical manipulation and their small livers would not have produced enough liver cells to permit sodium membrane permeabilities. Primarily for these reasons a larger mammalian, Rattus norvegicus and a larger reptilian, Amphibolurus vitticeps species were chosen to repeat the previous experiments. Once again both species were selected to have similar body weights and the reptilian species to have a high preferred body temperature (37°C; Bartholomew and Tucker, 1963), since both these factors influence metabolism.

In repeating the work of the previous comparison with the new species there are additional advantages apart from larger body and tissue sizes. Comparison of the results obtained with the new species to those previously obtained should presumably strengthen the comparison and additional work not previously attempted can be done using the new species. In this new study lung and skeletal muscle (gastrocnemius) were added to the comparison of tissue mitochondrial membrane surface area determinations and cytochrome oxidase
activity measurements. Also the cytochrome oxidase activities of the six mammalian and reptilian tissue homogenates were expressed both per gram (wet weight) of tissue and per gram of tissue protein. In the previous comparison (Else and Hulbert, 1981) the relative enzymatic activities of the mitochondria from the mammal and reptile were unknown. Isolated liver mitochondria from a mammalian and a reptilian species have previously been shown to have similar oxygen consumptions per mg of mitochondrial protein (Cassuto, 1971). To test whether this is true for other tissues and other species, mitochondria from the six mammalian and reptilian tissues were isolated and their cytochrome oxidase activities (per mg of mitochondrial protein) measured and compared. The lipid:protein ratios of the isolated mitochondria were also determined. Apart from determining the metabolic capacities of the mammalian and reptilian tissues the amount of energy used by liver, kidney and brain tissues were measured using "in vitro" tissue oxygen consumptions. In particular the amounts of energy used by the tissues in the pumping of sodium were measured and compared between the mammalian and reptilian species.
2.2 Animals

The mammal used in the comparison was the rat *Rattus norvegicus* and the reptile, a thermophilic lizard *Amphibolurus vitticeps*. The rats used were housed in rat cages at room temperature (approximately 25°C) with ad libitum food (Allied Feeds) and water. The lizard species used had a preferred body temperature range of 30°-40°C (Lee and Badham, 1963) and were maintained in temperature controlled cabinets (82x71x50 cm) at 37°±2°C for several weeks before any experiments were performed. The lizards were provided with ad libitum water and a varied diet of fruit, vegetables, dog food, mealworms and cockroaches. All animals were in good health and maintained weight. They were subjected to a 12:12 light:dark photoperiod.

2.3 Chemicals and Materials

Crystalline ethylenediaminetetra-acetic acid (EDTA, 99%) and ethyleneglycol-bis-(amino-ethyl)-tetra-acetic acid (EGTA, 98%), acid free Hepes and ascorbic acid, salt free cytochrome C (horse heart), lecithin (phosphatidyl-choline type IX-E, egg yolk), Tris-base (A.R.) and ouabain (strophanthin-G) were all obtained from Sigma Chemical Company. The agar was from Oxoid Limited and the bovine serum albumin (B.S.A., fraction V) was from the Commonwealth Serum Laboratories.
2.4 Methods

2.4.1 Electron Microscopy of Isolated Mitochondria and Tissues

To examine the purity of the mitochondrial pellets and the state of the mitochondria samples of rat liver, kidney and skeletal muscle (gastrocnemius) mitochondrial pellets were resuspended in 1ml of 2½% glutaraldehyde fixative in 0.1M sodium cacodylate buffer and 0.175M sucrose (pH = 7.4). One ml of each mitochondrial suspension was added to 2mls of solidifying 3% agar to give a solid 2% agar block with suspended fixed mitochondria. These blocks were cut into smaller blocks and processed as normal tissue blocks (see Methods in Chapter 4). The liver, heart, kidney, brain, lung and skeletal muscle (gastrocnemius) tissues from two rats and two lizards were processed for stereological analysis using the methods as set out in Chapter 4.

2.4.2 The Measurement of Tissue and Mitochondrial Cytochrome Oxidase Activity

The rats were killed by a blow to the head, the lizards, by decapitation. The liver, heart, kidneys, lungs, brain and gastrocnemius muscles were removed, weighed and the carcass frozen for later tissue composition determination. All six tissues were placed in 50ml of ice cold preoxygenated (95% O₂, 5% CO₂) isolating medium (250mM Sucrose, 0.5mM EGTA, 2mM Heps, 0.5g/l B.S.A., and pH = 7.4 with KOH). The tissues were cut into small pieces with scissors and weighed into enough cold isolating medium to make 10% tissue homogenates. Homogenates were prepared using a Polytron
at half speed for 15 seconds. Approximately 10% of each tissue homogenate was rehomogenized (maximum speed for 15 seconds) and used for tissue cytochrome oxidase activity measurements. The remaining 90% of each tissue homogenate was used to isolate mitochondria. While the isolation of the mitochondria from the six tissues was taking place the cytochrome oxidase activity of the tissue homogenates were measured.

2.4.3 Isolation of Mitochondria

All centrifugation was carried out at 0-5°C on a Sorval RC-5B Refrigerated Superspeed Centrifuge. Homogenates were centrifuged at 600g for five minutes, the supernatants removed and placed in fresh ice cold centrifuge tubes and recentrifuged for five minutes at 14,500g. The supernatants were poured off and the walls of the tubes wiped to remove any fat and broken mitochondria. The pellets were resuspended in ice cold mitochondrial wash media (250mM Sucrose, 0.5mM EDTA, 2mM Hepes, 0.5g/l B.S.A., and pH = 7.4 with KOH) using cold glass (20ml) tissue homogenizers. The crude mitochondrial homogenates were centrifuged at 14,500g for five minutes, the supernatants removed and the pellets gently resuspended in 4ml of wash media in small (5ml), cold glass tissue homogenizers. These mitochondrial preparations were then used to determine mitochondrial cytochrome oxidase activities.
2.4.4 Measurement of Cytochrome Oxidase Activity

Cytochrome oxidase activity was measured using the methods of Wharton and Griffiths (1962). Oxygen uptakes were measured with two Hansatech oxygen electrodes and recorded on a two channel Houston Omniscribe recorder. The incubation media contained 20mM potassium phosphate, 0.1mM EDTA, 0.05mM Cytochrome C, 20g/120ml lecithin and 20mM ascorbic acid (pH = 7.4). A suspension of lecithin at 20mg/ml was freshly prepared in distilled water, polytroned at maximum speed for 1 minute and centrifuged at 40,000 g and 5°C for 20 minutes. Three different homogenate concentrations (10, 20 and 50μl in 1.5ml) were used to correct for the autoxidation rate of the ascorbic acid by extrapolating to zero tissue concentration. To begin the reaction, ascorbic acid was added (20μl) upon thermal equilibration of the incubation media with the homogenate to give a final volume of 1.5ml. All tissue and mitochondrial homogenates and suspensions were assayed in the same order for all animals. All experiments were carried out at 37°C and in duplicate for both tissue and mitochondrial preparations. Samples of each preparation were taken for the determination of protein content. This allowed determination of cytochrome oxidase activity in units of nmoles O₂·mg⁻¹ of (wet) tissue·min⁻¹, nmoles O₂·mg⁻¹ of tissue protein·min⁻¹ and nmoles O₂·mg⁻¹ of mitochondrial protein·min⁻¹ which can be converted to μl O₂·mg⁻¹·hr⁻¹ multiplying by 1.344 (22.4 litres x 60 minutes/1000). This conversion allows for the direct comparison between "in vitro" tissue metabolism and cytochrome oxidase activities.
2.4.5 The Measurement of "in vitro" Tissue Metabolism and Associated Sodium Transport Metabolism

All animals were fasted for 48 hours before use. The rats were killed by neck dislocation followed by decapitation and the lizards, by decapitation. The liver, kidneys, brain, heart and lungs were removed and weighed. The remaining carcass was frozen for later complete tissue composition determination. The liver, kidneys and brain were placed in ice-cold preoxygenated (95% O₂, 5% CO₂) Ringers solution (135mM NaCl, 5mM KH₂PO₄, 1mM CaCl₂, 0.5mM MgCl₂, 5mM Tris base, 10mM glucose, and pH = 7.4). Tissue slices (approximately 250 μm thick) were prepared with a tissue slicer in the order: liver, kidney and brain. The tissue slices were placed in Warburg oxygen consumption flasks and connected to manometers, then flushed with oxygen for 20 seconds. Oxygen consumption rates were measured according to the methods of Umbreit et al. (1957). Care was taken to remove renal papillary tissue from the rat kidneys before slicing.

Normal "in vitro" metabolism was measured from tissue slices in Ringers solution. Sodium-independent metabolism was measured from tissue slices in Ringers solution plus ouabain (5mM). Therefore sodium-dependent metabolism was the difference between normal and sodium-independent metabolism. Ouabain is a specific inhibitor of Na⁺K⁺-ATPase and it is assumed that oxygen consumption in the presence of ouabain would include all aspects of energy use by the cells other than those associated with sodium transport. All preparations were carried out in duplicate. Preparation time from the death of the animal to the beginning of each experiment was kept constant at 60
minutes, this included a 15 minute pre-equilibration time. Oxygen consumptions were measured every 30 minutes for two hours at 37°C. After each experiment the pH of the solutions were checked, the slices blotted and placed in preweighed glass vials then reweighed to determine wet weights and then placed at 90°C for 24 hours for the determination of tissue dry weights.

2.4.6 Lipid Determination

Lipids from isolated mitochondrial suspensions were extracted by adding 1 volume of mitochondrial suspension to 7 volumes of methanol mixture and 14 volumes of chloroform. This was stirred for 15 minutes. The methanol and chloroform were separated by adding a one fifth volume of 0.73% NaCl to the solvents in a separating funnel. The chloroform phase was run off and the remaining solution was washed 3 times with chloroform. Residual water was removed from the chloroform by the addition of anhydrous Na₂SO₄. The chloroform with dissolved lipids was filtered (Whatman No.1) into preweighed glass conical flasks and slowly evaporated off using a rotavapour (Buchi) at 30°C. The conical flasks were dessicated (1 hour) and reweighed. The recovery of lipids using the method was assessed using phosphatidylcholine and was found to be 80±3% (mean value ± SEM, n = 11).

2.4.7 Protein Determination

The protein content of the tissue homogenates and mitochondrial suspensions were determined using the Lowry method (Lowry et al., 1951).
2.4.8 **Statistics**

Students t-test was used for the determination of all significant differences.
2.5 Results

The results from the measurement of mitochondrial volume densities, inner and cristae membrane surface areas from six mammalian and reptilian tissues are presented in Table 2.1. The major difference between the mammalian and the reptilian tissues appear to be in their mitochondrial volume densities. These all showed highly significant differences in homologous tissues between the two species. Significant differences are found for liver, heart and muscle tissues between the mammalian and reptilian inner mitochondrial membrane surface area values. These inner mitochondrial membrane surface area values are a function of mitochondrial number, size and shape. Within the mitochondria themselves, cristae membrane surface areas are significantly different in liver, kidney, heart and skeletal muscle between the mammal and reptile. In brain and lung mitochondria, very similar cristae membrane surface areas are found in both species. These differences between the mammal and reptile are very similar to those found in the previous comparison of a smaller mammalian and reptilian species (Else and Hulbert, 1981) and the values for the rat are very similar to the limited values available for liver, heart and skeletal muscle in the literature (see Table 2.8).

The values from Table 2.1 can be used to calculate the mitochondrial membrane surface areas per cubic centimetre of tissue (Eqn 4.2). Multiplying these values by their respective tissue weights from the animals used, the total tissue mitochondrial membrane surface areas can be found for each tissue. These calculated values
Table 2.1 A Comparison of Mitochondrial Volume Density, Cristae and Inner Mitochondrial Membrane Surface Area in Six Tissues from a Reptile, A. vitticeps and a Mammal, R. norvegicus.

<table>
<thead>
<tr>
<th></th>
<th>A. vitticeps</th>
<th>R. norvegicus</th>
<th>Signif. of Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Number of Animals</strong></td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td><strong>Body weight, (grams)</strong></td>
<td>248 ± 2</td>
<td>267 ± 32</td>
<td></td>
</tr>
</tbody>
</table>

**Mitochondrial Volume Density (%)**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>A. vitticeps</th>
<th>R. norvegicus</th>
<th>P-value (N, N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>2.21 ± 0.24</td>
<td>13.18 ± 0.76</td>
<td>&lt;0.01 (104, 104)</td>
</tr>
<tr>
<td>Kidney</td>
<td>10.44 ± 1.01</td>
<td>18.10 ± 1.77</td>
<td>&lt;0.01 (88, 88)</td>
</tr>
<tr>
<td>Brain</td>
<td>5.02 ± 0.36</td>
<td>6.55 ± 0.40</td>
<td>&lt;0.01 (100, 100)</td>
</tr>
<tr>
<td>Heart</td>
<td>18.13 ± 1.17</td>
<td>22.96 ± 1.05</td>
<td>&lt;0.01 (99, 99)</td>
</tr>
<tr>
<td>Lung</td>
<td>1.36 ± 0.19</td>
<td>3.14 ± 0.42</td>
<td>&lt;0.01 (91, 91)</td>
</tr>
<tr>
<td>Skeletal Muscle</td>
<td>2.15 ± 0.24</td>
<td>4.51 ± 0.29</td>
<td>&lt;0.01 (87, 87)</td>
</tr>
</tbody>
</table>

**Inner Membrane Surface Area (m².cm⁻³ of tissue)**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>A. vitticeps</th>
<th>R. norvegicus</th>
<th>P-value (N, N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>0.26 ± 0.03</td>
<td>1.12 ± 0.10</td>
<td>&lt;0.01 (27, 27)</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.91 ± 0.12</td>
<td>1.19 ± 0.18</td>
<td>N.S. (22, 22)</td>
</tr>
<tr>
<td>Brain</td>
<td>0.63 ± 0.06</td>
<td>0.70 ± 0.07</td>
<td>N.S. (25, 25)</td>
</tr>
<tr>
<td>Heart</td>
<td>1.52 ± 0.11</td>
<td>1.74 ± 0.09</td>
<td>&lt;0.05 (25, 25)</td>
</tr>
<tr>
<td>Lung</td>
<td>0.15 ± 0.02</td>
<td>0.20 ± 0.04</td>
<td>N.S. (24, 24)</td>
</tr>
<tr>
<td>Skeletal Muscle</td>
<td>0.29 ± 0.03</td>
<td>0.75 ± 0.06</td>
<td>&lt;0.01 (23, 23)</td>
</tr>
</tbody>
</table>

**Cristae Membrane Surface Area (m².cm⁻³ of mitochondria)**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>A. vitticeps</th>
<th>R. norvegicus</th>
<th>P-value (N, N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>13.31 ± 1.39</td>
<td>18.19 ± 0.84</td>
<td>&lt;0.01 (79, 79)</td>
</tr>
<tr>
<td>Kidney</td>
<td>29.06 ± 1.26</td>
<td>38.54 ± 1.30</td>
<td>&lt;0.01 (63, 63)</td>
</tr>
<tr>
<td>Brain</td>
<td>37.27 ± 1.68</td>
<td>37.04 ± 1.94</td>
<td>N.S. (49, 49)</td>
</tr>
<tr>
<td>Heart</td>
<td>48.56 ± 1.54</td>
<td>53.68 ± 2.15</td>
<td>&lt;0.01 (67, 67)</td>
</tr>
<tr>
<td>Lung</td>
<td>37.53 ± 2.16</td>
<td>37.55 ± 2.27</td>
<td>N.S. (39, 39)</td>
</tr>
<tr>
<td>Skeletal Muscle</td>
<td>42.83 ± 2.06</td>
<td>56.88 ± 3.63</td>
<td>&lt;0.01 (57, 57)</td>
</tr>
</tbody>
</table>

N. S. equals number of determinations
Values are mean ± determination values + S.E.M.
N.S. not significant
**Table 2.2** A Comparison of Mitochondrial Membrane Surface Area in Six Tissues from a Reptile, *A. vitticeps* and a Mammal, *R. norvegicus*.

<table>
<thead>
<tr>
<th></th>
<th><em>A. vitticeps</em></th>
<th><em>R. norvegicus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Number of Animals</strong></td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td><strong>Body Weight, grams</strong></td>
<td>248 ± 2</td>
<td>267 ± 32</td>
</tr>
</tbody>
</table>

**Mitochondrial Membrane Surface Area**

<table>
<thead>
<tr>
<th>Tissue</th>
<th><em>A. vitticeps</em></th>
<th><em>R. norvegicus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>0.6 ± 0.2</td>
<td>3.5 ± 0.3</td>
</tr>
<tr>
<td>Kidney</td>
<td>3.9 ± 0.3</td>
<td>8.1 ± 0.7</td>
</tr>
<tr>
<td>Brain</td>
<td>2.5 ± 0.1</td>
<td>3.1 ± 0.2</td>
</tr>
<tr>
<td>Heart</td>
<td>10.3 ± 1.1</td>
<td>14.5 ± 3.0</td>
</tr>
<tr>
<td>Lung</td>
<td>0.7 ± 0.1</td>
<td>1.5 ± 0.6</td>
</tr>
<tr>
<td>Skeletal Muscle</td>
<td>1.2 ± 0.3</td>
<td>3.2 ± 0.4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Tissue</th>
<th><em>A. vitticeps</em></th>
<th><em>R. norvegicus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>2.9 ± 0.5</td>
<td>41.1 ± 11.6</td>
</tr>
<tr>
<td>Kidney</td>
<td>3.0 ± 1.3</td>
<td>21.6 ± 4.9</td>
</tr>
<tr>
<td>Brain</td>
<td>1.0 ± 0.0</td>
<td>5.7 ± 0.3</td>
</tr>
<tr>
<td>Heart</td>
<td>7.2 ± 0.1</td>
<td>16.1 ± 4.0</td>
</tr>
<tr>
<td>Lung</td>
<td>1.3 ± 0.4</td>
<td>2.7 ± 1.1</td>
</tr>
<tr>
<td>Skeletal Muscle</td>
<td>108 ± 19</td>
<td>375 ± 60</td>
</tr>
</tbody>
</table>

Values are means ± S.E.M. (N=2)
for the six mammalian and reptilian tissues are given in Table 2.2. On average the mammalian tissues have a 2.5 fold greater amount (range 1.3-6.0) of mitochondrial membrane surface area per cubic centimetre of tissue. This difference is increased to an average 6 fold difference (range 2.1-14.0) when mitochondrial membrane surface area is expressed on a total tissue basis.

