PROTEIN TURNOVER AND STABILITY OF THE PROTEIN POOL DURING METABOLIC ARREST IN TURTLE HEPATOCYTES.

BY

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ABSTRACT.

Hepatocytes isolated from the western painted turtle (*Chrysemys picta bellii*) are capable of entering a period of metabolic suppression that is characterised by a coordinated and highly regulated reduction in rates of ATP synthesis and ATP demand. To examine the demand side this relationship, the studies described here investigated the partitioning of energy usage in metabolic suppression using protein turnover as an example of a highly regulated and energetically expensive cell process. Absolute rates of protein synthesis fell by 92% during 12h of anoxia at 25°C. Using an empirically determined cost of 5.2 ATPs per peptide bond the relative cost of protein synthesis was determined to be 24.4μmol ATP/g/h accounting for 28-36% of total ATP turnover. In anoxia, this fell to 1.6μmol ATP/g/h, constituting 25% of total anoxic ATP turnover. The energy dependence of proteolysis was assessed in labile and stable protein pools. During anoxia, labile protein half-lives increased from 24.7 to 34.4h, with stable protein half-lives increasing from 55.6 to 109.6h. Inhibitors of energy metabolism revealed that a large proportion of whole cell proteolytic rates was ATP-independent, with the majority of ATP-dependent proteolysis appearing in the stable protein pool. Consequently, the combined anoxic mean proteolytic suppression for both pools was 36%, but 93% of the ATP-dependent component was suppressed. ATP demand for normoxic ATP-dependent proteolysis was determined at 11.1μmol ATP/g/h, accounting for 21.8% of total ATP- turnover. In anoxia, this was suppressed by 93% to 0.73μmol ATP/g/h accounting for 12% of remaining ATP-turnover. Summation of anaerobic energy demand by proteolysis and protein synthesis accounts for about 40% of remaining ATP-turnover in metabolic suppression.

The final series of experiments tested the hypothesis that a heme-protein oxygen sensor is involved in modulating protein expression profiles. Cells incubated in anoxia consistently

expressed proteins of 83, 70.4, 42.5, 35.3 and 16.1kDa and suppressed proteins of 63.7, 48.2, 36.9, 29.5 and 17.7kDa. Except for the 70.4 kDa protein, this expression was not found during aerobic incubation with cyanide, used as a mimic of physiological anoxia. Incubation of cells with factors that affect protoporphyrin conformation (Co^{2+}, Ni^{2+}, CO) produced predictable changes in expression profiles for 42.5, 35.3, 17.7 and 16.1 kDa protein bands and incubation with a heme-synthesis inhibitor abrogated the response. Remaining suppressed proteins in anoxia demonstrated a predictable sensitivity to Co^{2+} and Ni^{2+}, but no effect with CO, possibly suggesting control by a protoporphyrin with different O_2 and CO binding kinetics. These results strongly suggest that one or more heme-protein based oxygen sensor mechanisms are present in turtle hepatocytes, which govern both positive and negative modulation of oxygen-sensitive protein profiles in anoxia.

Overall, these results demonstrate coordinated reductions in ATP demand by protein turnover, but also demonstrate that protein turnover rates constitute a large proportion of remaining ATP-turnover in anoxic metabolic suppression. An important signal for positive and negative changes in protein expression profiles on the anoxic transition appears to be oxygen itself, raising the suggestion that oxygen-sensing mechanisms may be important in the modulation certain cellular events during metabolic suppression.

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Chapter 1.

Principles and Mechanisms of Anoxia-tolerance.

Introduction.

Living tissues have four sustaining requirements: energy (ATP), reducing power (NADH), a selectively permeable membrane, and precursors for biosynthesis. At the most basic level, the inter-relationships between these components defines both the metabolic and physical characteristics of tissues and also, the ability of tissues to adapt to severe changes in the external environment (Atkinson, 1977).

Oxygen acts as the terminal electron acceptor in the aerobic production of ATP and NADH in a reaction that proceeds from glucose as follows:

$$\begin{array}{c} 10NADH \\ + \\ 10H^+ \\ + \\ 2FADH_2 \\ + \\ C_6H_{12}O_6 + 6O_2 + 38(36)ADP + 38(36)P_i \rightarrow 6CO_2 + 6H_2O + 38(36)ATP \\ \downarrow \\ 10NAD^+ \\ + \\ 2FAD^+ \\ + \\ 12H_2O \end{array}$$

The ratio of one mole of glucose to six moles of O_2 and CO_2 is fixed, as are the number of reducing equivalents generated per mole of O_2. The potentially variable factor within these equations is the yield of ATP which is determined by evolution rather than chemical constraints (Atkinson, 1977). Nevertheless, in most biological systems, about 40% of the free energy

yielded from complete glucose oxidation remains conserved in the form of 38 ATPs.

When oxygen is removed, the metabolism of glucose is entirely cytosolic and proceeds via a fermentation pathway where there is no net change in oxidation state (cytosolic redox is balanced). Lactate becomes the terminal electron acceptor in this pathway and the net reaction proceeds as:

$$C_6H_{12}O_6 + 2ADP + 2Pi \rightarrow 2lactate + 2ATP + 2H_2O$$

The energy yield of this anaerobic pathway is drastically lower than when it is combined with oxidative phosphorylation, above. Complete aerobic oxidation of glucose has a free energy change (ΔG^o) of -686 kcal/mol. Lactate demonstrates a ΔG^o change of -319.5 kcal/mol, therefore the net energy yield from anaerobic fermentation of glucose to 2 lactates is only -47 kcal/mol. Assuming that ATP has a ΔG^o of -7.3 kcal/mol, then anaerobic glycolysis transfers a mere 2.1% of the total free energy within a glucose molecule towards ATP, with 31% of the the overall energy yielded from lactate production conserved as ATP. Fermentation from glycogen enhances the conservation of energy yield to 47% by generating a further net gain of one more ATP per glucosyl unit liberated and subsequently fermented.

This overview of aerobic versus anaerobic energy production efficiencies serves to illustrate a basic, but important point: ***because of the inefficiency of energy yield from anaerobic pathways, the limits to life without oxygen are defined by the way in which the organism partitions energy supply against energy demand.*** The research presented in this thesis examines this relationship by taking protein turnover as an example of a compartmented, highly regulated and energetically expensive process, in an organism that displays a specialised ability to withstand long periods of anoxia, the western painted turtle (*Chrysemys picta bellii*). This species deals with the loss of ambient oxygen by entering a period of metabolic suppression

which involves a large-scale change in the way that energy is produced, partitioned amongst ATP-demanding processes, and subsequently utilised. To closely examine the mechanisms behind metabolic suppression, a turtle hepatocyte preparation has been developed which serves as a useful model system for studies aimed towards understanding the sub-cellular characteristics of anoxia-tolerance (Buck *et al.*, 1993b). The series of studies presented here utilise this preparation to examine the re-organisation, and relative importance of cellular processes, and in particular protein turnover, in the anoxic metabolic response. From this perspective, questions are broached as to how individual ATP demanding processes respond during the transition to anoxia, at what relative advantage to enhancing anoxic survival, and at what potential cost to cell function over long bouts of anoxia.

To establish the physiological and biochemical framework, the following literature review discusses the principles and mechanisms of anoxia tolerance, and in particular, metabolic suppression from the perspective of an organ-to-cell response. The aim is to demonstrate how an elegantly integrated series of innate adaptations, and changes in cellular organisation, combine to conserve all four of the essential components to sustaining life, but in the complete absence of oxygen.

Design of Metabolism in Facultative Anaerobes.

There are four conserved features of metabolic design in anoxia tolerant organisms that are advantageous to sustaining life without oxygen: 1) tissues possess large stores of fermentable substrate 2) the yield of ATP per mole of substrate is maximised, 3) the problems of metabolic end-product accumulation are circumvented and 4) organ systems require to integrate the anoxic metabolic response to sustain the specific needs of each tissue.

Storage of Fermentable Substrate. Glycogen hydrolysis provides a source of glucosyl units for fermentation through glycolysis. In vertebrate anaerobes, the principal storage site for glycogen is the liver which plays a central role in the provision of substrate for other tissues during periods of anoxia. In turtles, glycogen comprises 15% of the liver mass at a concentration of 860μmol glucose/g (high millimolar concentrations; Clark & Miller, 1973; Hochachka, 1982); in goldfish, this reaches as much as 1300μmol glucose/g (molar concentrations; Van den Thillart, 1982). This is in stark contrast to anoxia-intolerant species such as trout and rat where liver glycogen concentration is 235 and 210μmol glucose/g respectively (Hochachka, 1982).

Optimising Anaerobic ATP and H^+ Yields. The fermentation of glycogen to 2 lactates generates a net production of 3 ATP. However, the ATP yield of this pathways can be substantially improved by coupling glycolytic fermentation with other anaerobic ATP generating pathways. In parasitic helminths, fermentation of glucose to succinate increases the yield of ATP to 4 ATP per mole of glucose; further reduction to propionate increases the yield of this pathway to 6 ATP per mole of glucose. Stoichiometric coupling of this pathway to the reduction of aspartate to succinate, and leucine to isovalerate, generates a further 1 ATP each and enhances the classical glycolytic yield from glucose by over four fold (Hochachka, 1980). Bivalves also appear to possess the necessary metabolic machinery for this pathway but it appears to be principally utilised in the early stages of anoxia when aspartate concentrations are high (De Zwaan, 1983).

Turtles do not appear to utilise alternative fermentation pathways to any significant extent. In turtle hepatocytes over 10h of anoxia, lactate accumulates to 50mM and together with glucose mobilisation, accounts for almost all of the carbon loss from glycogen (Buck *et al.*, 1993b).

However, the ΔG° of lactate production and glucose release accounted for only 36% of anoxic heat flux measured by microcalorimetry re-issuing the question of whether other fermentative pathways might be active in anoxia (Buck *et al.*, 1993a). Subsequent measurement of succinate revealed an accumulation to only 2% of lactate concentrations indicating that the fumarate reductase pathway does not account for a significant proportion of the exothermic gap and is not an important alternative fermentative pathways in turtle liver. Microcalorimetry of turtle brain cortical slices also points to the lack of alternative fermentation pathways in this tissue as the exothermic gap was negligible during anoxia (Doll *et al.*, 1994).

Optimising the number of ATP produced per proton released helps to slow the rate of tissue acidification. The source of H^+ in anoxia is believed to result from the mismatch of protons consumed in fermentation versus protons produced by ATP hydrolysis (Hochachka and Mommsen, 1983). The stoichiometry of this relationship (glycogen fermentation *and* ATP hydrolysis) is always the same regardless of the fermentative pathway, favouring the net production of $2H^+$ per mole of glucosyl unit used and appears to be inviolable for numerous anaerobic systems (Hochachka and Mommsen, 1983).

The ratio of ATP synthesised per net H^+ produced in fermentation *can* be optimised, however. The fermentation of glucose to lactate generates a ratio of 1:1 for ATP synthesised to H^+ produced. If glycogen is the principal substrate, this ratio is improved to 1.5:1 and in the pathway from glucose to succinate and proprionate, the ATP:H^+ ratio increases to 2 and 3 respectively, per mole of glucosyl unit utilised (Hochachka and Somero, 1984).

Therefore, in situations where ATP demand is maintained during anaerobiosis, the utilisation of alternative fermentation pathways presents two principal advantages: 1) an increased yield of ATP per mole of substrate and 2) an increase in the yield of ATP per mole

of end-product (H^+) produced. Since the principal fermentative substrate in turtle tissues is glycogen, both ATP yield and overall ATP:H^+ are improved 33% over glucose fermentation to 2 lactates. However, with increasing time in anoxia, circulating glucose becomes the principle substrate for tissues such as heart and brain suggesting that both ATP yield and ATP:H^+ likely demonstrates a tissue specific decrease.

Minimising End-Product Accumulation. Despite potential improvements in the ratio of H^+ production to ATP turnover in anaerobic pathways, the overall stoichiometry of glycolytic H^+ utilisation to ATP hydrolysis and H^+ production still favours a net accumulation of protons (Hochachka and Mommsen, 1983). Therefore, in anoxia the accumulation of protons and lactate as metabolic end-products presents a problem since pathways allowing their complete oxidation, re-utilisation or excretion are O_2-dependent processes. Reducing potentially harmful accumulations of toxic metabolic end-products can be achieved by 1) metabolising the end-product into an excretable form, 2) increasing tissue tolerances to accumulations of the end-product by improving tissue buffering capacity and 3) reducing the rate at which the end-product is formed by suppressing metabolism.

Vertebrate anaerobes demonstrate all three mechanisms. Decarboxylation of lactate to ethanol and CO_2 (mechanism 1, above) has been characterised in goldfish and crucian carp (*Carassius* spp; Shoubridge & Hochachka, 1980; Johnston & Bernhardt, 1983). In goldfish, whole body + external lactate changes marginally from 0.2 to 6μmol/g over a 12h anoxic episode (Shoubridge & Hochachka, 1980). The apparent rate of lactate production accounts for only 17% of the metabolic needs of the animal and is low despite high activities of LDH within various tissues (Van den Thillart *et al.*, 1976). This apparent discrepancy is founded in the ability of these species to decarboxylate lactate to ethanol + CO_2 by alcohol dehydrogenase and

then excrete both products to the environment via the gills (Shoubridge & Hochachka, 1980). The advantage of this is clear: lactate accumulations are minimised over long periods of time, and the H^+ production associated with ATP hydrolysis is balanced with H^+ utilisation in fermentation resulting in no net yield of protons (Hochachka and Mommsen, 1983). This mechanism is so effective in goldfish that, despite possessing weak plasma buffering capacity, pH_i remains above neutrality for at least 3h of anoxia (Van den Thillart & Van Waarde, 1993).