The comparison of the cytochrome oxidase activities from six mammalian and reptilian tissues and their mitochondria are presented in Table 2.3. All tissue activities per mg of tissue show significant differences between the mammal and reptile. These values are similar to those found in the previous comparison (Else and Hulbert, 1981). These differences between the mammalian and reptilian enzyme activities are all reduced when the same activities are expressed per mg of tissue protein because all the mammalian tissues examined have significantly greater amounts of protein per gram of tissue (p<0.02) than the same reptilian tissues (see Table 2.4). In a previous comparison (Bennett, 1972), the measurement of liver and skeletal muscle cytochrome oxidase activities per mg of tissue protein showed 5 fold differences between a mammalian and three reptilian species. These same two tissues measured in this present study showed 2 fold differences between a mammalian and a reptilian species. The mammalian and reptilian mitochondrial cytochrome oxidase activities show both similarities in liver, kidney and brain mitochondria and large significant differences in heart, lung and muscle mitochondria (see Table 2.3). In both the mammal and the reptile, the heart and muscle mitochondria are very active compared to the other tissues.
TABLE 2.3  A COMPARISON OF TISSUE AND MITOCHONDRIAL CYTOCHROME
OXIDASE ACTIVITY IN SIX TISSUES FROM A REPTILE, A. vitticeps AND A
MAMMAL, R. norvegicus.

<table>
<thead>
<tr>
<th></th>
<th>A. vitticeps</th>
<th>R. norvegicus</th>
<th>SIGNIF. OF DIFF.</th>
</tr>
</thead>
<tbody>
<tr>
<td>NUMBER OF ANIMALS</td>
<td>6</td>
<td>6</td>
<td>N.S.</td>
</tr>
<tr>
<td>BODY WEIGHT, (grams)</td>
<td>340.4 ± 43.4</td>
<td>320.9 ± 32.8</td>
<td>N.S.</td>
</tr>
<tr>
<td>TISSUE CYTOCHROME OXIDASE ACTIVITY</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nmoles O₂·mg wet tissue⁻¹·min⁻¹</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LIVER</td>
<td>14.3 ± 4.4</td>
<td>37.0 ± 2.6</td>
<td>p &lt; 0.01</td>
</tr>
<tr>
<td>KIDNEY</td>
<td>19.9 ± 1.2</td>
<td>30.6 ± 1.1</td>
<td>p &lt; 0.01</td>
</tr>
<tr>
<td>BRAIN</td>
<td>12.2 ± 1.4</td>
<td>16.9 ± 1.3</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>HEART</td>
<td>21.1 ± 1.6</td>
<td>29.6 ± 0.9</td>
<td>p &lt; 0.01</td>
</tr>
<tr>
<td>LUNG</td>
<td>2.5 ± 0.2</td>
<td>9.4 ± 0.7</td>
<td>p &lt; 0.01</td>
</tr>
<tr>
<td>SKELETAL MUSCLE</td>
<td>6.7 ± 1.4</td>
<td>15.6 ± 1.2</td>
<td>p &lt; 0.01</td>
</tr>
<tr>
<td>nmoles O₂·mg protein⁻¹·min⁻¹</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LIVER</td>
<td>142.6 ± 31.9</td>
<td>226.3 ± 19.5</td>
<td>N.S.</td>
</tr>
<tr>
<td>KIDNEY</td>
<td>219.5 ± 16.0</td>
<td>246.3 ± 11.1</td>
<td>N.S.</td>
</tr>
<tr>
<td>BRAIN</td>
<td>230.3 ± 23.5</td>
<td>161.3 ± 14.7</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>HEART</td>
<td>251.3 ± 22.3</td>
<td>261.3 ± 12.1</td>
<td>N.S.</td>
</tr>
<tr>
<td>LUNG</td>
<td>42.0 ± 3.3</td>
<td>107.1 ± 8.7</td>
<td>p &lt; 0.01</td>
</tr>
<tr>
<td>SKELETAL MUSCLE</td>
<td>85.2 ± 21.6</td>
<td>136.2 ± 17.2</td>
<td>N.S.</td>
</tr>
<tr>
<td>MITOCYCHONDRIAL CYTOCHROME OXIDASE ACTIVITY</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nmoles O₂·mg protein⁻¹·min⁻¹</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LIVER</td>
<td>500 ± 121</td>
<td>571 ± 67</td>
<td>N.S.</td>
</tr>
<tr>
<td>KIDNEY</td>
<td>895 ± 117</td>
<td>649 ± 68</td>
<td>N.S.</td>
</tr>
<tr>
<td>BRAIN</td>
<td>464 ± 73</td>
<td>441 ± 54</td>
<td>N.S.</td>
</tr>
<tr>
<td>HEART</td>
<td>891 ± 172</td>
<td>1782 ± 152</td>
<td>p &lt; 0.01</td>
</tr>
<tr>
<td>LUNG</td>
<td>263 ± 37</td>
<td>531 ± 55</td>
<td>p &lt; 0.01</td>
</tr>
<tr>
<td>SKELETAL MUSCLE</td>
<td>751 ± 127</td>
<td>1532 ± 274</td>
<td>p &lt; 0.05</td>
</tr>
</tbody>
</table>

Values are means ± S.E.M.
N.S. not significant
### Table 2.4 Protein Content in Six Reptilian, A. vitticeps and Mammalian, R. norvegicus Tissues.

<table>
<thead>
<tr>
<th></th>
<th>A. vitticeps</th>
<th>R. norvegicus</th>
<th>SIGNIF. OF DIFF.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>NUMBER OF ANIMALS</strong></td>
<td>6</td>
<td>6</td>
<td>N.S.</td>
</tr>
<tr>
<td><strong>BODY WEIGHT, (grams)</strong></td>
<td>340 ± 43</td>
<td>321 ± 33</td>
<td></td>
</tr>
<tr>
<td>LIVER</td>
<td>*90 ± 10</td>
<td>165 ± 5</td>
<td><strong>P&lt;0.01</strong></td>
</tr>
<tr>
<td>KIDNEY</td>
<td>91 ± 5</td>
<td>126 ± 7</td>
<td><strong>P&lt;0.01</strong></td>
</tr>
<tr>
<td>BRAIN</td>
<td>53 ± 3</td>
<td>105 ± 1</td>
<td><strong>P&lt;0.01</strong></td>
</tr>
<tr>
<td>HEART</td>
<td>85 ± 4</td>
<td>114 ± 5</td>
<td><strong>P&lt;0.01</strong></td>
</tr>
<tr>
<td>LUNG</td>
<td>60 ± 5</td>
<td>90 ± 7</td>
<td><strong>P&lt;0.01</strong></td>
</tr>
<tr>
<td>SKELETAL MUSCLE</td>
<td>81 ± 6</td>
<td>120 ± 12</td>
<td><strong>P&lt;0.02</strong></td>
</tr>
</tbody>
</table>

Values are means ± S.E.M.

* mgs of protein per gram of tissue

N.S. not significant
but the mammalian mitochondria from these tissues are still twice as active as the same reptilian mitochondria.

No relationship could be found between mitochondrial cytochrome oxidase activities per mg of protein and mitochondrial membrane surface areas. This may be a result of the former values being expressed per mg of matrix protein as well as membrane protein. Examples of isolated rat liver, kidney and muscle mitochondria are shown in Plate 2.1. These isolated mitochondria electron micrographs were used to check the purity of the pellet and the condition of the mitochondria (all mitochondrial stereology was performed on mitochondria from tissue block preparations only). From the electron micrographs the mitochondria are predominantly in the condensed conformation or active State 3 respiration. This means that these mitochondria were working at maximum oxygen uptake and ATP production. The protein:lipid ratios for the isolated mitochondria from the six mammalian and reptilian tissues are given in Table 2.5. In general the mammalian mitochondria have slightly greater amounts of protein than lipid and in the reptilian mitochondria more lipid than protein. These differences are significant in the liver (p<0.02) and brain (p<0.01) mitochondria.

The "in vitro" oxygen consumptions of liver, kidney and brain tissues from the reptile and mammal are given in Table 2.6. The dry/wet weight percentages for the same tissues are in the Appendix, Table 2.1. These oxygen consumption values for the reptile are very similar to those for the same tissues in other reptiles of similar body weights measured at 32-37°C (Turner and Tipton, 1972 and Wong et al.,
Plate 2.1 Isolated Mitochondria

Liver

Kidney

Muscle
### Table 2.5 The Protein : Lipid Ratio of Mitochondria Isolated from Six Reptilian, A. vitticeps and Mammalian, R. norvegicus Tissues.

<table>
<thead>
<tr>
<th>Number of Animals</th>
<th>A. vitticeps</th>
<th>R. norvegicus</th>
<th>Signif. of Diff.</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIVER</td>
<td>54 : 46</td>
<td>78 : 22</td>
<td>p &lt; 0.02</td>
</tr>
<tr>
<td>KIDNEY</td>
<td>64 : 36</td>
<td>65 : 35</td>
<td>N.S.</td>
</tr>
<tr>
<td>BRAIN</td>
<td>32 : 68</td>
<td>46 : 54</td>
<td>p &lt; 0.01</td>
</tr>
<tr>
<td>HEART</td>
<td>59 : 41</td>
<td>45 : 55</td>
<td>N.S.</td>
</tr>
<tr>
<td>LUNG</td>
<td>38 : 62</td>
<td>53 : 47</td>
<td>N.S.</td>
</tr>
<tr>
<td>SKELETAL MUSCLE</td>
<td>35 : 65</td>
<td>55 : 45</td>
<td>N.S.</td>
</tr>
</tbody>
</table>

* values are mean relative percentages based on weight

N.S., not significant
1975). The rat values are also very similar to those values found by Ismail-Beigi and Edelman (1971) for kidney and brain but lower than they found for liver. All three tissues show significant differences (2-4 fold) in their rates of oxygen consumption between the mammal and reptile measured at the same temperature of 37°C. Since all the mammalian tissues measured consume significantly more oxygen per gram than the reptilian tissues, the amount of energy used on sodium-independent and sodium-dependent oxygen consumptions or metabolisms were also all significantly greater in the mammalian tissues (Table 2.6). The only exception to this was the sodium-independent metabolism of the brain. The mammalian and reptilian brain tissues show no differences in their sodium-independent metabolisms. This same phenomenon was also found in the previous comparison of a smaller mammalian and reptilian species (Hulbert and Else, 1981). The quantity of energy spent by the same amount of mammalian and reptilian tissue on Na⁺ dependent metabolism is 4-5 times greater in the mammal.

So far in considering the differences in the metabolic capacities and "in vitro" tissue metabolisms between the mammalian and reptilian tissues, the effects of tissue sizes has not been estimated. The tissue weights, expressed as percentages of the total body weights of the mammal and reptile are presented in Table 2.7. The liver, kidneys, brain, heart and skeletal muscle are all significantly (p<0.01) larger in the mammal than in the reptile. The lung showed no difference as a percentage of body weight between the mammal and reptile. When the effects of tissue size are combined with the
**Table 2.6** A Comparison of Total "In Vitro" Tissue Metabolism, Sodium Independent Metabolism and Sodium Dependent Metabolism in Three Tissues from a Reptile, *A. vitticeps* and a Mammal, *R. norvegicus.*

<table>
<thead>
<tr>
<th></th>
<th><em>A. vitticeps</em></th>
<th><em>R. norvegicus</em></th>
<th>Sign. of Diff.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Number of Animals</strong></td>
<td>6</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td><strong>Body Weights, (grams)</strong></td>
<td>323.5 ± 42.8</td>
<td>303.2 ± 26.6</td>
<td></td>
</tr>
<tr>
<td><strong>ul O₂ . mg dry weight⁻¹ . hr⁻¹</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total &quot;In Vitro&quot; Metabolism</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LIVER</td>
<td>1.04 ± 0.20</td>
<td>b4.51 ± 0.28</td>
<td>p &lt; 0.01</td>
</tr>
<tr>
<td>KIDNEY</td>
<td>7.19 ± 0.47</td>
<td>27.08 ± 0.77</td>
<td>p &lt; 0.01</td>
</tr>
<tr>
<td>BRAIN</td>
<td>a7.38 ± 0.88</td>
<td>c12.04 ± 0.92</td>
<td>p &lt; 0.01</td>
</tr>
<tr>
<td><strong>Sodium Independent Metabolism</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LIVER</td>
<td>0.69 ± 0.11</td>
<td>b2.48 ± 0.19</td>
<td>p &lt; 0.01</td>
</tr>
<tr>
<td>KIDNEY</td>
<td>3.22 ± 0.41</td>
<td>11.51 ± 0.57</td>
<td>p &lt; 0.01</td>
</tr>
<tr>
<td>BRAIN</td>
<td>a5.64 ± 1.57</td>
<td>c5.46 ± 0.81</td>
<td>N.S.</td>
</tr>
<tr>
<td><strong>Sodium Dependent Metabolism</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LIVER</td>
<td>0.36 ± 0.14</td>
<td>b2.03 ± 0.31</td>
<td>p &lt; 0.01</td>
</tr>
<tr>
<td>KIDNEY</td>
<td>3.96 ± 0.55</td>
<td>15.51 ± 0.96</td>
<td>p &lt; 0.01</td>
</tr>
<tr>
<td>BRAIN</td>
<td>a1.74 ± 0.79</td>
<td>c6.58 ± 0.53</td>
<td>p &lt; 0.01</td>
</tr>
</tbody>
</table>

Values are means ± S.E.M.

a N=5, b N=9, c N=7

N.S. not significant
### Table 2.7 Percent Body Composition in a Reptile, *A. vitticeps* and a Mammal, *R. norvegicus*.

<table>
<thead>
<tr>
<th>Number of Animals</th>
<th>9</th>
<th>10</th>
<th>Signif. of Diff.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body Weight (grams)</td>
<td>304 ± 34</td>
<td>310 ± 24</td>
<td>N.S.</td>
</tr>
<tr>
<td>Liver</td>
<td>2.84 ± 0.42</td>
<td>4.21 ± 0.11</td>
<td>p &lt; 0.01</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.41 ± 0.04</td>
<td>0.89 ± 0.04</td>
<td>p &lt; 0.01</td>
</tr>
<tr>
<td>Brain</td>
<td>0.13 ± 0.01</td>
<td>0.69 ± 0.04</td>
<td>p &lt; 0.01</td>
</tr>
<tr>
<td>Heart</td>
<td>0.29 ± 0.01</td>
<td>0.40 ± 0.01</td>
<td>p &lt; 0.01</td>
</tr>
<tr>
<td>Lung</td>
<td>0.81 ± 0.04</td>
<td>0.68 ± 0.05</td>
<td>N.S.</td>
</tr>
<tr>
<td>Skeletal Muscle</td>
<td>34.58 ± 1.94</td>
<td>42.82 ± 0.90</td>
<td>p &lt; 0.01</td>
</tr>
<tr>
<td>Stomach</td>
<td>1.12 ± 0.10</td>
<td>0.49 ± 0.03</td>
<td>p &lt; 0.01</td>
</tr>
<tr>
<td>Intestines</td>
<td>1.51 ± 0.10</td>
<td>2.04 ± 0.15</td>
<td>p &lt; 0.02</td>
</tr>
<tr>
<td>Reproduction</td>
<td>0.66 ± 0.13</td>
<td>1.37 ± 0.31</td>
<td>N.S.</td>
</tr>
<tr>
<td>Fur and/or Skin</td>
<td>21.21 ± 1.28</td>
<td>20.00 ± 0.55</td>
<td>N.S.</td>
</tr>
<tr>
<td>Other</td>
<td>36.42 ± 1.43</td>
<td>25.94 ± 1.25</td>
<td>p &lt; 0.01</td>
</tr>
</tbody>
</table>

Values are percent of body weight, means ± S.E.M.; N.S. not significant

* Body weights are expressed minus fat and stomach contents
differences already present between the mammalian and reptilian
tissues, cumulative differences (using values from Tables 2.2, 2.3,
2.4, 2.6 and 2.7) such as those shown in Figure 2.1 for each tissue
are evident. The values for the cytochrome oxidase activities and "in
vitro" metabolism for the tissues are in comparable units of ml O₂
consumed per total tissue per hour. For the total mitochondrial
membrane surface area per tissue, differences ranging from 2-14 fold
are found between the mammal and reptile. In the cytochrome oxidase
activities total tissue differences of 2-7 fold are found between the
six mammalian and reptilian tissues and in the three organs measured
by the "in vitro" method, organ metabolisms differ by 3-9 fold between
the mammal and reptile.
Figure 2.1 Total mitochondrial membrane surface areas, cytochrome oxidase activities and "in vitro" tissue metabolisms in six mammalian and reptilian tissues.
2.6 Discussion

All differences found in this present study from the measurement of mitochondrial membrane surface areas, cytochrome oxidase activities and "in vitro" tissue metabolisms show increased metabolic capacities and increased energy use in the mammal compared to the reptile. It would appear from this comparison that during the evolution of mammals from reptiles there has been an increase in the metabolic capacity of the tissues. One of the major increases has been an increase in the mitochondrial volume density and to a lesser extent an increase in the mitochondrial membrane surface area density within the mitochondria of the tissues. A review of the small body of data available (apart from that presented in this study) for mitochondrial volume and surface area densities in mammals and ectothermic vertebrates, of similar size to the animals used in this present study is given in Table 2.8. The data from this table shows large differences in the mitochondrial volume densities between mammalian and ectothermic vertebrate tissues. Further support for increased mitochondrial volume densities in mammalian tissues is found in a comparison in which a mammalian species was found to have far greater numbers of mitochondria than several ectothermic vertebrate species per gram of brain tissue (Wahbe et al., 1961). An increase in mitochondrial membrane surface area density by either increases in volume or internal membrane surface area densities of mitochondria would presumably increase the metabolic capacities of the tissues to produce energy.

Measurement of the cytochrome oxidase activities of the tissues (per mg of tissue) show similar differences between the mammal and
TABLE 2.8 Mitochondrial Parameters in Mammalian and Ectothermic Vertebrate Tissues.

<table>
<thead>
<tr>
<th>Tissue Species</th>
<th>Body Weight (grams)</th>
<th>Mitochondrial Volume Density (%)</th>
<th>Mitochondrial Membrane, S, A. Surface of cell volume m².cm⁻³ of tissue</th>
<th>Mitochondrial Membrane, S, A. Surface m².cm⁻³ of mitochondria</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Toad</td>
<td></td>
<td>11.8</td>
<td>-</td>
<td>-</td>
<td>HULBERT &amp; POPHAM, UNPUB.</td>
</tr>
<tr>
<td>Rat</td>
<td>174</td>
<td>16.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.69</td>
<td>23.0</td>
<td>LOUD (1968)</td>
</tr>
<tr>
<td></td>
<td>220</td>
<td>20.0</td>
<td>3.30</td>
<td>9.5</td>
<td>WEIBEL ET AL. (1969)</td>
</tr>
<tr>
<td></td>
<td>24.9</td>
<td>24.9</td>
<td>1.58</td>
<td>14.2</td>
<td>REITH (1973)</td>
</tr>
<tr>
<td>Heart</td>
<td>220</td>
<td>31.8</td>
<td>11.70</td>
<td>31.45</td>
<td>REITH &amp; FUCHS (1973)</td>
</tr>
<tr>
<td>Skeletal Muscle</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frog (Sartorius)</td>
<td></td>
<td>1.0 – 1.6</td>
<td>-</td>
<td>-</td>
<td>MOBERLY &amp; EISENBERG (1975)</td>
</tr>
<tr>
<td>Rat (Medial Gastrocnemius and Soleus)</td>
<td>500</td>
<td>4.86</td>
<td>-</td>
<td>-</td>
<td>STONNING &amp; ENGEL (1973)</td>
</tr>
<tr>
<td>Guinea Pig (White Vastus)</td>
<td>430</td>
<td>6 – 16</td>
<td>-</td>
<td>-</td>
<td>EISENBERG ET AL. (1974)</td>
</tr>
<tr>
<td>Dwarf Mongoose</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>MATHIEU ET AL. (1981)</td>
</tr>
</tbody>
</table>

<sup>a</sup> of cytoplasmic volume
reptile as found by the measurement of mitochondrial membrane surface areas, this suggests that the number of cytochrome oxidase units rather than the specific activities per se have increased during the evolution of the mammals. This is supported in a comparison of heart mitochondria from fish (Wilson et al., 1979) in which the cytochrome oxidase content was approximately 3 times less than in beef heart mitochondria although the oxygen consumption per mole of cytochrome oxidase were similar in the mammal and ectotherms. This comparison ignores any allometric effects which would probably increase the difference in the cytochrome oxidase content of the heart mitochondria between the mammal and ectothermic vertebrates. Cytochrome oxidases of elasmobranchs are half the size and contain 4 rather than 8 metal atoms compared to mammalian oxidases. The spectral properties, subunit compositions and functional properties are similar in both mammals and ectotherms (Wilson et al., 1980), this suggests that the reptilian cytochrome oxidase should also be similar to the mammalian oxidase in its structural and functional properties.

Both the measurement of mitochondrial membrane surface area per cubic centimetre of tissue and cytochrome oxidase activities of the tissues give similar 2-3 fold differences between the mammal and reptile. When the larger relative tissue size of the mammal is included into the comparison the metabolic capacities of the mammalian tissues are increased to approximately 5 times those in the reptile. The standard metabolism of the rat (0.80 ml O$_2$.gram$^{-1}$.hr$^{-1}$, Kibler, 1963) is also 5 times greater than for the lizard, A. vitticeps (0.17 ml O$_2$.gram$^{-1}$.hr$^{-1}$; Wilson, 1974).
Maximum metabolism is primarily muscle metabolism (Jansky, 1965) and the maximum metabolism of the rat (5.8 mls O\textsubscript{2}. gram\textsuperscript{-1}. hr\textsuperscript{-1}; Seeherman et al., 1981) is approximately nine times greater than that of the lizard, *A. vitticeps* (0.64 mls O\textsubscript{2}. gram\textsuperscript{-1}. hr\textsuperscript{-1}; Wilson, 1974). This maximal value for the lizards may be less than the true value since it was obtained from the manual and electrical stimulation of restrained animals. If this is the case it would reduce the nine fold difference in maximal metabolism between the mammal and the reptile. The differences between the mammal and reptile in their total skeletal muscle mitochondrial membrane surface areas and cytochrome oxidase activities measured in this study are 3.5 fold and 2.9 fold respectively. These differences are made up of smaller cumulative differences. In the skeletal muscle, the mitochondrial membrane surface area difference between the mammal and reptile is made up of the mammalian skeletal muscle having 25\% more internal mitochondrial membrane surface area density and these mitochondria occupying 200\% more of the cellular volume than reptilian skeletal muscle mitochondria (Table 2.1). The difference in the skeletal muscle cytochrome oxidase activity is a result of the mammalian skeletal muscle having 230\% more activity, for this particular enzyme, than reptilian skeletal muscle (Table 2.3). Both of these measured differences are then further increased with the additional effect of the increased skeletal muscle mass of the mammal compared to the reptile (Table 2.7). The muscle mitochondria in the mammal also have twice the cytochrome oxidase activity per gram of mitochondrial protein compared to the reptilian muscle mitochondria. These differences have been found using data from the gastrocnemius muscle and total skeletal muscle weights (this extrapolation is justified in the discussions of Chapters 4 and 5).
Not only is the skeletal muscle mass greater in the mammal but so are all the other major organs. Where is the compensating mass? The major compensating tissue in this comparison seems to be the skeleton. Unfortunately the skeletal weights are not available for a direct comparison but are the major component of the remaining tissue (other) in Table 2.7.

The decreased cytochrome oxidase activities between the mammal and reptile when expressed per mg of tissue protein is the result of an increased protein content in all the mammalian tissues examined (Table 2.4). The increased protein content of the mammalian compared to the reptilian tissues is interesting in light of some functions served by proteins, i.e., enzymes and transport proteins. It is possible that the differences in protein content between mammalian and reptilian tissues are a reflection of increased enzyme and transport units in the mammal associated with an increased metabolic rate.

Isolated mitochondria from the tissues of the mammal also show increased protein:lipid ratios in all tissues except heart, this suggests that within the mitochondria of the mammal there are more enzymes per gram of lipid than in reptile mitochondria. In the mammalian heart the greater cytochrome oxidase activity contradicts the reduced protein:lipid ratio in the mitochondria compared to the reptilian heart mitochondria. It may be that in mammalian heart mitochondria the cytochrome oxidase is disproportionately increased in relation to other mitochondrial enzymes and thus there is a dramatic increase in cytochrome oxidase activity without a corresponding increase in mitochondrial protein. This is supported by the
observations that beef heart mitochondria have greater amounts of cytochrome oxidase than other cytochromes (c₁ and b) compared to fish heart mitochondria with similar or reduced amounts of cytochrome oxidase compared to other cytochromes (Wilson et al., 1979).

Isolated mitochondria from the liver of the mammal and reptile used in this present study agree with the observations of Cassuto (1971), the "in vitro" oxygen consumption per gram of protein of isolated mitochondria from the liver of a reptile and mammal are the same when measured at 37°C, this also applies to brain and kidney mitochondria but not to isolated mitochondria from lung, heart and muscle between the mammal and reptile. Also, the isolated mitochondria from different tissues of the same species show large differences in their cytochrome oxidase activities. In both the mammal and reptile the most active mitochondria are from the skeletal muscle, heart and kidney tissues and the less active mitochondria from the liver, brain and lung tissues. These same sort of differences have previously been observed between isolated heart and liver mitochondria from a turtle (Privitera and Mersmann, 1961) and in fish (Irving and Watson, 1976).

The tissue metabolism of the three mammalian and reptilian tissues measured reflect the large differences found at the organismal level. The amount of energy spent on Na⁺ transport by the mammalian and reptilian tissue were measured using ouabain. The use of ouabain to estimate sodium transport has been criticized (Himms-Hagen, 1976) because it promotes the intracellular gain of Na⁺ and loss of K⁺ (Elshove and van Rossum, 1963 and Kleinzeller and Knotkova, 1964).
The observed decrease in oxygen consumption may be partially due to possible inhibitory effects of increased Na⁺ and decreased K⁺ on other aspects of cellular metabolism. The use of Na⁺ free (choline chloride or sucrose) enriched K⁺ media as a substitute for ouabain has been found to elicit the same decrease in oxygen consumption as found with ouabain (Asano et al., 1976) and thus negates this criticism. This is assumed to be the case in this study. The mammal and reptile spend a similar proportion of their respective tissue metabolism to transport Na⁺ out and K⁺ into their cells. As a result, the actual amount of energy used is 4-5 times greater in a gram of mammalian tissue compared to a gram of reptilian tissue. The possible reasons for this difference are the subject of the next chapter.

In summary, all the major tissues measured in this study from the mammal both have and use greater metabolic capacities than the same reptilian tissues. Metabolic capacities of the tissues measured by both total mitochondrial membrane surface area and cytochrome oxidase activity show the mammal to possess approximately 5 times the metabolic capacity of the reptile. The measurement of total tissue energy use in the mammal and reptile, assessed using "in vitro" tissue oxygen consumptions, show the mammalian tissues use 7 times more energy than the reptilian tissues to maintain cellular processes within the tissues. The same mammal and reptile organismal metabolisms measured at the same body temperature (37°C) for standard (Kibler, 1963 and Wilson, 1974) and maximum (Seeherman et al., 1981 and Wilson, 1974) metabolism show 5 and 9 fold differences in the mammal compared to the reptile respectively. The differences in the
capacity to produce energy at the cellular level in the mammal and reptile therefore appear to be well matched to the differences in the use of energy both at the cellular and organismal level in both species.
CHAPTER 3

SODIUM PERMEABILITY OF ISOLATED CULTURED LIVER CELLS:

A COMPARISON OF ENDOTHERMIC AND ECTOTHERMIC VERTEBRATES
3.1 Introduction

Reptiles and mammals use similar proportions of their "in vitro" metabolism to maintain Na⁺ and K⁺ gradients. From Chapter 2 and a previous comparison (Hulbert and Else, 1981), mammals and reptiles of the same body weight at the same temperature use approximately 40% of their "in vitro" metabolism in various tissues to pump Na⁺ and K⁺. Sodium and potassium pumping is associated with the Na⁺K⁺-ATPase or "Na⁺ pump" which maintains low intracellular Na⁺ and high K⁺ against the inverse physiological conditions outside the cell of high Na⁺ and low K⁺ as shown in Figure 3.1. These intracellular conditions are beneficial to protein synthesis by ribosomes and to the internal economy of the cell as a number of enzymes require K⁺ for maximum activity, pyruvate kinase being an example (Lehninger, 1975).

The "in vitro" metabolism of mammalian tissues is 4 fold higher than for reptilian tissues (Hulbert and Else, 1981, and Chapter 2). Consequently mammals spend 4 times more energy on Na⁺ and K⁺ pumping in various tissues than reptiles. In the liver particularly, this difference is 5 fold. To explain the difference in the amount of energy used by the Na⁺ pump of mammals and ectothermic vertebrates there are several possible explanations.

Firstly, the Na⁺ pumps of mammals and ectothermic vertebrates may have different efficiencies and/or stoichiometrics. The mammalian Na⁺ pump may transport less Na⁺ and K⁺ per molecule of ATP used or less K⁺ per Na⁺ ion transported. This is contrary to the small body of
Figure 3.1  Schematic view of the Na\textsuperscript{+}K\textsuperscript{+}-ATPase. ATP is synthesised through the oxidation of substrates in the mitochondria. ATP hydrolysis by the Na\textsuperscript{+}K\textsuperscript{+}-ATPase drives the transport of Na\textsuperscript{+} and K\textsuperscript{+} against their ionic gradients. This causes the passive movement (*) of these ions back down the gradient sustaining the effect.
information available which suggests average values of 18 Na⁺ ions transported per oxygen molecule consumed and 3 Na⁺ and 2 K⁺ ions transported per ATP molecule dephosphorylated for various mammalian, reptilian and amphibian tissues (Bonting and de Pont, 1977; Weiner and Maffly, 1980 and Lazaro and Balaban, 1981). Alternatively mammals may not maintain the same Na⁺ and K⁺ gradients across their cell membranes as ectotherms. However gradients for both Na⁺ and K⁺ of approximately 120mM are reported in the tissues of reptiles and mammals (Bentley, 1971; Prosser, 1973 and Minnich, 1979). This is similar to the gradients found in some amphibians and fish (Bentley, 1971 and Prosser, 1973). A further possibility is the cells of mammals may be smaller or highly convoluted increasing the cell membrane surface area: cell volume ratio and increasing the net influx of Na⁺ and efflux of K⁺ per gram of tissue, increasing the energy needed for pumping. To account for the 4 fold difference in energy expenditure the mammalian cells would need to be 8 times smaller. In my own observations of various mammalian and reptilian tissues (Else and Hulbert, 1981 and Chapters 2 and 5), no such obvious differences in cell sizes or cell membrane convolutions are apparent. A final alternative is the cell membranes of mammals may be more permeable or "leaky" to Na⁺ and/or K⁺ than ectothermic cell membranes. This would increase the influx and efflux of these ions and increase the amount of energy spent on maintaining similar gradients. No information is available concerning this possibility.

This chapter examines the permeability of Na⁺ across the cell membrane of isolated cultured liver cells from a mammalian, a reptilian and an amphibian species. The mammal and reptile used are
the same species used in Chapter 2. The amphibian used is the cane toad, *Bufo marinus*. The amphibian has been added to the comparison to increase the extent of the comparison to ectotherms in general. Primary monolayer culture was chosen as the system to study the permeability of liver cells to Na\(^+\). This system combines the stability and long term viability of cell culture with the diverse and differentiated features of the intact parent liver (Bissell, 1976). It is possible that in primary culture isolated liver cells that attach to the substratum and remain viable can recover from injury incurred during isolation (Bonney, 1974) and regain normal ATP levels (Bissell *et al.*, 1973). In brief, primary monolayer culture of liver cells is potentially the ideal approach for the study of liver "in vitro" (Bissell, 1976). It offers the best system for the comparative study of a single organ and in the context of this study the liver has the added benefit of being a major thermogenic organ. To the best of my knowledge primary monolayer cell culture or any other cell culture has rarely been attempted with reptilian cells and never before has this technique been used to directly compare cellular physiology different vertebrate groups.
3.2 Animals

The rats (*Rattus norvegicus*) and the lizards (*Amphibolurus vitticeps*) were maintained as previously described in Chapter 2. The toads, *Bufo marinus* were supplied commercially and housed in the same sized cabinets as the lizards (87x71x50 cm) but with an inground pool (28x41x12 cm) of slow flowing fresh water. The toads were kept at 25°C in a 12:12 light:dark photoperiod with mealworms provided.