Turtles deal with the problem of H^+ and lactate end-product accumulation by combining effective mechanisms for buffering H^+ with metabolic suppression, exhibiting mechanisms 2) and 3) above. In anoxic dives lasting up to 6 months at 3°C, plasma lactate rises to 200mM indicating a significant metabolic acidosis, yet plasma pH falls gradually over this time from pH 7.9 to a range between pH 7.6-7.2 (Herbert & Jackson, 1985; Jackson & Heisler, 1983; Ultsch & Jackson, 1982). Despite possessing exceptionally high intrinsic concentrations of plasma HCO_3^- (40mequiv/l), the non-bicarbonate buffering capacity of turtle blood is no different from anoxia-intolerant species and as such, the disappearance of HCO_3^- only accounts for about 20% of the lactate load (Jackson & Ultsch, 1982). The shortfall in plasma buffering capacity is compensated by an increase in the concentrations of K^+, Ca^{2+} and Mg^{2+}, which, together with decreasing anion concentrations (HCO_3^- and Cl^-), serve to balance the large anionic lactate load (Jackson & Ultsch, 1982). On the cation side, the most remarkable changes occur in plasma Ca^{2+} and Mg^{2+} concentrations which rise to 68 and 26mM respectively. Together, these compensate for more than 50% of the lactate charge imbalance (Jackson & Heisler, 1982). The source of these cations appears to be the shell and long-bones which are rich in $Ca(HCO_3)_2$ and $Mg(HCO_3)_2$ (Jackson, 1993). With bicarbonate as the accompanying anion, mobilisation of Ca^{2+} and Mg^{2+} also serves to buffer H^+ associated with lactate

production.

Further enhancing tolerance to metabolic end-product accumulation, especially on recovery, is the ability to store toxic metabolic end-products in low tissue concentrations. Despite a large plasma lactate accumulation in turtles, skeletal muscle and liver lactate only accumulates to about 35% of that in the extracellular fluid (Jackson & Heisler, 1983). Turtles therefore appear to be capable of "storing" tissue derived lactate at high concentrations in blood plasma and minimise excessive intra-tissue accumulations. In addition, turtle bladder epithelium possesses an H^+ATPase activity which operates to alkalinise or acidify the urine (Youmans & Barry, 1989; Fritsche *et al.*, 1991). Whether this H^+ sequestering activity is active during anoxia has not been determined but since the bladder can occupy as much as one third of the body cavity volume when full, it represents a potentially important site for sequestering H^+.

By far the most significant mechanism to sustaining life without oxygen is the ability to suppress the rate of ATP demand and, in so doing, enter a period of metabolic suppression. This has the dual effect of slowing both the rate at which fermentable substrate is utilised and at which metabolic end-products accumulate. When metabolic suppression is combined with metabolic design features that optimise ATP yield, minimise H^+ production, tolerate end-product accumulation, all in the presence of large stores of fermentable substrate, anoxic survival time can be extended many fold.

Amongst vertebrates, the ability to suppress metabolism by various degrees is widespread amongst ectothermic and endothermic species in extreme environments (reviewed by Hochachka & Guppy, 1987). However the most profound suppression in metabolic rate is found in various species of freshwater turtle. In the red-eared slider (*Pseudemys scripta elegans*) heat output measured by direct calorimetry falls 85% during an anoxic dive at 24°C (Jackson, 1968). In

the western painted turtle (*Chrysemys picta bellii*), biochemical measures of anoxic metabolic depression demonstrate a significant temperature dependent component, ranging from 84% of normoxic metabolic rate at 15°C (Q_{10}=3.7), to 91% at 3°C (Q_{10}=9.7). Over a temperature range of 20-3°C, the combined effects of anoxia and increasing Q_{10} result in a 99.5% reduction in metabolic rate from normoxic metabolism at 20°C (Herbert & Jackson, 1985). In the northern range of these species (lat 54°) where winter lasts from 4-6 months, turtles hibernate by burrowing into anoxic mud at the bottom of ice-frozen ponds, where ambient temperatures are constant at 3-4°C (Ultsch, 1989). Therefore in the wild, the combined effects of metabolic suppression and Q_{10} extend survival time several fold beyond what could be achieved through increased tolerance to end-product accumulation and sequestration of large stores of fermentable substrate alone. To this end, Hochachka (1986) has calculated that dealing with metabolic acidosis, optimising ATP yield and possessing large glycogen stores could account for a 3-4 fold increase in survival time. However, by combining these factors with metabolic suppression, survival time in turtles is extended by 60 fold.

Integrating Organ Systems in Metabolic Suppression. The final requirement for survival as an anaerobe, which is particularly relevant during metabolic suppression, is to establish an optimal arrangement of remaining metabolism between organ systems. In whole turtle forced submergence studies, the general tissue response on entry into anoxia appears to involve a brief activation of glycolysis (Pasteur effect) followed by a decrease in glycolytic rate as on-board oxygen supplies become completely exhausted (Clark & Miller, 1973; Kelly & Storey, 1988). The reversal of the Pasteur effect, coincident with tissue anoxia, marks the entry into true metabolic suppression.

Entry into anoxia requires a significant metabolic readjustment within organ systems which

favours reduced, but sustained tissue function during metabolic suppression. In turtles at 3°C, heart rate falls 80% from 1.8 to 0.4 beats/min, contractility of atria and ventricles falls by 50% and there is a significant decrease in cardiac output (Wasser *et al.*, 1990; Jackson, 1987). There is also a redistribution of blood flow which favours perfusion of central organs, such as heart and especially brain (3.5 fold increase over normoxic controls) over splanchnic organs (liver, kidney, gut) and skeletal muscle (Davies, 1989). Since arterial pressure is maintained, the basis for this re-distribution of blood supply seems to be a local increase in vascular resistance. Potentially of great importance in this regard is the role that endothelial cells play in the release of autocoids (affect local vascular tone) and factors that alter the capillary proliferation. The release of these substances is regulated by changes in local oxygen concentrations through a number of "oxygen sensing" mechanisms (discussed later). This has lead to the conceptual development of the vascular system as a systemic oxygen sensor (Pohl, 1990).