3.3 Chemicals and Materials

Collagenase (type 1), heparin (sodium salt, grade II), gentamicin, ouabain (strophanthin-G), trypan blue and Hepes (H-3375) were obtained from Sigma Chemical Company. Dulbeccos (dried powder) culture media, penicillin-streptomycin and non-essential amino acids were all cell culture quality and obtained from the Commonwealth Serum Laboratories. Plastic cell culture incubation wells, plastic coverslips and pure collagen (Vitrogen) were all supplied by Flow Laboratories. Radioactive $^{22}$Na salt and $^{3}$H-inulin were supplied by Amersham Radiochemicals. The Sterivex GS (pore size, 0.22μm) and Swinex (47mm, dia) filter units, adaptors, bell housings and AP15 prefilters were purchased from Millipore. Sagital (60 mg/ml) from May and Baker Laboratory Chemicals, ammonia (A.R. grade) and Folin reagent from Ajax Chemicals. The toluene, Permablend and Triton X-100 used in the liquid scintillation cocktail were all liquid scintillation grade and supplied by Packard.
3.4 Methods

The methods used in the isolation and monolayer culture of liver cells were those of Seglen (1972 and 1973), Bissell (1976) and Elliot et al., (1976), in the measurement of the passive permeability of sodium those of Scharschmidt and Stephens (1981).

3.4.1 The Isolation of Liver Cells

Liver cells were isolated from non-regenerating livers using the perfusion apparatus shown in Figure 3.2. The animals were weighed on a Sartorius (1265 MP) balance and injected with 1 to 1.5 ml of a heparinized (8 mg.ml⁻¹) sagital (42 mg.ml⁻¹) solution. A U-shaped incision was cut through the skin, muscle and connective tissue, ventrally from the peritoneal to the pleural cavity. The liver was perfused via the hepatic portal vein, with the perfusate exiting from the posterior vena cava. The initial perfusate was a calcium free Krebs-Henseleit medium (122mM NaCl, 25mM NaHCO₃, 4.76mM KCl, 1.2mM KH₂PO₄) which was passed through the liver, without recirculation, to clear blood and calcium. The final perfusate, a Krebs-Henseleit medium with calcium and collagenase added (2.6mM CaCl₂, and 50 mg.100ml⁻¹ collagenase) was recirculated for varying periods of time as set out in Table 3.1 for each species. All media used were adjusted to a pH of 7.4 and maintained at 37°C in equilibrium with 95% O₂, 5% CO₂ throughout the perfusion.

The perfused livers were removed from the animals (where appropriate, gall bladders tied off and removed) and the membraneous
Figure 3.2 Liver perfusion apparatus.
sac covering the cells teased away with a pair of forceps. The cells were gently agitated free with the aid of a pair of plastic spoons in Ca\textsuperscript{2+} free Krebs-Henseleit media and passed through a 250\textmu m plastic mesh grid. The cells were then centrifuged and isolated as set out in Table 3.1.

3.4.2 The Primary Culture of Isolated Liver Cells

Plastic coverslips (24x32mm) coated with 0.13mg of collagen (polymerized in NH\textsubscript{3} for 2 hours, washed with glass distilled water 5x30 minutes and sterilized in U.V. for 2 hours) were plated with liver cells in 26x33mm incubation wells with final adherent cell protein densities as shown in Table 3.1. The cells were cultured in Dulbecco's modified Eagles media supplemented with 20% boxer calf serum (suckling calves) and single strength non-essential amino acids. Antibiotics; penicillin, streptomycin and gentamycin were used at concentrations; of 50, 50 and 20 \mu g.ml\textsuperscript{-1} of culture media respectively. Mammalian cell culture media have previously been used to culture fish (Wolf and Quimby, 1969), amphibian (Rafferty, 1976) and reptilian (Wolf et al., 1960) cells. Dulbecco's media has been shown to stimulate attachment and division of cells from fish, a frog and a turtle, and calf serum has been selected as the serum of choice for poikilothermic cell culture (Wolf et al., 1960 and Wolf and Quimby, 1969). The major precaution taken, in this study, was to reduce by half the usual level of antibiotics (for both the mammal and ectotherms) to avoid any possible toxic effects to the cells of the liver cells from the ectotherms (Wolf and Quimby, 1969). The ectotherms also needed longer periods of time for cell adhesion compared to the
### Table 3.1 The Preparation of Isolated Liver Cells and Monolayer Culture from a Mammal, *R. norvegicus*, a Reptile, *A. vitticeps* and an Amphibian, *B. marinus*.

<table>
<thead>
<tr>
<th></th>
<th><em>R. norvegicus</em></th>
<th><em>A. vitticeps</em></th>
<th><em>B. marinus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Perfusion</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flow rate (mls/min)</td>
<td>25 - 30</td>
<td>10 - 15</td>
<td>10 - 15</td>
</tr>
<tr>
<td>Initial perfusate</td>
<td>Ca(_{2+}) free Krebs-Henseleit medium</td>
<td>5 - 10</td>
<td>10 - 20</td>
</tr>
<tr>
<td>Final perfusate</td>
<td>5 mm Ca(_{2+}), 0.05% collagenase</td>
<td>20 - 30</td>
<td>50 - 60</td>
</tr>
<tr>
<td>Final perfusate recirculated volume/ perfusion (mls)</td>
<td></td>
<td></td>
<td>500 - 900</td>
</tr>
<tr>
<td><strong>Isolation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial centrifugation medium</td>
<td>Ca(_{2+}) free Krebs-Henseleit medium</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Number of washes</td>
<td></td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td>Force (g)</td>
<td></td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Time (mins)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Culture</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Culture medium</td>
<td>Dulbecco's modified medium</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Incubation time (mins)</td>
<td></td>
<td>0.10 - 0.78</td>
<td>0.06 - 0.22</td>
</tr>
<tr>
<td>Cell protein per coverslip (mgs)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell viability</td>
<td>Trypan blue exclusion microscope observations</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
mammalian liver cells. This characteristic was previously described by Wolf et al. (1960), that monolayers of frog and turtle cells form more slowly. The rat and lizard liver cells in this present study were all cultured for 5 hours but the cells from 5 of the 6 toads used were cultured for 23 hours. However, the results from the cells of the one toad cultured for 5 hours are indistinguishable from the other 5 animals. Media sterilization was carried out using an AP15 prefilter in a 47mm diameter Swinex unit connected to 0.22 μm pore Sterivex final filter with bell housing, in a sterile air flow cabinet. Cells were incubated at room temperature in a 95% O₂, 5% CO₂ environment.

3.4.3 Measurement of the Passive Permeability of Sodium across the Cell Membrane of Liver Cells

Coverslips with adherent cells were preincubated for 1 hour in a balanced electrolyte solution (150mM NaCl, 0.8mM MgSO₄, 1.2mM CaSO₄, 0.86mM K₂HPO₄, 0.14mM KH₂PO₄, 10mM Heps, adjusted to a pH of 7.4 with KOH) either with or without ouabain (1mM). The coverslips used to measure the passive permeability of sodium were incubated with ouabain, the controls were incubated without ouabain. Ouabain (1mM) has been shown to inhibit rat liver cell Na⁺K⁺-ATPases (Ismail-Beigi and Edelman, 1971 and Ismail-Beigi et al., 1979) and similar ouabain concentrations (0.5-1mM) have been used to inhibit both reptilian intestinal epithelium (Gilles-Baillien et al., 1979) and Bufo marinus urinary bladder and liver cell membrane Na⁺K⁺-ATPases (Rossier et al., 1979).
After preincubation, cell viabilities were checked as set out in Table 3.1 for each species. Coverslips with adherent liver cells were transferred to incubation wells containing 3ml of balanced electrolyte media with $^{22}\text{Na}$ at 0.5 μCi.ml$^{-1}$ (Ci = $3.7 \times 10^{10}$ Bq) either with or without ouabain (1mM). Coverslips were incubated for 30 seconds, 1, 5, 10, 30, 45 and 60 minutes for the mammal and 1, 10, 30 and 60 minutes for the ectotherms (N.B. 1 minute incubation for the ectotherms only for coverslips without ouabain.) All incubations were performed in duplicate and all experiments were completed within 75 minutes from the start of the incubation with the radioisotope. One coverslip with adherent liver cells in each experiment was also incubated for 60 minutes in identical balanced electrolyte media with $^{3}\text{H}$-inulin at 0.3μCi.ml$^{-1}$ (1Ci = $3.7 \times 10^{10}$ Bq) either with or without ouabain (1mM).

One set of collagen coated coverslips without cells were incubated (in duplicate) in balanced electrolyte media with $^{22}\text{Na}$ or $^{3}\text{H}$-inulin for 1, 10, 30 and 60 minutes to test the wash sequence. All coverslips after incubation were subject to a wash sequence, 5 washes of 30 second duration in 20ml of ice cold balanced electrolyte media. This wash procedure removes the rapidly exchangeable extracellular $^{22}\text{Na}$ (Scharschmidt and Stephens, 1981). Samples of the wash sequence for all 60 minute incubations were taken. After the wash sequence the coverslips were cut with scissors and placed in 2mls of Lowry's base solution (0.1M, NaOH and 0.189M, Na$_2$CO$_3$) in gamma scintillation counting vials. The radioactivity and protein content of each coverslip were then measured ($^{22}\text{Na}$-Packard Modumatic VI Auto Gamma Model 5360; $^{3}\text{H}$-Packard Liquid Scintillation Spectrometer System
Tri-cab Model 2425). The radioactivity of two 20μl samples of each incubation medium were also determined to allow the calculation of the uptake of sodium in nmole Na⁺.mg⁻¹ of liver cell protein. 

3.4.4 Electron Microscopy

Freshly isolated liver cells from the mammal and reptile were subject to electron microscope examination. The preparation of these liver cells was based on the method used by Chapman et al. (1973). The liver cells were fixed in 2.5% glutaraldehyde, buffered in 0.08 M sodium cacodylate with 0.175M sucrose (pH 7.4) for 1 hour. Washed 4x30 min in 0.2M sodium cacodylate and stained with 2% W/V osmium tetroxide in 0.1M sodium cacodylate for 45 minutes, at pH 7.4. Washed with 2% W/V sodium acetate and poststained with 2% W/V uranyl acetate in 50% ethanol for 30 minutes.

The cells were dehydrated in an ethanol series (70-100%) for 40 minutes then in 100% acetone (2x10 mins). The cells were infiltrated with 1:1 and 1:9 acetone : Spurrs resin and pure resin for 30 minutes, then pure resin for 60 minutes at 60°C and finally set in resin, 8 hours at 80°C. Section preparation, further post staining and electron microscopy is as described in the methods of Chapter 4.

3.4.5 Scintillation Counting

All counts were counted for enough time to give coefficients of variation of less than 5%, except for 30 second and 1 minute incubation coverslips which were counted for enough time to give
coefficients of variation of less than 10% (England and Miller, 1969). The efficiencies of the scintillation counters were determined from d.p.m. versus c.p.m. of standard solutions. The gamma counter was found to count \(^{22}\text{Na}\) with an efficiency of 61%. The beta counter was found to count \(^{3}\text{H}\) with an efficiency of 61%. The liquid scintillation cocktail used consisted of toluene: Triton X-100, 2:1 with 4 g of Permablenid III per litre. Water was added to the scintillation cocktail used in small samples to bring all samples to parity.

3.4.6 Protein Determination

Protein content was determined using the methods of Lowry et al. (1951).

3.4.7 Statistics

Students t-test was used for the determination of all significant differences.
3.5 Results

The mean body weights ± SEM, liver cell viabilities and the sodium uptake in nmole Na⁺·mg⁻¹ of protein of the six animals used from each species are given in Table 3.2. The viabilities of the rat liver cells were determined from the percentage of the cells excluding trypan blue (0.2% W/V soln. for 5 minutes). All viabilities were determined just prior to incubation with radioisotope. No differences were found in the viabilities of the cells isolated with or without ouabain. The viabilities of the ectothermic liver cells were not able to be determined using trypan blue, this was due to their large and abundant storage vesicles and in some cases pigmentation which obscured the nucleus of the cells used as a marker to indicate dye exclusion. Alternatively the cells from 4 of the 6 animals used from both species (only 4 were used due to lack of coverslips) were examined under the microscope and the general appearance of the membrane interfaces, shape and roundness of the cells used as indicators of viability. Berry (1976, p.144) has suggested that these characteristics may serve as better indicators of viability than the exclusion of trypan blue. The cells from both ectothermic vertebrates examined appeared in excellent condition with well rounded shape and intact membrane borders. The viabilities of the ectothermic liver cells with and without ouabain appeared to be similar to the percentage viabilities found for the mammalian liver cells using trypan blue.

The low yields of reptile liver cells seemed to be associated with the fat stores in the livers of these animals. Reduction of
TABLE 3.2 SODIUM UPTAKE AND VIABILITY OF LIVER CELLS ISOLATED FROM A MAMMAL, *R. norvegicus*, AND ECTOTHERMIC VERTEBRATES, *A. vitticeps* AND *B. marinus*.

<table>
<thead>
<tr>
<th></th>
<th><em>R. norvegicus</em></th>
<th><em>A. vitticeps</em></th>
<th><em>B. marinus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>NUMBER OF ANIMALS</strong></td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td><strong>BODY WEIGHT (grams)</strong></td>
<td>316 ± 38</td>
<td>309 ± 19</td>
<td>158 ± 17</td>
</tr>
</tbody>
</table>

**CONTROL INCUBATIONS (WITHOUT OUABAIN)**

<table>
<thead>
<tr>
<th><strong>VIABILITY</strong></th>
<th><strong>SODIUM UPTAKES</strong> (nmole Na⁺·mg⁻¹ of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>86 ± 3% Well rounded shape</td>
</tr>
<tr>
<td><strong>INCUBATION TIME</strong> (minutes)</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>18.2 ± 5.0</td>
</tr>
<tr>
<td>1</td>
<td>26.0 ± 5.9</td>
</tr>
<tr>
<td>5</td>
<td>60.6 ± 9.8</td>
</tr>
<tr>
<td>10</td>
<td>79.4 ± 12.7</td>
</tr>
<tr>
<td>30</td>
<td>101.6 ± 10.3</td>
</tr>
<tr>
<td>45</td>
<td>102.8 ± 6.6</td>
</tr>
<tr>
<td>60</td>
<td>97.8 ± 6.3</td>
</tr>
</tbody>
</table>

**PERMEABILITY INCUBATIONS (WITH OUABAIN)**

<table>
<thead>
<tr>
<th><strong>VIABILITY</strong></th>
<th><strong>SODIUM UPTAKES</strong> (nmole Na⁺·mg⁻¹ of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>83 ± 3% Well rounded shape</td>
</tr>
<tr>
<td><strong>INCUBATION TIME</strong> (minutes)</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>16.8 ± 3.1</td>
</tr>
<tr>
<td>1</td>
<td>29.2 ± 5.7</td>
</tr>
<tr>
<td>5</td>
<td>100.2 ± 20.1</td>
</tr>
<tr>
<td>10</td>
<td>155.7 ± 24.3</td>
</tr>
<tr>
<td>30</td>
<td>286.6 ± 40.4</td>
</tr>
<tr>
<td>45</td>
<td>339.1 ± 51.1</td>
</tr>
<tr>
<td>60</td>
<td>346.4 ± 51.3</td>
</tr>
</tbody>
</table>

Values are means ± S.E.M.  

a Number of animals (n) =1; b n=3; c n=4
these stores by starvation aided the collagenase action and increased the yield of cells. The toad livers produced abundant cell yields using the perfusion method.

Validation of the wash technique is given in Figures 3.3 and 3.4. These figures are semilog plots and show the removal of $^{22}\text{Na}$ and $^3\text{H}$-inulin (as percentages of the loss of radioactivity from the coverslips in the first wash) from 60 minute incubations of collagen coated coverslips without cells, Figure 3.3 and collagen coated coverslips with adhered liver cells, Figure 3.4. The wash sequence uses the principle that the fastest rate of exchange is the extracellular component followed by the intracellular component (Kleinzeller et al., 1962). In Figure 3.3, $^{22}\text{Na}$ and $^3\text{H}$-inulin show similar wash out rates, these rates were the same as found for 1, 10 and 30 minute incubation wash sequences. The major difference is that $^{22}\text{Na}$ washes out faster than the $^3\text{H}$-inulin, this was observed in all washes (Figure 3.4). For this reason extracellular $^{22}\text{Na}$ was not determined since the $^{22}\text{Na}^+/^3\text{H}$ ratio of the incubation media would not reflect the $^{22}\text{Na}^+/^3\text{H}$ ratio of the coverslips after the wash sequence. Since extracellular $^{22}\text{Na}^+$ would be expected to be found in both control (no ouabain) and ouabain incubations, subtraction of control incubations from ouabain incubations should cancel out this error. In addition the coverslips incubated in $^{22}\text{Na}$ with or without ouabain show significant differences in their $^{22}\text{Na}$ content. Coverslips incubated in $^3\text{H}$-inulin show no statistical differences between control and ouabain incubations (Figure 3.4) which suggests the same extracellular space and thus extracellular Na$^+$ content in both incubations.
Figure 3.3  Loss of radioactivity during the wash procedure from collagen coated coverslips without cells after incubation for 60 minutes with (●) $^{22}$Na$^+$ and (■) $^3$H-inulin.
Figure 3.4 Loss of radioactivity during the wash procedure from collagen coated coverslips with adherent liver cells from R. norvegicus, A. vitticeps and B. marinus after 60 minutes of incubation with (●) $^{22}$Na$^+$ and (■) $^{3}$H-inulin, with (□) and without (□) ouabain.

Values are means ± S.E.M.