Brain activity is also reduced: EEG and evoked potential activity fall by 50-80% (Feng *et al.*, 1988; Pérez-Pinzón *et al.*, 1992), action potential thresholds increase for Ca^{2+} and Na^{+} and postsynaptic transmission is depressed (Pérez-Pinzón *et al.*, 1992). The overall effect is to reduce, but not inhibit, the ATP demanding activity of the brain, a phenomenon that has been termed spike arrest (Sick *et al.*, 1993). Skeletal and smooth muscle also become quiescent. Entry into anoxia is associated with the cessation of movement (Ultsch, 1989) and although there have been no studies of gut motility in turtles during metabolic arrest, the accompanying starvation is probably associated with a reduction in gut motility and possibly gut length (noted in other reptiles - Secor & Diamond, 1994 & pers. comm.).

The systemic response to anoxia is largely based on fuel economy and during metabolic

suppression, the liver plays a central role ensuring a supply of plasma glucose that meets demand. In turtles, entry into anoxia is associated with a 50 and 60 fold increase in the plasma concentrations of adrenaline (Ad) and noradrenaline (NA) respectively. Subsequent activation of hepatic β-adrenergic receptors leads to an increase in total glycogen phosphorylase activity and the percentage in the active "a" form. The resulting mobilisation of glucose from glycogen increases plasma glucose 6 fold over controls in a 4h period of anoxia (Keiver & Hochachka, 1991). Corticosterone levels remain low during anoxia but increase dramatically on re-oxygenation and are suggested to pay a role in enhancing glycogen deposition and lactate metabolism after a bout of anoxia (Keiver *et al.*, 1992).

Organ-wide metabolic suppression, mobilisation of hepatic glucose and a redistribution in systemic blood flow are important factors in glucose sparing for fermentation by vital organs such as brain and heart and probably aids in sustaining glycogen concentrations within these tissues over the anoxic period. The importance of liver glucose mobilisation to sustained heart function has been demonstrated in studies which show that depleted cardiac glycogen supplies do not affect heart function so long as liver glycogen stores are present (Daw *et al.*, 1967). Therefore, vital organs possess a 3-way back-up system: metabolic suppression (80% in brain), large intrinsic stores of glycogen, and liver-derived plasma glucose. Glucose delivery is assured by relative increases in the perfusion of these organs which also serves to remove metabolic end-products.

A final and remarkable feature of anoxic metabolism that is demonstrated by all tissues, is the maintenance of ATP concentrations and cellular energy charge on entry into, and during anoxic metabolic suppression (Kelly & Storey, 1988). As discussed later, the maintenance of ATP concentrations are central to sustaining cell function and avoiding acute and lethal forms

of cell damage. ***To achieve this, the metabolic changes that occur during the transition to anoxia and metabolic suppression must be balanced and highly coordinated by a single signalling event.*** The next section discusses signalling mechanisms that may be at the root of transducing a change in environmental O_2 to a cellular response.

Signalling Mechanisms.

The signal that links changes in O_2 concentrations to events in metabolic suppression must act through a mechanism that can achieve a global and synchronous change in ATP demand and ATP synthesis, and that can also coordinate this change between organ systems. Hormones can be dismissed as the primary signal in this response since anoxic metabolic suppression has been demonstrated in numerous isolated systems without the input of exogenous effectors. Although pH is a potent modulator of cellular metabolism in invertebrate systems (Artemia embryos for example), in vertebrates, it can also be dismissed as the primary signalling mechanism since intracellular pH (pH_i) falls on a different time-scale to metabolic changes. Metabolic suppression is also associated with little alteration in [ATP], [ADP] or [AMP] and changes in metabolite concentrations are not sufficient to cause a 90% drop in enzyme flux along a pathway (cf-Kelly & Storey, 1988). What then, relays changes in environmental O_2 to invoke a systemic and controlled metabolic suppression?

Figure 1 demonstrates the change in oxygen uptake versus oxygen concentration in the liver. The K_m of this curve is about 170μM O_2 and is more than 170 times the K_m for O_2 at cytochrome c oxidase in liver tissue ($<1\mu$M O_2;Longmuir, 1957). Inset is the relationship between oxygen concentration and oxygen uptake in isolated hepatocytes and isolated mitochondria (Yoshihara *et al.*, 1988). Most significant is the observation that in intact

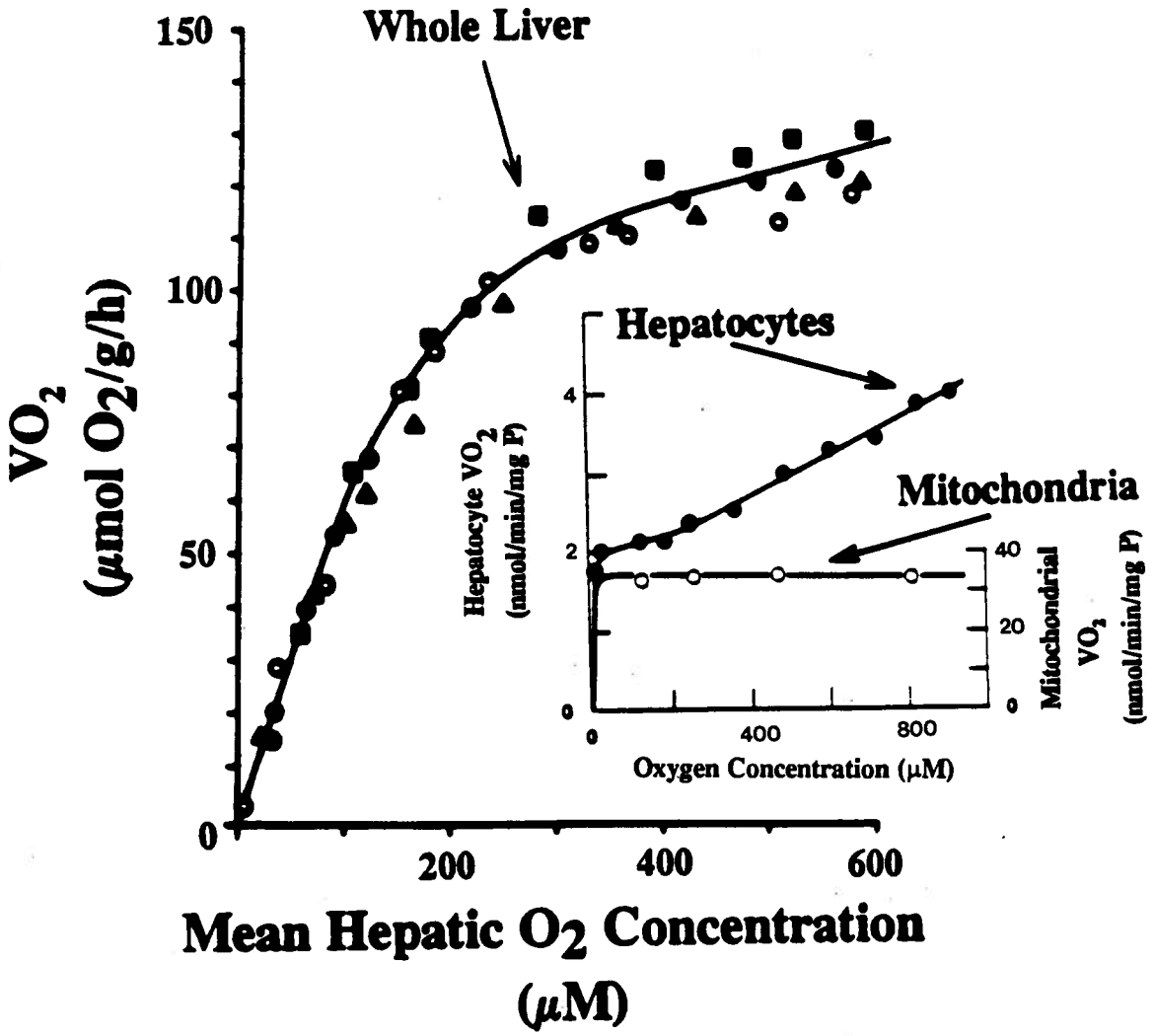


FIGURE 1. Relationship between oxygen uptake and oxygen concentration in whole liver, isolated hepatocytes and isolated mitochondria. Figure is adapted from Yoshihara *et al.*, (1988). *Abbreviation*: P - protein.

hepatocytes, VO_2 changes by more than 50% over an oxygen concentration range where isolated mitochondrial VO_2 remains constant. Assuming that the gradient of oxygen from the exterior of the cell to the mitochondrion is small (about 6μM; Jones *et al.*, 1990) this suggests that hypoxia-associated changes in metabolism occur whilst O_2 is still saturating at the mitochondrion. In isolated, contracting dog gracilis preparations, evidence presented suggests that during hypoxia, O_2 modulates ATP supply and demand in such a way that ATP synthesis and ATP demand remain balanced and coordinated with one-another (Hogan *et al.*, 1992; Arthur *et al.*, 1992). ATP turnover rates also show a direct, linear correlation with O_2 supply when O_2 is still well above limiting concentrations at the mitochondrion (reviewed by Hochachka, 1994). ***Evidence such as this strongly suggests that O_2 itself acts as the global signalling mechanism on entry into anoxia.***

There are four principal mechanisms for cellular O_2-sensing that have been presented in recent years. These include 1) a nitric oxide (NO) model where hypoxia-induced increases in $[Ca^{2+}]_i$ activate NO-synthase with a subsequent increase in cGMP and protein-level phosphorylation (King *et al.*, 1993), 2) an H_2O_2 generating NAD(P)H oxidase model where changes in the redox state of glutathione, or H_2O_2 effects on guanylate cyclase activation lead to changes in channel efflux and gene expression (Acker *et al.*, 1994), 3) a high km oxygenase model where the activity of a rate limiting oxygenase is modulated through its high sensitivity to $[O_2]$ (Thurman *et al.*, 1993), and 4) a heme protein oxygen receptor model (discussed later) where changes in the O_2-dependent conformation of a membrane bound heme-protein transduce changes in extracellular O_2 to a cellular response through a second messenger pathway (Goldberg *et al.*, 1988). The exact nature of the heme-protein receptor is currently vague but an interesting consideration is the potential for its association with other cellular signalling

mechanisms. For instance the cytosolic form of guanylate cyclase, the enzyme responsible for cGMP synthesis, possesses a protoporphyrin sub-unit which is activated and modulated by NO (Ignarro, 1989).

The most significant feature of these kinds of O_2 signalling mechanisms is that by virtue of their extreme sensitivity to changes in O_2 concentrations, they demonstrate the potential to bring about subsequent changes in cellular functions well before O_2 is truly limiting to cell function. Therefore, the potential exists for scaling metabolic events with decreasing changes in O_2. All of these signalling mechanisms could have a potential role in coordinating the disappearance of O_2 with metabolic suppression and since hypoxia-anoxia occurs in all tissues at approximately the same time, they provide a means for systemic signalling.

Whatever the mechanism, there is mounting evidence for wide scale protein-level phosphorylation in metabolic suppression (see next section). Therefore transduction of external O_2 changes likely involves a receptor mechanism at some level.

As mentioned above, a characteristic feature of tissues capable of arresting metabolism is the long-term maintenance of steady ATP concentrations. However, a number of studies have noted that in response to declining p_{O_2} there is a small and transient reduction in ATP concentrations that appears universal amongst tissues (Kelly & Storey, 1988; Nilsson & Lutz, 1992; Land *et al.*, 1993) and results in the catabolism of a small amount of adenine nucleotide beyond AMP. Studies on turtle brain slices find a transient increase in extracellular adenosine from 1 to 21 μM over 100 min of anoxia (Nilsson & Lutz, 1992) which coincides with the release of inhibitory neurotransmitters and the time of down-regulation of metabolic rate found for the whole animal. Although these changes in adenosine concentrations are acute (half-life of adenosine in blood is a few seconds), there could be a role for this metabolite in effecting

local metabolic changes during the transition to metabolic suppression. As discussed below, there is strong evidence to suggest that enzyme activity can be modulated through phosphorylation/dephosphorylation reactions during metabolic suppression. A current working hypothesis for the induction of this phosphorylation involves the binding of adenosine to the adenosine A_2 receptor to activate protein kinase C (PKC) through the diacylglycerol second messenger system. PKC directly, or indirectly through increased $[Ca^{2+}]_i$, could phosphorylate glycolytic enzymes and membrane associated proteins bringing about a coordinated suppression of metabolism. However, the means by which metabolism *stays* suppressed once adenosine concentrations have abated remain unknown.

Whether the link between environmental anoxia and metabolic suppression lies in O_2 itself or a combination of effects such as those found with adenosine remains unclear. Whatever the mechanism, there are a number of well defined cellular responses that ensue which are discussed in the next section.

Mechanisms of Metabolic Suppression.