* p<0.01
The uptake of sodium by the liver cells of rat, lizard and toad with and without ouabain are presented in Figure 3.5. The amount of sodium present in the rat and ectothermic vertebrates (lizard and toad) liver cells after 60 minute incubations are without ouabain 98 and 90-83nmole Na+ mg⁻¹ of protein and with ouabain 346 and 142-200 nmole Na+ mg⁻¹ of protein respectively. The values for the rat are compared to the values available from other studies in Table 3.3. The values obtained in the present study for the mammal are similar to those obtained by Berthon et al. (1980) and higher than the values obtained by Scharschmidt and Stephens (1981). Although the values differ between the present study and the study of Scharschmidt and Stephens, there are similarities. The difference between the control and ouabain incubations over the 60 minutes of incubation differ by the same proportional amounts in both studies, the control incubations level off after 20-30 minutes and the ouabain incubations are levelling off after 60 minutes of incubation in both studies. Using the value of 2.76 ml of liver cell.gram⁻¹ of liver cell protein (Scharschmidt and Stephens, 1981), the conversion of uptake in nmole Na+ mg⁻¹ of protein to apparent intracellular concentration in mM is possible. The rat liver cells of Scharschmidt and Stephens after 60 minutes of incubation are levelling off with an intracellular concentration of 32 mM Na⁺ with ouabain and in the control incubation at 6.5 mM Na⁺, far lower than expected. In the present study for the rat liver cells the ouabain incubations are levelling off after 60 minutes with an intracellular concentration of 125 mM Na⁺, similar to the concentration of the incubation media (150 mM Na⁺) and in the control incubations at 36 mM Na⁺.
Figure 3.5  Passive sodium uptake of liver cells from the rat, *R. norvegicus*, lizard, *A. vitticeps* and amphibian, *B. marinus* with (●) and without (○) ouabain (1 mM).

Values are means ± S.E.M.
### Table 3.3 The Sodium Content of Isolated Rat Liver Cells Incubated for One Hour With and Without Ouabain (1 mM) at 38°C: A Comparison of Different Studies.

<table>
<thead>
<tr>
<th>Study</th>
<th>Incubation</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n mole Na⁺. mg⁻¹ of protein</td>
<td>CONTROL</td>
</tr>
<tr>
<td>ELSE (1984)</td>
<td>98</td>
<td>346</td>
</tr>
<tr>
<td>Nonregenerating liver perfusion</td>
<td></td>
<td>5 hour culture</td>
</tr>
<tr>
<td>Primary monolayer culture</td>
<td></td>
<td>²²Na⁺ incubation</td>
</tr>
<tr>
<td>5 washes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BERTHON ET AL. (1980)</td>
<td>78</td>
<td>226</td>
</tr>
<tr>
<td>Nonregenerating liver perfusion</td>
<td></td>
<td>Suspended liver cells in</td>
</tr>
<tr>
<td>Suspended liver cells in</td>
<td></td>
<td>cell culture medium</td>
</tr>
<tr>
<td>cell culture medium</td>
<td></td>
<td>²²Na⁺ incubation</td>
</tr>
<tr>
<td>Silicone oil method</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SCHARSCHMIDT AND STEPHENS (1981)</td>
<td>18</td>
<td>90</td>
</tr>
<tr>
<td>Regenerating liver perfusion</td>
<td></td>
<td>Primary monolayer culture</td>
</tr>
<tr>
<td>Primary monolayer culture</td>
<td></td>
<td>44 - 50 hour culture</td>
</tr>
<tr>
<td>²²Na⁺ incubation</td>
<td></td>
<td>8 washes</td>
</tr>
</tbody>
</table>
Assuming the extracellular $^{22}\text{Na}^+$ in the control and ouabain incubations to be the same, taking the control incubation $^{22}\text{Na}^+$ content away from the ouabain incubation $^{22}\text{Na}^+$ content, the rate of passive sodium uptake can be calculated. This passive uptake of the mammalian, reptilian and amphibian liver cells is shown in Figure 3.6. Considering the uptake of sodium by the liver cells to be linear for the first ten minutes, this being the first measured value common to both the mammal and ectothermic vertebrates, rates of 7.6 and 2.3-1.5 nmole Na$^+$.mg$^{-1}$ protein.min$^{-1}$ are found for the mammal and ectothermic vertebrates respectively. Converting these values to $\mu$ mole Na$^+$.gram$^{-1}$ of liver cells.min$^{-1}$ (using 2.76 of liver cells.gram$^{-1}$ of liver cell protein) the rates are 2.8 and 0.8-0.5 $\mu$ mole Na$^+$.gram$^{-1}$ of liver cells.min$^{-1}$ for the mammal and ectothermic vertebrates respectively.

The 2.8$\mu$ mole Na$^+$.gram$^{-1}$.min$^{-1}$ found for the rat liver cells is slightly higher than 1.9$\mu$ mole Na$^+$.gram$^{-1}$.min$^{-1}$ found by Claret and Mazet (1972) and lower than the rates of 5.6 and 8.9$\mu$ mole Na$^+$.gram$^{-1}$.min$^{-1}$ found by Scharschmidt and Stephens (1981) and Berthon et al. (1980) respectively. The rate of sodium uptake estimated for the first 10 minutes of incubation for the mammalian liver cells in this present study is probably an underestimate. The uptake of Na$^+$ by cultured liver cells being linear only for the first 90 seconds (Scharschmidt and Stephens, 1981). The values for the uptake of sodium of the ectothermic vertebrates seem not to be underestimated to the same extent as obvious from Figure 3.6. The rate of sodium uptake of the ectothermic vertebrates may in fact be slightly overestimated by assuming the same amount of liver cell protein per
Figure 3.6 Passive sodium uptake of liver cells from a mammal, R. norvegicus; a reptile, A. vitticeps and an amphibian, B. marinus. Values are means±S.E.M.
gram of liver cells. In Chapter 2, the reptilian liver had less protein per gram than the mammalian liver, this could increase the value of 2.76 ml liver cells gram⁻¹ liver cell protein for the ectotherms and result in a decreased value for sodium entry in μ mole Na⁺ gram⁻¹ liver cells min⁻¹.
3.6 Discussion

In the liver the differences in the amounts of energy used by the Na\(^+\) pump between the mammal and ectothermic vertebrates seems to be largely accountable to relative differences in the "leakiness" of the cell membranes. The mammalian liver cell plasma membranes are 4-5 times more permeable to Na\(^+\) than ectothermic vertebrates liver cell plasma membranes. Sodium enters the mammalian liver cells at 2.8 \(\mu\)mole Na\(^+\).gram\(^{-1}\).min\(^{-1}\) and in ectothermic liver cells at 0.5-0.8 \(\mu\)mole Na\(^+\).gram\(^{-1}\).min\(^{-1}\). This difference in the Na\(^+\) permeability of the liver cell membranes between the mammal and ectothermic vertebrates is very similar to the difference in the amount of energy spent on pumping sodium between the two groups.

The mammalian liver cells contain far more sodium after the inhibition of the Na\(^+\)K\(^+\)-ATPase with ouabain than both ectothermic vertebrate liver cells but similar amounts of sodium without any inhibition of the pump (Figure 3.5). After 60 minutes of incubation with ouabain the sodium content of the mammalian liver cells is approaching equilibrium but both the reptilian and amphibian liver cells are still linear in their uptake of sodium and far from any equilibrium after sixty minutes of incubation with ouabain. All the control incubations (i.e., without ouabain) show no differences between the mammal and the ectothermic vertebrates. Conversely the ouabain incubations all show significant differences for the 10 \((p<0.01)\), 30 \((p<0.01)\) and 60 \((p<0.05)\) minute incubations between the mammal and both ectothermic vertebrates. The reptile and amphibian show no statistical difference between them for any
incubation. An example of a freshly isolated mammalian and reptilian liver cell before culture are given in Plate 3.1. From these electron micrographs it is obvious that the mammalian liver cell is larger than the reptilian liver cell. This was generally observed of all mammalian liver cells examined, and is contrary to the expected size of the cells based on energy use. The mammalian liver cells appear to have a reduced surface area : volume ratio compared to the reptilian liver cells and therefore would not be expected to spend extra energy on sodium pumping as a result of this parameter.

Changes in membrane permeabilities are part of the normal functioning of organisms and many hormones have been shown to affect membrane permeabilities. Insulin increases mammalian Na+K+-ATPase activity by a postulated rapid change in the permeability of the cell membrane to Na+ (Clausen and Kohn, 1977 and Fehlmann and Frychet, 1981). Glucagon increases Na+K+-ATPase activity by increasing the permeability of the cell membranes to K+ (Fehlmann and Frychet, 1981). ATP and noradrenaline cause a rapid loss of K+ in isolated guinea pig hepatocytes, this is believed to be due to a rise in the K+ permeability of the liver cell membrane triggered by an increase in cytosolic Ca2+ (Burgess et al., 1981), this is not found in rat hepatocytes but is in other mammals. Aldosterone increases the permeability of Na+ across the toad colon and bladder (Cofré and Crabbé, 1967). Thyroid hormones increase the Na+ and K+ permeabilities of mammalian skeletal muscle cells (Asano, 1977). Thyroid hormones also increase the tissue oxygen consumption in mammals (Ismail-Beigi and Edelman, 1971), reptiles (Wilhoft, 1966; Turner and Tipton, 1971 and Wong et al., 1975) and amphibians (Packard
Plate 3.1 Isolated Liver Cells
×6,360

Rat

Lizard
and Packard, 1973). In mammals this increased oxygen consumption can be attributed to varying degrees (depending upon the tissue, animal and method used), to the increased activity of the Na⁺ pump (Ismail-Beigi and Edelman, 1971). This increased Na⁺ pump activity has been shown to be a result of an increase in the number of Na⁺K⁺-ATPase pumps (Rahimifar and Ismail-Beigi, 1977). The increased Na⁺ pump activity slightly decreases the intracellular Na⁺/K⁺ ratio increasing the Na⁺ and K⁺ gradients across the cell membranes (Ismail-Beigi and Edelman, 1973).

Comparing the effects of thyroid hormones with the increased Na⁺ permeability of the mammal compared to the ectothermic vertebrates it suggests that the number of pumps should be greater in the mammal. If the number of Na⁺ pumps per unit of mammalian membrane is greater than for reptilian or amphibian membrane, then both ribosomal activity and RNA synthesis should also be expected to be higher in the mammal. Regulation of the number of Na⁺K⁺-ATPase units appears to be modulated by removal rather than synthesis (Pollack et al., 1981), this mechanism being highly efficient in allowing for rapid responses to changing conditions. This strategy also means the maintenance of additional Na⁺K⁺-ATPase units above that normally needed. The rate of synthesis and degradation of the Na⁺K⁺-ATPase units for mammals should therefore also be expected to be greater than for the ectothermic vertebrates.

The characteristic properties of the Na⁺K⁺-ATPases from mammals, reptiles, amphibians and a fish measured from diverse tissue types are very similar as shown in Table 3.4. If the Na⁺K⁺-ATPases in the mammal and ectothermic vertebrates are the same or functionally very
<table>
<thead>
<tr>
<th>Reference</th>
<th>Tissue</th>
<th>Optimum Na+ K+ ATP</th>
<th>Na+ K+ ADP</th>
<th>Mg2+ ATP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Westenfelder et al. (1980)</td>
<td>-</td>
<td>- 0.40 1.9 I.5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Jurpers &amp; Donning (1969)</td>
<td>-</td>
<td>- 0.9 I.6 8.0 0.5</td>
<td>- 0.5 - 1.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Verwey &amp; Elsasser (1973)</td>
<td>-</td>
<td>- 0.65 I.5 0.5 1.0 3.1</td>
<td>- 0.38</td>
<td>- 0.9 6.0</td>
</tr>
<tr>
<td>Aas &amp; Elsasser (1976)</td>
<td>-</td>
<td>- 2.6 0.2 1.1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Kranke et al. (1971), L. Elsasser (1979)</td>
<td>-</td>
<td>- 3.9 2.3 0.3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Hessen et al. (1972)</td>
<td>-</td>
<td>- 5.0 0.5 - 1.0 0.35</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Gillies &amp; Baines et al. (1975)</td>
<td>-</td>
<td>2.5 - 3.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Park &amp; Hone (1967)</td>
<td>-</td>
<td>- 3.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Harris (1967), Corrie &amp; Donning (1968)</td>
<td>-</td>
<td>- 2.2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Donning (1966)</td>
<td>-</td>
<td>- 2.2</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 3.14: Properties of the Sodium Pump from Mammalian and Ectothermic Vertebrate Tissues.
similar as is suggested from their properties, the amount of Na⁺ pumped out and K⁺ pumped in by the Na⁺K⁺-ATPase is directly comparable between the mammal and ectothermic vertebrates by its activity. Crude plasma membrane fractions from rat livers give Na⁺K⁺-ATPase activities of 11 μMPi.mg⁻¹ protein.hr⁻¹ (Ismail-Beigi and Edelman, 1971). Similar crude plasma membrane fractions isolated from Bufo marinus livers give Na⁺K⁺-ATPase activities of 1 μMPi.mg⁻¹ protein.hr⁻¹ (Rossier et al., 1979). Although some of this large difference may be partially explained by the use of different membrane isolation methods (Emmelot and Bos, 1962 and Lo et al., 1976), the difference is presumably too large to be explained by the use of different methods alone. These values represent the maximal capacity of the cells to pump Na⁺ across the liver membrane. If the Na⁺ pumps of mammals and ectothermic vertebrates are functionally the same, the increased activity of the mammalian Na⁺K⁺-ATPase is probably due to an increased number of Na⁺ pumps in the membrane of the mammal as previously suggested. An increase in the number of Na⁺ pumps could maintain similar gradients with a "leaky" membrane at the expense of increased energy utilization as found in the mammalian tissues. Unfortunately there is no data available on the numbers of Na⁺ pumps in any comparable mammalian, reptilian or amphibian tissues.

In the evolutionary transition from ectothermic vertebrates to mammals there has been an increase in heat production. In animal tissues heat is produced as a byproduct in the formation of ATP by mitochondria and in the hydrolysis of ATP and degradation of any products incorporating free energy from ATP. Augmented heat
production depends on increasing the rate of the overall process. So what is the advantage of increased Na⁺ permeability in the mammals compared to the ectothermic vertebrates? If Na⁺ transport was linked to substrate cotransport, the expense of Na⁺ pumping would be easily offset by the energy obtained from the substrate, particularly when active. The only tissues to show such coupled transport are the small intestine and kidney tubule epithelial cells (Lehninger, 1975).

What is the advantage of increased Na⁺ permeability in other tissues, such as the liver? The only obvious advantage offered by high Na⁺ permeabilities seems to be augmented heat production. There have been two recent attempts to relate the evolution of endothermy and homeothermy in mammals with the Na⁺ pump as a major heat source in the evolution of mammals. The actual contribution of the Na⁺ pump to heat production in vivo is controversial (Folke and Sestoft, 1977 and Ismail-Beigi et al., 1979). Heat produced by the Na⁺ pump would presumably make its maximal contribution in resting animals as muscle thermogenesis may diminish its relative contribution in active animals. Nevertheless regardless of the Na⁺ pumps contribution, be it large or small, in mammals 5 times more heat is produced than in the reptiles per gram of liver tissue as a result of the Na⁺ pump activity. This increased energy use in the liver can be explained as primarily a result of increased Na⁺ permeability in the mammal. Since other tissues from mammals also show increased sodium dependent metabolism compared to the ectotherms (Hulbert and Else, 1981 and Chapter 2), the liver may serve as an example of increased cell membrane permeabilities in other mammalian tissues compared to the
tissues of ectothermic vertebrates. Similarly Na⁺ may serve as an example of other ions and their increased permeabilities in mammalian tissues.
CHAPTER 4

ENERGY METABOLISM AND BODY SIZE IN MAMMALS:
AN ALLOMETRIC EXAMINATION
AT THE LEVEL OF TISSUES AND MITOCHONDRIA
4.1 Introduction

Two of the major influences on metabolism are body size and phylogeny. This chapter will examine the effect of body size on mitochondrial parameters in mammals. The general relationship between standard energy metabolism and body weight can be described by the allometric equation

\[ M = aW^{0.75} \]

where \( M \) is metabolic rate, \( W \) is body weight and "\( a \)" is a constant, depending on the units of \( M \) and \( W \). The allometric relationship between metabolism and body size is also seen at different levels of organization. Mammals show the influence of the allometric relationship between metabolism and body size in their ecology (Calder, 1983) and suborganismal physiological rates such as cardiac output (Holt et al., 1968), renal glomerular filtration rate and renal plasma flow (Edwards, 1975), as well as at the subcellular level of organization in rates of protein synthesis and RNA synthesis (Munro and Downie, 1964) and mitochondrial parameters, such as volume density (Smith, 1956 and Mathieu et al., 1981), oxygen consumption (Smith, 1956), enzyme content (Drabkin, 1950) and enzyme activities (Kunkel et al., 1956 and Emmett and Hochachka, 1981). The ecological, physiological, cellular and subcellular levels all reflect the effect of body size on metabolism.

The cellular organelles responsible for the consumption of oxygen and the production of ATP are the mitochondria. Mitochondria contain all the enzymes associated with the mechanisms of oxidative
phosphorylation in their inner and cristae membranes and the enzymes of the tricarboxylic acid cycle in the mitochondrial matrix (Lehninger, 1975). In Chapter 2 the metabolic difference between a "cold-blooded" reptile and "warm-blooded" mammal (of the same body weight and body temperature) were found to be reflected in the difference in the total mitochondrial membrane surface areas of their tissues. However, this previous study did not examine the influence of an animal's body size on these suborganismal aspects of metabolism.