There are three principal components of metabolic suppression that are highly conserved between forms (i.e. caused by differing environmental conditions) and species in which this kind of dormancy occurs. Firstly, ATP synthesis and ATP demand are coordinately reduced with the effect that ATP concentrations ([ATP]) and cellular energy charge remain high and constant. Secondly, ion gradients and the plasma membrane potential are conserved. Lastly, tissues are maintained functionally viable during long periods of metabolic suppression such that their phenotypic characteristics and active function are not compromised on re-oxygenation and recovery. ***Successful survival of anoxia by metabolic suppression is therefore a balance of***

conflicting demands: reduced ATPase and synthase activity versus the need to expend energy in the stabilisation of the membrane potential and the turnover of functionally important proteins. This section discusses current concepts of how energy supply, membrane functions and anabolic processes are controlled to achieve a reversible suppression in activity in hand with a balance between energy supply and demand.

Reducing Enzyme Fluxes. The first conserved feature of metabolic suppression, reduced ATP supply and demand, is associated with a coordinated inhibition of rate controlling, regulatory proteins. Slowing the rate at which ATP is both synthesised and hydrolysed requires a coordinated, large scale depression of enzyme activity, with remaining flux through enzyme pathways finely tuned to the specific requirements of the tissue. The rate at which substrate (S) fluxes to a product (P) through an enzyme, is expressed as $V_{max}=k_{cat}.e_o$, where V_{max} is the maximum rate of S→P flux through an enzyme, k_{cat} is the turnover number of S→P per catalytic site and e_o is the enzyme concentration. This relationship states that the principal enzyme flux control mechanisms affect changes in V_{max} either through changes in enzyme catalytic efficiency (k_{cat}) or by altering the absolute concentration of active catalytic sites (e_o).

On entry into metabolic suppression, the principal routes for changing k_{cat} are via covalent modification and allosteric regulation. Covalent modification of enzyme activity occurs by the binding of phosphate and directly influences enzyme catalytic efficiency. Because phosphorylation is controlled by signal transduction pathways associated with cAMP and cGMP-dependent protein kinases, this also has the potential to be influenced by exogenous signals such as hormones.

In anoxia-tolerant tissues, anoxia generally results in significant, tissue-specific changes in the kinetic constants for rate limiting glycolytic enzymes, pyruvate kinase (PK),

phosphofructokinase (PFK), and also for glycogen mobilisation through glycogen phosphorylase (GPase). In turtle liver, PFK demonstrates a 1.5 fold increase in K_m for ATP and a 2.9 fold decrease in inhibitory constant (I_{50}) for citrate. This tissue also demonstrates a 5 fold increase in the amount of GPase in the active "a" form (Keiver and Hochachka, 1991). Heart shows the opposite pattern with a 2 fold decrease in K_m for ATP and a 1.7 fold increase in I_{50} for citrate (Brooks and Storey, 1989). PK also demonstrates the same general trend for both tissues. Changes in glycolytic intermediates during anoxia in turtle tissues demonstrate that altered enzyme kinetic characteristics are correlated with a tissue specific glycolytic response: liver tissue exhibits enhanced glycogenolysis and glucose release, and heart and brain show an increased capacity for fermentation of exogenous glucose (Kelly & Storey, 1988; Brooks & Storey, 1989).

Evidence that covalent modification by enzyme phosphorylation may be at the root of this kind of response comes from in vitro comparisons of kinetic constants from purified aerobic and anoxic forms of PFK and PK. In the goldfish, significant shifts in the isoelectric focusing points (pI) of PK and PFK isolated from liver, red, and white muscle have been detected indicating a larger charge is present in the anoxic enzyme forms. However, the increase in pI is only associated with changes in kinetic properties of PK and PFK from the liver (Rahman & Storey, 1988). Subsequent in vitro treatment of the purified enzymes with protein kinase or alkaline phosphatase demonstrates same-direction, and similar magnitudes of change in the kinetic constants for the purified enzymes and the anoxic enzymes (Rahman & Storey, 1988). In turtles, PFK and PK-phosphorylation events are yet to be investigated, however it seems that there is an increase in ^{32}P-protein interactions during anoxia in liver and brain (Brooks & Storey, 1993a).

The most compelling evidence for phosphorylation-mediated covalent modification during metabolic suppression in vivo comes from studies on PK from the channelled whelk (*Busycotypus canaliculatum*). In anoxia, there is a 50% increase in ^{32}P bound to total cellular protein. Purification of PK from these crude extracts reveals a phosphorylated anoxic form of the enzyme which demonstrates a 34% lower V_{max}, 90% increase in the K_m for phosphoenolpyruvate, and increased inhibitory constants for alanine and ATP (Plaxton & Storey, 1984a and b). Incubation of the anoxic form of PK with alkaline phosphatase reverts the kinetic properties of the enzyme back to the aerobic state (Plaxton & Storey, 1984b). Recently, the protein kinase responsible for this effect was identified as a cGMP-dependent protein kinase G (Brooks & Storey, 1990) supporting the possibility of receptor-mediated control of metabolic suppression.

Allosteric modulation of enzyme activity occurs by the association of a ligand with a specific, modulatory binding site which is separate from the enzyme catalytic site. This affects a change in enzyme tertiary and quaternary structure which influences the properties of the enzyme catalytic site. The role of fructose 2,6-bisphosphate (F-2,6P_2) as an allosteric regulator has been extensively examined during metabolic suppression in whelk tissues. PFK-1 catalyses the glycolytic reaction between fructose 6-phosphate and fructose 1,6-diphosphate by transphosphorylation using ATP as a substrate. This is a non-equilibrium reaction which is catalysed in the gluconeogenic direction by fructose 1,6-bisphosphatase (F1,6P_2ase). The control of the rate of flux through glycolysis versus gluconeogenesis in the upper part of the pathway is controlled by the relative states of activation of these two enzymes (Hers & Hue, 1983). F2,6P_2 is a potent activator of PFK-1, and synergistically inhibits F1,6P_2ase with AMP, and as such, enhances the potential for glycolytic flux over gluconeogenesis at this locus (Hue

and Rider, 1987). Concentrations of $F2,6P_2$ are regulated by a bifunctional enzyme which is governed by phosphorylation through a cAMP-dependent protein kinase to favour either the synthesis of $F2,6P_2$ when dephosphorylated (PFK-2 activity) or its catalysis to F6P when phosphorylated ($F2,6P_2$ase activity). In anoxic whelk tissues, there is a 4-fold drop in PFK-2 activity with resulting, significant tissue-specific reductions in $F2,6P_2$ concentrations (Bosca & Storey, 1991). This together with an anoxic phosphorylated form of PFK-1 that is less responsive to $F2,6P_2$, ensures that glycolytic activation by $F2,6P_2$ is removed at the PFK locus.

In turtle and goldfish, the change in $F2,6P_2$ is tissue-specific but inconsistent between the same tissues from these two species. Goldfish show a significant increase in brain and heart $F2,6P_2$ and large reductions in liver, kidney and muscle (Storey, 1988). In turtles however, red muscle shows a large increase in $F2,6P_2$ whereas in heart, it decreases (Brooks and Storey, 1989). Interpreting these results is difficult, however the authors suggest that increases in $F2,6P_2$ are correlated with the relative activation of glycolysis in more metabolically active tissues (Storey, 1988).

The final mechanism for modulating enzyme activity during metabolic suppression involves the binding of enzymes to sub-cellular structures. In a review by Srere (1987), the cellular milieu is envisaged as a relatively organised environment where enzymes along pathways bind to sub-cellular structures and are arranged in close proximity to each other. Hypothetically, within this ordered system, substrates could be channelled from one enzyme to another increasing the efficiency of catalysis along a pathway by removing the randomness of substrate diffusion. Another possibility suggested by this model is that enzyme pathways could be located locally to where their product is in highest demand.

There is good evidence to suggest that glycolytic enzymes do reversibly bind to cytoskeletal

structures in a number of tissues, and that the reversible nature of this binding correlates with changes in glycolytic rate. In trout white muscle for example, exercise to exhaustion produced an associated increase in PFK, aldolase, GAPDH and phosphoglycerate kinase (PGK) binding to the particulate fraction (Brooks & Storey, 1988). However, in anoxia tolerant organisms, the evidence for enzyme binding in the control of glycolysis is inconsistent between systems. In the channelled whelk ventricle, activities of hexokinase (HK), PFK, aldolase, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and PK all exhibit a reduction in binding to the particulate fraction during anoxia (Plaxton & Storey, 1986), but this is not the general rule for all tissues. In whelk foot muscle, HK and PFK exhibit increases in binding whereas all other enzymes measured exhibit a decrease (Plaxton & Storey, 1986). In turtle brain, the activities of HK, PFK, GAPDH, aldolase, phosphoglycerate kinase and PK all exhibited significant increases in binding according to the assay method used (Duncan & Storey, 1992). Based on studies which associate changes in glycolytic flux with altered enzyme binding profiles, these authors conclude that binding enhances the rate of substrate passage through glycolysis. Therefore during metabolic suppression, increased enzyme binding in turtle brain would seem rather anomalous and is explained on the basis of situating the glycolytic pathway close to the source of ATP demand in the cell.

Although changes in enzyme binding to the particulate fraction have been demonstrated in association with anoxia, no study to date has assigned any real, measurable, metabolic significance to this phenomenon. Studies examining the effects of binding interactions between purified glycolytic enzymes and F-actin on enzyme kinetics have been examined in vitro and tend to show inconsistent results. PFK activity is enhanced with binding according to its phosphorylation state whereas aldolase, GAPDH and PK all showed an inhibition on binding

(Reviewed by Brooks & Storey, 1993c). However, the concept of substrate channelling through bound enzymes is also weakened by the fact that enzymes are rarely at equilibrium where channelling would work best. Differences in the equilibrium constants between enzymes would result in accumulations of products that would lead to product inhibition at points along the pathway. Whether this truly is a significant mechanism involved in modulating metabolic flux during metabolic suppression remains vague.

Where enzyme binding to cell structures may have some true significance is in the modulation of effective enzyme concentration. Changing e_o requires a system that can accommodate the rapid and reversible masking of enzyme catalytic sites in response to an external stimulus such as phosphorylation. A good example of such a system is found in the erythrocyte transmembrane anion transporter known as band 3 protein (reviewed by Low, 1986). This protein possesses a rod-shaped cytoplasmic domain that binds, and in so doing inhibits, a number of glycolytic enzymes, notably GAPDH, aldolase and PFK. These glycolytic enzymes are released through the phosphorylation of a tyrosine group near the N-terminus of the band-3 cytoplasmic domain with a resulting increase in e_o and glycolytic rate (Harrison *et al.*, 1991). Other means for increasing e_o can occur through mechanisms such as ligand-induced association-dissociation of enzyme subunits (Kurganov, 1983) or synthesis or degradation of existing protein (Hargrove & Schmidt, 1989). However, for affecting rapid changes in metabolic flux rates, when energy consumption must be conserved, the latter models represent less attractive means for changing effective enzyme concentration.

A significant problem with the entire analysis above is the lack of demonstrated cause and effect. The timing, magnitude and collective effect of phosphorylation events, changes in allosteric effector concentrations and alterations in enzyme binding have not been demonstrated

to be causative in bringing about the coordinated metabolic suppression of the magnitude described in vivo. A recent theory put forward by Hochachka and Matheson (1992) provides a framework for effecting large scale changes in metabolic rate that may prove useful in future studies of regulation in metabolic suppression and activation. In systems that undergo large and rapid, positive or negative changes in metabolic rate (eg hummingbird flight muscle or metabolically suppressed tissues) changes in k_{cat} such as those described above, have been hypothesised to be insufficient in bringing about the orders of magnitude change in metabolic activity required. Furthermore in each case, and especially in metabolically suppressed systems, the change in metabolic activity occurs whilst concentrations of regulatory metabolites and allosteric effectors are relatively constant (Hochachka *et al.*, 1991; Hogan *et al.*, 1992). This prompted Hochachka and Matheson (1992) to suggest that a primary mechanism for large scale changes in flux through a pathway, in response to a change in oxygen availability, primarily involves a change in the concentration of enzyme catalytic sites (e_o) rather than a change in the turnover number of S to P per catalytic site (k_{cat}). Within this scenario, large changes in metabolic flux are accommodated primarily through changes in e_o, with allosteric and covalent modulation performing the fine tuning of flux through the changed number of catalytic sites. As a framework for studying mechanisms of reducing metabolic flux in tissues of anoxia tolerant organisms, Hochachka and Matheson's theory is particularly attractive since it proposes a mechanism where large positive and negative changes in metabolic flux can be accommodated, then finely tuned to the specific needs of the tissue.

Reducing Membrane Ion Fluxes. The membrane potential is generated by the active pumping of charged ions against their electrochemical gradient and provides the kinetic energy for cellular processes such as solute transport, amino acid and carbohydrate uptake, ATP

synthesis, cellular communication and so forth (reviewed Harrison & Lunt, 1980). A loss of this charge imbalance across the membrane results in a depolarisation of the cell membrane resulting in the rapid opening of voltage-gated Ca^{2+} channels, activation of phospholipases A_1 and A_2, rupture of the cell membrane and ultimately cell death (see later).