The purpose of this chapter is to see if the relationship between mitochondrial parameters from various tissues of mammals of different body size are similar to the relationship between whole animal metabolism and body size. Many previous studies dealing with the problems of allometric scaling in mammals have either drawn conclusions from the literature (Holliday et al., 1967; Prothero, 1979 and 1982) or from single tissue studies (Smith, 1956 and Mathieu et al., 1981). In this present study the respiratory active mitochondrial membrane surface areas of the liver, lung, heart, brain, kidney and skeletal muscle (gastrocnemius) are examined. The mammals had a 100 fold weight range and represent diverse mammalian groups including three placental, two marsupial and a monotreme species. These animals are used to show body size related mitochondrial parameters in mammals. Tissue weights, volume (assumed weight) specific mitochondrial membrane surface areas and total tissue mitochondrial membrane surface areas are compared allometrically. As well, this present study examines the total mitochondrial membrane surface areas for the summated tissues and attempts to relate them to the standard and maximal organismal levels of energy metabolism in mammals.
4.2 Materials

4.2.1 Animals

The placental mammals used were the mouse (*Mus musculus*; body weights 27g and 41g) the rat (*Rattus norvegicus*; 235g and 298g) and the rabbit (*Oryctolagus cuniculus*; 1870g and 2067g). The marsupial mammals were the brown antechinus (*Antechinus stuartii*; 18g and 23g) and the bandicoot (*Perameles nasuta*; 1145g and 1835g) whilst the monotreme mammal examined was the echidna (*Tachyglossus aculeatus*; 1486g). All the marsupials and monotremes were captured in the Wollongong area whilst the placental mammals were from captive colonies. All animals were in good health, whilst in captivity they were provided with food daily, *ad libitum* water and a 12:12 light:dark photoperiod.

4.2.2 Chemicals

Glutaraldehyde (10% soln) and sodium cacodylate (97%) were obtained from BDH Chemicals. Ethanol, acetone, lead nitrate and sodium acetate were all A.R. grade and supplied by Ajax Chemicals. Sodium citrate (99%) was supplied by May and Baker Laboratory Chemicals and osmium tetroxide from Johnson Malthey Chemicals. Uranyl acetate and Spurrs resin kits were purchased from Polaron.
4.3 Methods

The methods used to determine the organ weights, relative mitochondrial volume densities and membrane surface areas are essentially those used by Else and Hulbert (1981) and Weibel (1969). Two animals from each species (except Tachyglossus aculeatus, where only one individual was available) were used. The following tissues: liver, kidney, brain, heart, lung and skeletal muscle (gastrocnemius) were removed from all animals except Mus musculus, which only liver, kidney, brain and heart data were available. These values are those reported previously (Else and Hulbert, 1981) and were not obtained as part of the work towards this thesis but are included for a more complete interpretation of the data. Only liver, kidney and heart data were available from either one or the other of the two Antechinus stuartii used. For the skeletal muscle, the tissue weight includes all skeletal muscle on the animal's body, however the mitochondrial parameters were only measured on gastrocnemius muscle, assuming this muscle to be representative of total body skeletal muscle.

4.3.1 Determination of Tissue Weights and the Preparation of the Tissues

Animals were killed either by decapitation or an injection of sagital. The six tissues: liver, kidney, brain, heart, lung and skeletal muscle (gastrocnemius) were quickly removed (the carcasses were frozen for later complete body composition determinations), weighed (Sartorious 1265 MP balance), and random samples placed in ice cold 2.5% glutaraldehyde fixative in 0.1M sodium cacodylate buffer and 0.175M
sucrose (pH = 7.4) in which they were diced to 0.25mm³ and fixed for 4 hours. The samples were washed in 0.1M sodium cacodylate buffer overnight, then fixed in 2% W/V osmium tetroxide in 0.1M sodium cacodylate buffer (pH = 7.4) for 4 hours, rinsed in 2% W/V sodium acetate and bulk stained in 2% W/V uranyl acetate. The tissue blocks were then dehydrated in an ethanol series (30-100% dry) over 3 hours, transferred to 100% acetone (dry, 2x15 minutes) and infiltrated with 1:1 and 1:9 acetone:Spurrs resin for 1 hour and 12 hours respectively. The tissue blocks were then placed at 60°C for 1 hour in pure resin and finally cured in resin at 60°C overnight. Sections were post stained with lead citrate (Reynolds, 1963).

4.3.2 Sampling and Sectioning of Tissues

For each tissue, ten (12 in the case of the liver) ultrathin sections (60-150 nm) were cut, each section was cut from a separate random tissue block. Sections were cut on an LKB 8800 III ultramicrotome using glass knives and supported on 200μm copper mesh grids.

4.3.3 Electron Microscopy and Stereology

From each section two electron micrographs at x6,200 and x53,000 were taken with a Jem 100U electron microscope (at 120KV). The x6,200 micrographs were used to determine relative mitochondrial volume densities and inner mitochondrial membrane surface areas. The x53,000 micrographs were used for the determination of cristae membrane surface areas. Relative volume densities of the mitochondria
(V\textsubscript{v}) were estimated by projecting (Kodak, Carousel S Projector) electron micrographs onto a 28cm square screen with a 100 point lattice test system. The surface area densities (S\textsubscript{V\textsubscript{i}}) of the mitochondrial membranes were found from the projection of electron micrographs onto similar screens and counting the number of intersections (I\textsubscript{i}) of the membranes with six diameters of known length within a circular test grid, using the equation

\[
S_{V_i} = \frac{2I_i}{L_f}
\]  
Eqn. 4.1

where S\textsubscript{V\textsubscript{i}} equals the surface area per volume ratio (m\textsuperscript{2}.cm\textsuperscript{-3}), from Weibel (1969). Cristae membrane surface areas (S\textsubscript{V\textsubscript{c}}), determined from within the mitochondria using the x53,000 electron micrographs are in units of m\textsuperscript{2} of cristae membrane per cm\textsuperscript{3} of mitochondria. Inner membrane surface areas (S\textsubscript{V\textsubscript{im}}) determined from the x6,200 micrographs and therefore relative to the tissue volume, were in units of m\textsuperscript{2} of inner mitochondrial membrane per cm\textsuperscript{3} of tissue and are a function of both the size, shape and number of the mitochondria. The mitochondrial membrane surface area per cm\textsuperscript{3} (S\textsubscript{V}) for each tissue was determined using the following equation

\[
S_V = \left(\frac{S_{Vc}.V_v}{100}\right) + S_{Vim}
\]  
Eqn. 4.2

where S\textsubscript{V\textsubscript{c}} equals cristae surface area densities (m\textsuperscript{2}.cm\textsuperscript{-3} of mitochondria), S\textsubscript{V\textsubscript{im}} equals inner membrane surface area densities (m\textsuperscript{2}.cm\textsuperscript{-3} of tissue) and V\textsubscript{v} equals mitochondrial volume densities (% of tissue volume). The units of S\textsubscript{V} equal the m\textsuperscript{2} of mitochondrial membrane per cubic centimetre of tissue. Total tissue mitochondrial membrane surface areas (m\textsuperscript{2}) were derived by multiplying S\textsubscript{V} by respective tissue weights (assuming the specific weight of the tissues to be that of water).
4.3.4 Statistics

Equations used for the biostatistical analysis were obtained from Zar (1974). All allometric relationships were determined and fitted using least squares linear regression analysis on a Texas SR-51-II calculator.
4.4 Results

4.4.1 Tissue Weights

The weights of the liver, kidney, brain, heart, lung and skeletal muscle (total body) for each animal are plotted in Figure 4.1 and the corresponding allometric equations are given in Table 4.1. The allometric exponents found in this study for each tissue are very similar to those found by other authors. The liver exponent (0.87) is the same as that found by Brody (1945) and similar to that found (0.89) by Prothero (1982). The kidney exponent (0.78) is lower than found (0.85) by Brody (1945). The brain exponent (0.69) is the same as found by Brody (1945) and slightly less than found (0.72) by Harvey and Bennett (1983). The heart exponent (0.83) is between 0.77 (Addis and Gray, 1950) and 0.98 (Brody, 1945; Stahl, 1967 and Prothero, 1979). The lung at 0.90 is slightly less than that found by Brody (1945) and Stahl (1967) at 0.99. The skeletal muscle exponent (1.01) is lower than that reported by Heusner (1964) at 1.14.

4.4.2 Mitochondrial Volume Densities and Membrane Surface Areas

The values for each animal's liver, kidney, brain, heart, lung and skeletal muscle (gastrocnemius) mitochondrial volume densities are plotted in Figure 4.1 and the allometric equations are given in Table 4.1. The mitochondrial membrane surface areas of the six tissues per cm^3 or gram of tissue (derived using equation 4.2) and per total tissue are plotted in Figure 4.2 and the allometric equations are given in Table 4.1.
Figure 4.1 The relation between body weight and tissue weight (upper graph) and percent mitochondrial volume densities (lower graph) in six mammalian tissues.
Figure 4.2 The relation between body weight and mitochondrial membrane surface areas, per cubic centimetre of tissue [upper graph] and per total tissue [lower graph] in six mammalian tissues.
<table>
<thead>
<tr>
<th>Tissue</th>
<th>Mitochondria</th>
<th>Density (%)</th>
<th>Volume (£)</th>
<th>Membrane Surface Area (£)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skeletal muscle</td>
<td>7</td>
<td>10</td>
<td>12</td>
<td>9.6</td>
</tr>
<tr>
<td>Lung</td>
<td>7</td>
<td>10</td>
<td>12</td>
<td>9.6</td>
</tr>
<tr>
<td>Heart</td>
<td>9</td>
<td>10</td>
<td>12</td>
<td>9.6</td>
</tr>
<tr>
<td>Brain</td>
<td>9</td>
<td>10</td>
<td>12</td>
<td>9.6</td>
</tr>
<tr>
<td>Kidney</td>
<td>10</td>
<td>10</td>
<td>12</td>
<td>9.6</td>
</tr>
<tr>
<td>Liver</td>
<td>10</td>
<td>10</td>
<td>12</td>
<td>9.6</td>
</tr>
</tbody>
</table>

For each tissue, the table shows the number of animals and the correlation coefficient. The correlation is significant at 0.05 level (p < 0.05).
4.5 Discussion

In a review of the relation between metabolic rate, body weight and organ size, Holliday et al., (1967) made some simple observations. They noted that as mammals become larger their metabolically active internal organs become relatively smaller. This is shown in our present study with all the internal tissues (except skeletal muscle) having weight related allometric slopes of less than one (Table 4.1 and Figure 4.1). Also noted by Holliday et al. was that some of the highly active internal organs (e.g. liver and kidney) have lower metabolic rates per gram in mammals of increasing size. They postulated that the lower basal metabolic rate per gram in larger mammal species is due to these two factors.

Relating mitochondrial capacities to oxygen consumption of the tissues then we should expect similar relationships. Total tissue mitochondrial capacities should have an allometric exponent of approximately 0.75 and per unit weight an exponent of approximately -0.25. The mitochondrial membrane surface area per cubic centimetre of tissue for the six tissues examined produced allometric slopes (Table 4.1 and Figure 4.2) ranging from -0.24 in the liver to -0.07 in the lung. Using the data of Kleiber (1941) and Krebs (1950) for "in vitro" tissue metabolism in mammals, exponents of -0.24 for the liver, -0.10 for the lung, -0.06 and -0.07 for the kidney and brain respectively are obtained per unit weight over a broad body weight range.
It is of interest that when the tissues are ranked in order of the highest to the lowest oxygen consumption per gram (Krebs, 1950) for the rat and rabbit, these two species being the only two mammals common to both studies, show the same rank order when we compare their mitochondrial membrane surface areas per gram of tissue found in this present study. It is also of interest that at least for the rat and rabbit, the tissues which receive the highest cardiac outputs (Neutze et al., 1968; Sasaki et al., 1971), also show the highest mitochondrial membrane surface areas.

The mitochondrial volume densities of the tissues and mitochondrial membrane surface area densities per cubic centimetre of mitochondria both show negative allometric exponents for all six tissues measured. The exponents significantly different from zero are for mitochondrial volume densities liver, kidney and heart and for the mitochondrial membrane surface area densities per cubic centimetre of mitochondria liver and brain (Table 4.1). These exponents all suggest decreasing mitochondrial capacities per gram of tissue with increasing body size and this is shown in the mitochondrial membrane surface areas per gram of tissue for all six tissues measured with liver, kidney, brain and heart all showing allometric exponents significantly less than zero (Table 4.1).

The values found for the (heart and liver) mitochondrial membrane surface areas and mitochondrial volume densities in the rat are very similar to the limited values available in the literature (Reith, 1973; Reith and Fuchs, 1973 and Reith et al., 1973; also see Table 2.8). The theoretical limit for the ratio of mitochondrial membrane
surface area to mitochondrial volume \((m^2.cm^{-3} of mitochondria)\) has been calculated as 133 \(m^2.cm^{-3}\) (Srere, 1982). The highest values found in this study for any single determination of mitochondrial membrane surface area per cubic centimetre of mitochondrial were 114, 114 and 105 \(m^2.cm^{-3}\) in \textit{A. stuartii} heart mitochondria, rat heart mitochondria, and rat muscle mitochondria respectively. These values are unusually high, generally the values for other samples from these tissues as well as other tissues from these and other species were far below these upper limits. In all tissues, it was found that the mitochondria of small animals were more densely packed with mitochondrial membranes than the corresponding tissue from large animals (Table 4.1). The exponents ranged from -0.08 in heart mitochondria to -0.17 in lung mitochondria. In gastrocnemius muscle mitochondria the exponent was -0.13 and thus smaller animals had a greater amount of membrane in their muscle mitochondria than large animals, this is in contrast to the findings of Hoppeler et al., (1981). Hoppeler et al., concluded that there is no difference in the density of internal mitochondrial membranes in skeletal muscle as a result of changing body size from the study of 3 species (in some cases 2 species only) and different muscles from that used in this study. In this present study all six mammalian tissues showed decreasing internal mitochondrial membrane densities with increasing body size and these changes were significant in liver and brain mitochondria (see Table 4.1). An alternative to increasing mitochondrial capacities of the tissues by increasing the internal mitochondrial membrane surface area densities of the mitochondria is to increase the tissue mitochondrial volume densities, this was found in all the small mammals measured in this present study. The
mitochondrial volume densities per gram of tissue increased with decreasing body weight (Figure 4.1 and Table 4.1).

The total tissue mitochondrial membrane surface areas for the six tissues examined (Table 4.1 and Figure 4.2) have allometric slopes ranging from 0.55 for the kidney up to 0.78 for skeletal muscle (using total skeletal muscle weight and gastrocnemius mitochondrial data). These exponents for the tissues are similar to those for summated total tissue (Davies, 1961) and organismal oxygen consumptions (Kleiber, 1961). In comparing mitochondrial membrane surface areas to the oxygen consumption of an organism, it is important to know what tissues are consuming oxygen or alternatively what tissues are using a major part of their mitochondrial capacities. Considering standard metabolism firstly, it is the active internal organs which are major contributors. For example, the liver, heart, brain and kidney, weigh only 6% of the total body weight in man but account for approximately 70% of the total oxygen consumption at rest (Drabkin, 1950). Obviously the internal organs are much more active in comparison to the muscle, yet presumably they still do not use their full metabolic capacity under the conditions that standard metabolism is measured. To use their total capacity would leave no potential for further activity above that of resting levels. If we assume that the tissues use a constant proportion of their oxygen consuming capacity and therefore total mitochondrial capacity in each animal, we would expect similar allometric exponents to standard metabolism when the same four internal organs mitochondrial surface areas are summated for each mammal. When this is done the allometric equation is obtained:
Summated Organ Mitochondrial Membrane Surface Area (m²)  

\[ = 3.04W^{0.59} \]  

(W in grams, \( r = 0.98 \))  

When the standard organismal oxygen consumption values for the same five species, taken from the literature (Lee, 1929; Kibler, 1963; Dawson and Hulbert, 1970; Dawson and Grant, 1980 and Else and Hulbert, 1981) and corrected for a body temperature of 37°C (Q₁₀ assumed 2.5) is also plotted allometrically (see Figure 4.3) we get the equation  

Standard Metabolism (m₁₀₂.hr⁻¹)  

\[ = 6.17 W^{0.62} \]  

(W in grams, \( r = 0.99 \))  

The similarity between the two exponents is interesting since standard oxygen consumption normally scales allometrically with an exponent approximating 0.75. The mammals used in the present study have produced a lower allometric oxygen consumption exponent because of the peculiar phylogenetic mix of species but it is an exponent that is very similar to the summated organ mitochondrial membrane surface area exponent. These suggest total mitochondrial membrane surface area is a realistic measure of metabolic capacity and supports the assumption that the major internal organs are using a constant proportion of their mitochondrial capacity at rest.  

As previously noted, total skeletal muscle weight is the only tissue examined with an allometric slope greater than one. Since body weight equals the sum of its tissue weights and in general, tissue weights have exponents less than one then there must be at least one tissue which has a tissue weight exponent greater than one. Skeletal muscle is one of these compensating tissues. Skeletal muscle mitochondrial membrane surface area per gram has an allometric slope of -0.23 (Table 4.1 and Figure 4.2), which is similar to that of other
tissues (liver and kidney particularly). Total skeletal muscle weight has an allometric slope of 1.01 and therefore this produces a total mitochondrial membrane surface area exponent of 0.78, the highest for any of the tissues examined.

One possible limitation of this study is the use of the gastrocnemius muscle as representative of skeletal muscle in general. Mammalian skeletal muscles are composed (to varying degrees) of three different types of muscle fibres: fast glycolytic (FG), fast oxidative glycolytic (FOG) and slow oxidative (SO) fibres. These three different fibre types have different metabolic profiles that are largely related to the different amounts of mitochondria in the different fibre types (Peter et al., 1972). In the guinea pig the white vastus muscle (which is composed overwhelmingly of FG fibres) has a mitochondrial volume of 1.9% cell volume (Eisenberg and Kuda, 1975) whereas the soleus muscle (composed predominantly of SO fibres) has a mitochondrial volume of 4.85% of cell volume (Eisenberg, Kuda and Peter, 1974).

Arians et al. (1973) surveyed the relative fibre composition of the hind limb muscles in five species of mammals (15-31 different muscles per species). The average percent composition of all these muscles was 46:29:25 (FG:FOG:SO) whilst the average percent composition of the gastrocnemius muscle in these five mammals was 55:25:20 respectively. Hoppeler et al. (1981) in their examination of mitochondrial volume densities in twenty different muscles in the wildebeest and dik-dik found an average mitochondrial volume of 3.85% and 4.21% of cell volume in the respective species. Their values for
the gastrocnemius muscle were 2.32% and 3.27% of cell volume in these respective species. Testing the predictive nature of the allometric equation found in this present study from gastrocnemius mitochondrial volume densities, the predicted volume densities for the wildebeest and the dik-dik gastrocnemius muscles are 2.89% and 3.84% respectively.

The conclusion to be reached from both these studies is that the gastrocnemius is a mixed muscle whose relative composition is not drastically different from the "average" muscle but that its use as a representative muscle probably slightly underestimates the relative amount of mitochondria in the total body musculature.

There is a good body of information concerned with the allometric scaling of mitochondrial volume densities ($V_v$) for different skeletal muscles in mammals. The skeletal muscles M. semitendinosus, M. longissimus dorsi, M. vastus medialis and diaphragm have been shown to scale for mitochondrial volume densities with slopes of -0.23, -0.16, -0.14 and -0.06 respectively per gram of muscle (Mathieu et al., 1981). All of these slopes were found to be significantly less than zero. From the animals used in this present study, the mitochondrial volume densities for the gastrocnemius produce an exponent of -0.09 (Table 4.1, Figure 4.1) similar to those for other skeletal muscles. The total volume of mitochondria for three of the four skeletal muscles examined by Mathieu et al. (1981) (longissimus, not given) scaled allometrically with exponents of 0.80, 0.82, and 0.81. The mitochondrial volume data from the present study similarly scales with an exponent of 0.92 (using total body skeletal muscle weight). This exponent is reduced to 0.78 when the additional effect of membrane surface area is considered.
Skeletal muscle is the major contributor to maximal organismal oxygen consumption. When an organism is active, it is presumably the skeletal muscle that uses the bulk of the oxygen consumed. If the total mitochondrial membrane surface area of the organs including the lung are added to those of skeletal muscle for each mammal and compared allometrically (see Figure 4.3), the equation we obtain is:

\[
\text{Summated Tissue Mitochondrial Membrane Surface Area (m}^2) = 6.85W^{-0.76} \quad (W \text{ in grams, } r = 0.77)
\]

The values for maximum oxygen consumption for the same four species examined is not available. The maximum rate of oxygen consumption found by Taylor et al. (1980) for 22 species of mammals (7g to 203Kg) is described by the equation:

\[
\text{Maximum Metabolism (m}^{102}.\text{sec}^{-1}) = 1.94W^{-0.79} \quad (W \text{ in kgs, } r = 0.99)
\]

The 0.76 and 0.79 exponents for summated tissue mitochondrial membrane surface area and maximum metabolism are greater than those for summated organ mitochondrial membrane surface area and standard metabolism, this is caused by the influence of the skeletal muscle.

The summation of the mitochondrial membrane surface areas from the various tissues is presented graphically for the rat in Figure 4.4. From this graph, it can be seen that the muscle mitochondrial membrane surface area is the overwhelming contributor to the total mitochondrial membrane surface area being responsible for 82% of the total in the rat. In general a mammal's maximal oxygen consumption is approximately 10 times its standard metabolism. Total mitochondrial membrane surface area for the muscle and major internal organs is 5-16 times that of the total mitochondrial membrane surface area of the internal organs alone for the individual mammals used. The mammals
Figure 4.3 The relation between body weight and standard (open symbols) and maximum oxygen consumptions, summated organs (liver, kidney, brain and heart) and summated organs plus skeletal muscle (liver, kidney, brain, heart, lung and skeletal muscle) mitochondrial membrane surface areas (closed symbols) in mammals.
A comparison of mitochondrial membrane surface areas of the summated organs and summated total tissues in the rat.
with the lowest differences were the laboratory animals, the rat and rabbit at 5-6 times, the highest were the wild animals, the echidna (*T. aculeatus*) and bandicoot (*P. nasuta*) at 7-16 times. This trend has also been found in the standard and maximal oxygen consumption of wild and domestic animals (Taylor *et al.*, 1980). The monotreme and marsupials fit well on the mammal line for most of the parameters measured. They show no tendency toward any reduction of total mitochondrial capacity similar to those found in reptiles (Chapter 5).

In summary the major points of this discussion are that the tissue weights have allometric slopes (except for total skeletal muscle) of less than one but generally greater than that for organismal oxygen consumption (i.e., they are generally between 0.75 and 1.00). Mitochondrial surface area per cm$^3$ of tissue, as "in vitro" tissue oxygen consumption, have negative allometric slopes (excluding skeletal muscle) between -0.07 and -0.24 and this results in total tissue mitochondrial membrane surface area exponents of between 0.55 and 0.67 for all tissues. These are obtained by multiplying tissue mitochondrial membrane surface area per cm$^3$ for each tissue by respective tissue weights. Summating the total tissue mitochondrial membrane surface area of those organs most responsible for standard metabolism, we obtained an allometric slope of 0.59. The standard metabolism allometric slope for the same mammals (values from the literature) used to determine the mitochondrial capacities is 0.62. Summating the same organs and adding lung and total skeletal muscle mitochondrial membrane surface areas, we obtain an allometric slope of 0.76, a result of the large mitochondrial capacity of the skeletal muscle. From the literature (Taylor *et al.*, 1980) the allometric slope of maximum metabolism for mammals is 0.79.
Table 4.2 The allometric equations of summated tissue weights and mitochondrial membrane surface areas in mammals.

<table>
<thead>
<tr>
<th>Summated Organs</th>
<th>Liver</th>
<th>Kidney</th>
<th>Brain</th>
<th>Heart</th>
<th>Total Tissue Weight (g)</th>
<th>Mitochondrial Membrane Surface Area (m²) per total of tissues</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>9</td>
<td></td>
<td></td>
<td></td>
<td>0.18W₀.₈₃(⁺₀.₁₁, ₀.₉₉)</td>
<td>3.04W₀.₅₉(⁺₀.₁₁, ₀.₉₈)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Summated Tissue</th>
<th>Liver</th>
<th>Kidney</th>
<th>Brain</th>
<th>Heart</th>
<th>Lung</th>
<th>Skeletal Muscle</th>
<th>Total Tissue Weight (g)</th>
<th>Mitochondrial Membrane Surface Area (m²) per total of tissues</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.₅₁W₁.₀₀(⁺₀.₃₁, ₀.₉₆)</td>
<td>6.₈₅W₀.₇₆(⁺₀.₇₃, ₀.₇₇)</td>
</tr>
</tbody>
</table>

n = number of animals
( ) Within the brackets firstly the ± 95% confidence limit for each exponent and secondly the correlation coefficient.
* Denotes exponents statistically significant from 0 (p < 0.05)
Finally, in order to obtain some relative idea of the surface areas examined in this present study, I would like to point out that the total summated tissue mitochondrial membrane surface area in the two rats studied (mean body weight of 266g) averaged 460 m². This is slightly larger than the size of a basketball court. Similarly, the total summated tissue mitochondrial membrane surface area of the larger bandicoot measured in the present study (body weight of 1.8Kg) is equivalent to the surface area of twelve basketball courts.
CHAPTER 5

AN ALLOMETRIC COMPARISON OF THE METABOLIC CAPACITIES OF
MAMMALIAN AND REPTILIAN TISSUES: THE EFFECTS OF PHYLOGENY
AND THE EVOLUTION OF ENDOTHERMY
5.1 Introduction

As well as body size a major influence on metabolism is phylogeny. Both mammals (Kleiber, 1961 and Dawson and Hulbert, 1970) and ectothermic vertebrates (Hemmingsen, 1960; Bennett and Dawson, 1976 and Taigen, 1983) show the same general relationship between standard metabolism and body size, i.e., \( M = aW^{0.75} \). The differences between the two groups of animals lies in the level of their total organismal oxygen consumptions. The mammals consume vastly greater amounts of oxygen (5-10x) than similar sized ectotherms measured at the same body temperatures (Dawson and Hulbert, 1970 and Bennett and Dawson, 1976). These differences are shown in the "a" constants (or elevations) which are higher for mammals than for ectothermic vertebrates.

As previously noted for mammals (Chapter 4, Introduction) the relationship between body size and oxygen consumption shows effects at many different levels of organization from ecological and physiological, through to cellular and subcellular levels. Similar allometric relationships between organismal metabolism and body size are also reflected at different levels within the biology of the ectothermic vertebrates. As a result of the phylogenetic differences between the mammals and reptiles the elevations or "a" constants show phylogenetic related effects at the different levels of organization between the mammals and ectothermic vertebrates.

The field metabolic rates of lizards during the active season are
allometrically correlated with body mass \( W^{0.80} \) but are twenty times lower than for similar sized mammals (Nagy, 1983). Respiratory variables such as heart weight in mammals (Stahl, 1967 and Prothero, 1979) and fish (Poupa et al., 1981) scale allometrically with similar slopes but the mammals have larger hearts as indicated by the 5 times higher value of their "elevation" constant. The pulmonary surface area in mammals and reptiles scale with exponents of 0.75 and for amphibians as 0.80 (Tenney and Tenney, 1970), yet the pulmonary diffusing capacity in a mammal appears to be about five times greater than for a similar sized reptile at the same body temperature (Glass and Johansen, 1981).

In the transport and delivery of oxygen to the tissues reptiles have lower haematocrits, lower blood oxygen capacities and lower oxygen affinities than similar sized mammals (Pough, 1980). The ectothermic vertebrates also have reduced heart rates compared to mammals (Clark, 1927 and Templeton, 1970), although heart rates scale allometrically with slopes of -0.25 in mammals (Schmidt-Nielsen, 1979) and -0.21 in reptiles (Licht, 1965). In an intraspecies comparison of fish and an interspecies comparison of mammalian aerobic enzymes, activities scale (per gram of tissue) with negative slopes characteristic of aerobic metabolism whereas in both groups glycolytic enzymes do not (Somero and Childress, 1980 and Emmett and Hochachka, 1981). Yet the aerobic enzyme capacities of mammalian tissues have been shown to be greater than those for similar sized reptiles measured at the same temperature (Bennett, 1972; Else and Hulbert, 1981; and Chapter 2).
In the evolution of mammals from reptiles there appears to have been a transition from low to high metabolic capacities and all its associated activities correlated. In Chapter 2 in a comparison between a single mammalian and reptilian species, of similar body weight and preferred body temperature, large differences in the mitochondrial capacities of the tissues from each species were found. These differences always showed increased metabolic capacities in the mammalian tissues compared to the same reptilian tissues. In a further comparison in Chapter 4 the mitochondrial capacities of six tissues, measured by determining the mitochondrial membrane surface areas, from six diverse mammalian species over a 100 fold weight range showed allometric exponents characteristic of those for aerobic metabolism of the whole animal. In this present chapter the mitochondrial capacities of the same six tissues from 4 reptilian species representing 3 reptilian subclasses and a 100 fold weight range are examined. Mitochondrial volume densities, membrane surface area densities, tissue weights, tissue specific and total tissue mitochondrial membrane surface areas are determined and compared allometrically. There are no previous comparisons that are concerned with the suborganismal allometric scaling of metabolic variables in reptiles available other than for lung (Tenney and Tenney, 1970) and blood parameters (Pough, 1980).

Since it is impossible to examine the original ancestral reptiles it is necessary to work with contemporary representatives. Many of the reptilian groups present today are almost as isolated by time from one another as they are from the early group of reptiles whose descendants are now the mammals. The inference is that if the various
reptilian groups display similar metabolic characteristics then those characteristics are probably ancestral. Subsequent differences in the metabolic capacities between mammals and reptiles are probably a result of the ectothermic to endothermic transition.
Animals

The reptilian species used in the comparison are the lizards, Amphibolurus nuchalis (body weights 29g and 36g) and Amphibolurus vitticeps (246g and 249g), the freshwater tortoise, Chelodina longicollis (575g and 582g) and freshwater crocodile, Crocodylus johnstoni (242g and 2286g). The lizards were maintained in temperature controlled boxes (as previously described in Chapter 2) at 37±2°C. The freshwater tortoises were kept at room temperature (25°C) in a large filtered, aerated pool with access to a large dry pen area. The crocodiles were obtained from Sydney University. The lizards and tortoises were fed daily on a varied fruit, meat, mealworm, cockroach diet. They were supplied with ad libitum water and a 12:12 light:dark photoperiod. The mammals used are the same animals used in Chapter 4, pictures of the animal species used are provided in Plates 5.1 and 5.2.
Plate 5.1 Reptiles

Amphibolurus nuchalis
Amphibolurus vitticeps
Chelodina longicollis
Crocodylus johnstoni
Plate 5.2 Mammals

Mus musculus  Antechinus stuartii  Rattus norvegicus

Perameles nasuta  Tachyglossus aculeatus  Oryctolagus cuniculus
5.3 Method

Two animals from each species were used to determine the mitochondrial volume and membrane surface area densities and tissue weights. The tissues used were the liver, kidney, brain, heart, lung and skeletal muscle (gastrocnemius muscle) except for A. nuchalis where only liver, kidney, brain and heart values are available. These values for A. nuchalis are those previously reported (Else and Hulbert, 1981) and are not included as part of the work of this thesis but are used to increase both the extent and confidence of the comparison. For skeletal muscle the mitochondrial parameters were measured only on the gastrocnemius muscle but muscle weight includes all skeletal muscle. The methods used in the tissue preparation, sampling, sectioning, microscopy and stereology are as described in the Methods of Chapter 4.

5.4 Statistics

Equations used for the biostatistical analysis were obtained from Zar (1974). Mammalian and reptilian allometric slopes were tested for significant differences. In the two only cases where the slopes of the mammalian and reptilian regressions were found to be different no further statistical analysis was performed. When the mammalian and reptilian slopes were found to be parallel (i.e. statistically the same slopes), the elevations were tested for significant differences. Percentage differences (Table 5.2) between the mammalian and reptilian mitochondrial parameters were calculated from common slopes (where slopes statistically parallel) and adjusted mean scores (Kleinbaum and Kupper, 1978).
5.5 Results

The liver, kidney, brain, heart and skeletal muscle tissues are all larger in mammals than in reptiles. These results are shown in Figure 5.1 and the corresponding allometric equations for the reptiles are given in Table 5.1 (and for the mammals in Table 4.1). In all figures each point represents the data from a single animal.

The lung is the only organ to have a similar weight in both the mammals and reptiles, although the reptiles include two aquatic species. The reptilian lung allometric slope value of 0.73 is less than 1.0 found by Tenney and Tenney (1970). The allometric slope for the heart weight of the reptiles, 0.77 is less than that found for sea fish at 0.89 (Poupa et al., 1981). The skeletal muscle exponent of 1.09 is very similar to those found for intraspecific comparisons in teleost fish with exponents ranging from 0.90 up to 1.21 (Somero and Childress, 1980).

The mitochondrial volume densities of the six mammalian and reptilian tissues are compared in Figure 5.2 and the allometric equations for the reptilian tissues given in Table 5.1. Reptilian liver, kidney and heart tissues all scale with negative slopes as do all the mammalian tissues (except brain, slope = 0), with the mammalian liver, kidney and heart scaling with negative slopes significantly different from zero. All six tissues show statistically parallel slopes between the mammals and reptiles. The liver, kidney and heart also show significantly different elevations in
Figure 5.1 A comparison of mammalian [○] and reptilian [●] tissue weights.
Figure 5.2 A comparison of mammalian [O] and reptilian [●] tissue mitochondrial volume densities.
mitochondrial volume densities per cm$^3$ of tissue between the mammals and reptiles. The average difference between the mammalian and reptilian tissues is a 60% increase in the mitochondrial volume density in the mammalian tissues.

Within the mitochondria the internal membrane surface area density differs only slightly between the mammals and reptiles. The mitochondrial membrane surface area densities per cm$^3$ of mitochondria for the mammalian and reptilian tissue are compared allometrically in Figure 5.3, the corresponding allometric equations for the reptilian tissues are given in Table 5.1. The liver and brain of the mammals show negative slopes significantly different from zero whereas none of the reptilian tissues show slopes significantly different from zero. The mitochondrial membrane surface area densities per cm$^3$ of mitochondria for the mammalian and reptilian tissues show no statistical differences in slope (with the exceptions of brain and heart mitochondria) or elevation values (Table 5.1).

Using data for mitochondrial volume densities, cristae and inner membrane surface area densities, the mitochondrial membrane surface area for the six reptilian tissues have been determined using equation 4.2. These values are compared to the mammalian values in Figure 5.4 and the allometric equations for the reptilian tissues are given in Table 5.1. All the mammalian tissues scale with negative slopes and those for liver, kidney, brain and heart tissues are significantly different from zero (see Chapter 4, Table 4.1). None of the reptilian tissue slopes are significantly different from zero. All the mammalian and reptilian slopes are statistically parallel, with
Figure 5.3 A comparison of mammalian [○] and reptilian [●] mitochondrial membrane surface area densities.
Figure 5.4 A comparison of mammalian (〇) and reptilian (●) tissue mitochondrial membrane surface areas.

The differences in the mitochondrial membrane surface area per g of tissue between the mammals and reptiles increase when the effect of tissue size is included. Yet the mammalian mitochondrial membrane surface area for the mammalian skeletal muscle. For the mammals the tissues which show significantly different lines are with the mammalian skeletal muscle. For the reptiles, kidney and heart although the tissues at similar body weight are of some interest.
liver, heart and kidney all showing statistically different elevations between the mammals and reptiles. The differences in the mitochondrial membrane surface areas per cubic centimetre of tissue between the mammals and reptiles range from 10% greater in the brain to 110% greater in the liver.

The differences in the mitochondrial membrane surface area densities per gram of tissue between the mammals and reptiles are further increased when the effect of tissue size is included. Total tissue mitochondrial membrane surface area for the mammalian and reptilian tissues are compared in Figure 5.5. The allometric equations for the reptilian values are given in Table 5.1. All slopes for both the mammals and the reptiles are positive and parallel and all significantly different from zero with the one exception of mammalian skeletal muscle. For the mammals the tissues which show exponents significantly less than one are liver, kidney, brain and heart, and for the reptiles kidney, brain and heart. Although the slopes are parallel for total tissue mitochondrial membrane surface area, the liver, kidney, brain and heart tissues all show statistically different elevations between the mammals and reptiles.

Examples of the electron micrographs of echidna and crocodile tissues and their mitochondria are given in Plates 5.3 to 5.6. These animals are used as representatives of the mammals and reptiles simply because they are of similar body size and may be of more interest than other species measured. One interesting features is the slight concentric packing of the cristae within the reptilian mitochondria. This feature was not obvious in mammalian mitochondria examined but was very common in reptilian mitochondria.
Figure 5.5 A comparison of mammalian [O] and reptilian [●] total tissue mitochondrial membrane surface areas.
### Table 5.1: The Allometric Equations for Tissue Masses

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Mitochondrial Membrane Surface Area</th>
<th>Mitochondrial Membrane Surface Area in Tissues of Replicates</th>
<th>Mitochondrial Membrane Surface Area in Tissues of Replicates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart</td>
<td>1.10 W 0.081</td>
<td>18.30 W 0.098</td>
<td>14.30 W 0.102</td>
</tr>
<tr>
<td>Brain</td>
<td>2.80 W 0.057</td>
<td>16.50 W 0.071</td>
<td>20.50 W 0.076</td>
</tr>
<tr>
<td>Kidney</td>
<td>4.60 W 0.088</td>
<td>19.30 W 0.099</td>
<td>17.50 W 0.099</td>
</tr>
<tr>
<td>Liver</td>
<td>6.20 W 0.095</td>
<td>16.90 W 0.095</td>
<td>15.80 W 0.094</td>
</tr>
</tbody>
</table>

### Table 5.2: The Allometric Equations for Tissue Masses and Mitochondrial Volume

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Mitochondrial Volume Density</th>
<th>Mitochondrial Volume Density in Tissues of Replicates</th>
<th>Mitochondrial Volume Density in Tissues of Replicates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart</td>
<td>2.40 W 0.082</td>
<td>18.30 W 0.098</td>
<td>14.30 W 0.102</td>
</tr>
<tr>
<td>Brain</td>
<td>5.30 W 0.101</td>
<td>16.50 W 0.071</td>
<td>20.50 W 0.076</td>
</tr>
<tr>
<td>Kidney</td>
<td>7.60 W 0.097</td>
<td>19.30 W 0.099</td>
<td>17.50 W 0.099</td>
</tr>
<tr>
<td>Liver</td>
<td>9.80 W 0.104</td>
<td>16.90 W 0.095</td>
<td>15.80 W 0.094</td>
</tr>
</tbody>
</table>
Plate 5.4 Tissues ×6,200

Echidna

Heart

Lung

Crocodile

Skeletal muscle
Plate 5.5 Mitochondria ×53,000

Echidna
Liver
Kidney
Brain

Crocodile
Plate 5.6 Mitochondria ×53,000

Echidna

Heart

Lung

Crocodile

Skeletal muscle
5.6 Discussion

In both mammals and reptiles the total mitochondrial membrane surface areas of the tissues show allometric exponents characteristic of those which describe metabolism of the whole animal. In the majority of tissues these exponents are made up of tissue weights with allometric slopes between 0.75 and 1.00 and weight specific tissue mitochondrial membrane surface area densities with slopes between -0.25 and zero. Although the slopes of the allometric equations describing total tissue mitochondrial membrane surface area are statistically the same, in the mammalian tissues the elevations are significantly higher, approximately 4 times higher in the liver, brain and kidney and 3 times higher in the heart. Not significant but also higher in the mammals is the skeletal muscle (2.2 times) and the lung (1.4 times) total tissue mitochondrial membrane surface areas Table 5.1 and 5.2.

These differences are not the result of any single "quantum" factor but are primarily a result of the mammals having relatively larger tissues with a greater proportion of their volume occupied by mitochondria. Internal mitochondrial membrane surface area densities i.e. per cm$^3$ of mitochondria show no large differences between the mammalian and reptilian mitochondria and therefore do not contribute overall to the observed differences. All differences measured for tissue sizes and mitochondrial parameters in mammalian and reptilian tissues regardless of how small the difference are always to the increased metabolic advantage of the mammalian tissues, Table 5.2. The factors which contribute to the observed differences between the
**Table 5.2 Relative Differences of Mitochondrial Parameters: Mammals Compared to Reptiles.**

<table>
<thead>
<tr>
<th>Organ</th>
<th>Inner Mitochondrial Membrane Surface Area (m² cm⁻³ of tissue)</th>
<th>Cristae Mitochondrial Membrane Surface Area (m² cm⁻³ of mito.)</th>
<th>Mitochondrial Volume Density (%) of cell volume</th>
<th>Mitochondrial Membrane Surface Area (m² cm⁻³ of tissue)</th>
<th>Total Tissue Membrane Surface Area (m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIVER</td>
<td>186%</td>
<td>103%</td>
<td>219%</td>
<td>213%</td>
<td>395%</td>
</tr>
<tr>
<td>KIDNEY</td>
<td>253%</td>
<td>106%</td>
<td>156%</td>
<td>156%</td>
<td>404%</td>
</tr>
<tr>
<td>BRAIN</td>
<td>374%</td>
<td>116%</td>
<td>133%</td>
<td>107%</td>
<td>439%</td>
</tr>
<tr>
<td>HEART</td>
<td>165%</td>
<td>142%</td>
<td>166%</td>
<td>174%</td>
<td>288%</td>
</tr>
<tr>
<td>LUNG</td>
<td>84%</td>
<td>156%</td>
<td>111%</td>
<td>148%</td>
<td>141%</td>
</tr>
<tr>
<td>SKELETAL MUSCLE</td>
<td>129%</td>
<td>119%</td>
<td>140%</td>
<td>172%</td>
<td>223%</td>
</tr>
</tbody>
</table>

Values are percentages with mammalian values expressed relative to a 100% reptilian value.

Percentage differences calculated from common slopes (where slopes statistically parallel) and adjusted mean scores.
mammalian and reptilian total tissue mitochondrial membrane surface areas also vary in their relative contributions depending upon the species.

The similarities between the allometric slopes for total tissue mitochondrial membrane surface area and standard and maximal metabolisms is interesting in light of the relationships which may be presumed to exist between mitochondria and aerobic metabolism. In reptiles standard metabolism (at 30°C) is best described by the allometric equation:

\[
\text{Standard Metabolism (mL_2.hr}^{-1}) = 0.28 W^{0.77}
\]

\[(W \text{ in grams, } r = 0.91, \text{ and } n = 44, \text{ Bennett and Dawson, 1976}).\]

Considering the largest and most active organs (liver, heart, kidney and brain) to be major contributors to standard metabolism and summating the mitochondrial membrane surface areas for these organs from each individual reptile measured in this present study, the allometric equation obtained is:

\[
\text{Summated Organ Mitochondrial Membrane Surface Area (m}^2) = 0.55 W^{0.67}
\]

\[(W \text{ in grams, } r = 0.96, \text{ and } n = 8)\]

The maximum metabolism of reptiles (at 30°C) is best described by the equation:

\[
\text{Maximum Metabolism (mL}_2\text{.hr}^{-1}) = 1.40 W^{0.82}\]

\[(W \text{ in grams, } r = 0.81, \text{ and } n = 14, \text{ Bennett and Dawson, 1976}).\]

Maximum metabolism is predominantly muscle metabolism (Jansky, 1965) and summating muscle and organ (liver, heart, kidney, brain and lung) mitochondrial membrane surface areas the allometric equation obtained for the reptiles is:
Summated Tissue Mitochondrial Membrane Surface Area (m²) = 0.30 W^{1.11}

(W in grams, r = 0.97, and n = 6)

The summated mitochondrial membrane surface areas show increased allometric slopes from summated organ to summated tissue mitochondrial membrane surface area, the same trend as found for standard and maximum organismal metabolism of reptiles.

The higher allometric slope for summated tissue mitochondrial membrane surface area is due to the effects of the skeletal muscle values and in particular the values from the freshwater reptile species. The largest of the two crocodiles had a large amount of skeletal muscle mass and the tortoises had extremely high mitochondrial volume densities in their gastrocnemius muscle. When isolating the gastrocnemius muscle from the tortoises it was noted that these muscles were extremely red. In a study of a single lizard species (Putnam et al., 1980) the red regions of the locomotory muscles were found to be small in relative mass (approximately 21%) but similar in muscle fibre number to white muscle regions. These red muscle regions were also found to have the highest number of fast oxidative glycolytic (FOG) and tonic fibres.

In mammals FOG fibres have the highest succinate dehydrogenase activity, cytochrome and myoglobin concentrations, also high glycogen concentrations and moderate lactate dehydrogenase activity. This indicates that FOG fibres should have only moderate glycolytic capacities but the highest aerobic potential compared to other muscle fibre types (Peter et al., 1972). The high aerobic capacity of these
### Table 5.3 The Allometric Equations for Summated Tissue Weights and Mitochondrial Membrane Surface Areas in Reptiles

<table>
<thead>
<tr>
<th>Summated Organs</th>
<th>Total Tissue Weight (grams)</th>
<th>Mitochondrial Membrane Surface Area (m²) per Total of Tissues</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>0.14 W 0.75*</td>
</tr>
<tr>
<td>LIVER</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>KIDNEY</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BRAIN</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HEART</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

N = number of animals

( ) Within the brackets firstly the ± 95% confidence limit for each exponent and secondly the correlation coefficient.

* Denotes exponents statistically significant from 0 (p < 0.05)
fibres should also be characterised by high mitochondrial volume or membrane densities and this appears to be the case in the rat. A comparison of red soleus and white gastrocnemius muscle shows mitochondrial surface area as a percentage of muscle fibre area in red fibres to be 6.5% and in white fibres 2.1% (Stonnington and Engel, 1973). This may also be the case in the tortoise gastrocnemius muscles which show high mitochondrial volume densities.

The allometric equation for total skeletal muscle in the reptiles as the mammals is based on gastrocnemius muscle mitochondrial parameters and total skeletal muscle mass. In a comparison of eleven separate locomotory muscles from a single lizard species (Putnam et al., 1980) the average muscle fibre composition was found to be FG:FOG:Tonic as 49:38:30 and for the gastrocnemius muscle of this species 54:32:14. These ratios for the lizard skeletal muscles are similar to the ratios for FG:FOG:SO found for mammalian skeletal muscles (Arians et al., 1973). In general the gastrocnemius is similar in its fibre composition to other skeletal muscles and probably as in mammals (Chapter 4, Discussion) its use as a representative muscle slightly underestimates the total aerobic capacity of the body musculature.

Over the weight range examined in this study reptiles in general have levels of maximal metabolism 6-7 times greater than their standard metabolism (Bennett and Dawson, 1976). Mammals also show similar differences between their standard and maximal metabolisms (see Chapter 4, Discussion). These differences between the standard and maximum metabolisms of the reptiles and mammals are shown in
Figure 5.6 Mammalian and reptilian standard and maximum organismal metabolisms.
Figure 5.6. This figure also shows that the general level of metabolism in mammals is approximately 7-20 times higher than in reptiles. In Figure 5.7 summated organ and summated tissue mitochondrial membrane surface areas for the mammals and reptiles are compared. The mammals and reptiles show 4-15 fold differences between their own summated organ and tissue mitochondrial membrane surface areas. The mammals also show 2-8 times greater summated organ and tissue mitochondrial membrane surface areas than for the reptiles, see Figure 5.7. These differences are not as large as those found for organismal metabolism between mammals and reptiles. The additional part of this difference may possibly be made up of increased enzyme numbers per unit of membrane in mammalian mitochondria.

Mitochondrial volume density is the major difference in the weight specific metabolism of tissues measured using mitochondrial parameters. It also appears from observation that mitochondrial volume densities are primarily a reflection of mitochondrial numbers per gram of tissue. This parameter therefore was a major factor involved in the evolution of endothermic capacities in mammals from reptiles and is a major factor involved in different weight specific metabolisms resulting from differences in body size. These differences may also be further combined with increases in enzyme number and activities within the mitochondria themselves of animals with high weight specific metabolisms.
Figure 5.7 A comparison of (○) mammalian and (■) reptilian (●■) organ (liver, heart, kidney and brain) and (○■) total tissue (liver, heart, kidney, brain, lung and skeletal muscle) mitochondrial membrane surface areas.
Summary

In the allometric equations relating total tissue mitochondrial membrane surface areas to body weight the exponents or slopes for reptiles are statistically the same as those for mammals. However, the coefficients or elevation values for the reptiles are always smaller, in the tissues measured, than for the mammals. In general, reptilian tissues have total mitochondrial membrane surface areas one quarter those found in mammalian tissues. These differences are the result of several smaller differences and it is therefore suggested from this present study that the evolution of endothermy was a gradual process rather than the result of any single "quantum" jump. These differences include an increase in the size of the major organs and skeletal muscle weight and increased mitochondrial volume densities in the tissues of mammals. All these factors show allometric relationships in both mammals and reptiles and together with internal mitochondrial membrane surface area densities characterize the allometric exponents of aerobic metabolism.

Differences in internal mitochondrial membrane density in the tissues of mammals and reptiles appear now from this present study to be of only minor importance. The major contributing factor to differences in the weight specific metabolism between the mammalian and reptilian tissues found in this study is tissue mitochondrial volume density. This same parameter is also primarily responsible for changes in weight specific mitochondrial capacities of tissues in both mammals and reptiles as a result of changing body size.
The monotreme and marsupial species examined in the present study showed high mitochondrial capacities similar to eutherian mammals and significantly higher than for similar sized reptiles. This suggests that the early mammals and therefore many of the later mammal-like-reptiles also had large endothermic capacities.

The large 4 fold difference in the amount of energy used by mammalian and reptilian tissues to pump Na⁺, measured in this present study, appears to be a result of differences in cell membrane permeabilities. Mammalian liver cells were found to be 5 times more permeable to Na⁺ than either reptilian or amphibian liver cells. This inefficiency in terms of relative energy use of the mammalian cells compared to the ectothermic cells is postulated as having evolved as a means of increasing heat production.
**APPENDIX TABLE 2.1** Percent Dry/Met Tissue Weight Values for Three Reptilian and Mammalian Tissues.

<table>
<thead>
<tr>
<th></th>
<th>A. vitticeps</th>
<th>R. norvegicus</th>
<th>SIGNIF. OF DIFF.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Number of Animals</strong></td>
<td>6</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td><strong>Body Weight (grams)</strong></td>
<td>324 ± 43</td>
<td>312 ± 28</td>
<td>N.S.</td>
</tr>
<tr>
<td><strong>Liver</strong></td>
<td>32.7 ± 3.7</td>
<td>15.2 ± 0.3</td>
<td><strong>P 0.01</strong></td>
</tr>
<tr>
<td><strong>Kidney</strong></td>
<td>13.9 ± 0.8</td>
<td>15.6 ± 0.9</td>
<td>N.S.</td>
</tr>
<tr>
<td><strong>Brain</strong></td>
<td>10.9 ± 0.6</td>
<td>11.7 ± 0.4</td>
<td>N.S.</td>
</tr>
</tbody>
</table>

Values are means ± S.E.M.

N.S. not significant

* High value due to intracellular fat stores


Fernel (1637) De Abditis Rerum Causis, II, 7; Opera, p.590.