The pumping of ions against an electrochemical gradient is an energetically demanding processes. In rat hepatocytes Na^+/K^+ ATPase activity accounts for 16% of cellular ATP demand, and in combination with Ca^{2+} and H^+ ATPases, this is extended up to 40% (Schneider *et al.*, 1990). Na^+/K^+ ATPase activity alone utilises 60% of total ATP demand in the mammalian brain, and in kidney accounts for as much as 80% (Erecinska & Silver, 1989; Brezis *et al.*, 1984). Clearly, ion pump ATPases constitute a significant ATP sink within the cell. Therefore, metabolic suppression in hand with stable ATP concentrations requires that rates of ATP turnover should be reduced by lowering ion pumping activity. This represents only half of the solution, however. Maintenance of the cell membrane potential demands that reduced ion pumping rates must be balanced at all times with opposing rates of ion flux as ions migrate along their respective electrochemical gradients. This flux of ions is controlled by a gating mechanism within trans-membrane channel proteins that are specific for individual ion species. The gating mechanism can seal the channel against further ionic conductance, however this is far from an all or nothing process. In inactive excitable tissues, inefficiencies in channel closure result in a statistical probability of a certain percentage of channels being open at any given time (Hille, 1984). This background conductance is countered by pump-ATPase activity which maintains a polarised membrane potential over the cell membrane. Therefore during metabolic suppression, without a mechanism to coordinate the reduction in ion pumping rates with opposing passive ion flux through channels, the cellular membrane potential would slowly

dissipate.

The channel arrest hypothesis (Lutz *et al.*, 1985; Hochachka, 1986) predicts that during metabolic suppression in anoxia-tolerant tissues, ***the membrane potential is maintained by decreased ion-pump ATPase activity with coordinated reductions in channel ion flux.*** This results in an overall reduction in the permeability of the cell membrane with sustained, but greatly diminished rates of ion flux. If the predictions of the channel arrest theory hold true, then anoxia should be associated with a reduction in the conductance of specific ion species across the plasma membrane with a resulting increase in membrane resistance and a steady membrane potential. Studies conducted on isolated turtle cerebellum demonstrate that the cellular transmembrane potential does indeed remain constant at -60 to -85 mV during an anoxic metabolic suppression (Pérez-Pinzón *et al.*, 1992a) which is estimated to be on the order of 80% in turtle brain (Lutz *et al.*, 1984; Chih *et al.*, 1989a). These studies also demonstrated that input resistance fell by 36% during anoxia suggesting that cell membranes in anoxic preparations are generally less leaky to ions. This has also been demonstrated in slices incubated with ouabain where K^+ leakage was significantly slowed in anoxic brains versus normoxic controls (Chih *et al.*, 1989b). Ca^{2+} flux through Ca^{2+}-channels also appears to be reduced during at least 5h of anoxia (Bickler, 1992). The evidence for channel arrest is not clear-cut for all brain regions, however. Pyramidal neurons from the turtle cortex demonstrate no apparent change in membrane resistance with anoxia (Doll *et al.*, 1991). Although these cells can survive at least 120min of anoxia, they appear to exhibit only a moderate 42% suppression in calorimetric heat-flux (Doll *et al.*, 1994) which may factor into a specific membrane and metabolic response of this brain region to anoxia.

Experiments in turtle hepatocytes also present evidence for channel arrest in metabolic

suppression. By using $^{86}Rb^+$ as a potassium analogue, it has been estimated that Na^+/K^+ ATPase activity is diminished by 75% during anoxia (Buck & Hochachka, 1994). Under the same conditions, hepatocyte membrane potential remains constant at -31mV and is apparently maintained independently of O_2 availability. Therefore, despite a large reduction in the ATP turnover specific to Na^+/K^+ ATPase activity, the membrane potential remains constant, an observation that is consistent with the channel arrest hypothesis.

A unifying observation from all of these studies is that despite a large metabolic suppression associated with constant [ATP] (Brain - Lutz *et al.*, 1984; Doll *et al.*, 1994, Kelly and Storey, 1988; Hepatocytes - Buck *et al.*, 1993), preservation of the membrane potential is dependent on energy provision through glycolysis. In each case there is a rapid depolarisation on administration of the GAPDH inhibitor, iodoacetate, and in the case of turtle brain, this also occurs during incubation without exogenous glucose (Doll *et al.*, 1991; Pérez-Pinzón *et al.*, 1992a and 1992b). Therefore, although the membrane potential is maintained during metabolic suppression, there is still a requirement for continued pump ATPase activity which is vital to the continued survival of the tissue.

How coordinated changes in rates of ion flux through channels and pump ATPases might occur remains a mystery. From the equation $V_{max}=k_{cat}.e_o$ discussed earlier, it appears that a reduction in ion flux could occur either through an effective concentration change of the functional proteins involved or through a change in their "catalytic" efficiency (efficiency of gating). There is some evidence to suggest that modulation of channel e_o occurs during anoxia in turtle brain. The density of both active and inactive Na^+ channels decreases by 42% on exposure of turtle brain slices to anoxia (Pérez-Pinzón *et al.*, 1992c). Moreover, there is a good possibility that phosphorylation-mediated inhibition of channel activity may be involved here

also. In turtle brain, anoxia results in an increase in the proportion of protein kinase C (PKC) isozymes I and II that are associated with hindbrain membranes. In the cerebellum, the opposite effect has been noted (Brooks & Storey, 1993b). This change in PKC distribution has been suggested to occur in associated with changes in membrane organisation during anoxia.

From the mammalian literature, a number of studies have noted that in rat brain, Na^+ and K^+ (Numann *et al.*, 1991), and in heart, Na^+ and Ca^{2+} (Schubert *et al.*, 1989), flux through the respective ion channels can be reduced by either direct administration of PKC or addition of phorbol esters to activate PKC. In liver cells, this has been developed further where transient opening of Na^+, K^+ and Ca^{2+} channels occurs by increases in $[Ca^{2+}]_i$, and deactivation by PKC (Fitz *et al.*, 1994). In this model, extracellular ATP mediated the increase in $[Ca^{2+}]_i$ through a 5'nucleotide receptor. Similarly, PKA and cGMP dependent protein kinases appear capable of modulating ion channel flux and G-proteins have been implicated in the control of ion channels through direct association with Na^+ and Ca^{2+} channel proteins (Schubert *et al.*, 1989).

Given the fact that changes in channel flux rates occur in response to progressive hypoxia, the recent finding that an oxygen-sensing mechanism modulates K^+ channel activity in brain is particularly interesting (Jiang & Haddad, 1994). In this study, progressive hypoxia deactivated K^+ channels in a manner that suggested the direct involvement of a metal binding protein that was not a Fe^{2+}-heme protein. The implications of this study are significant since it demonstrates a direct associate between ion flux, channel opening and oxygen concentration.

Alteration of pump-ATPase activity also occurs as the result of phosphorylation. Na^+/K^+ ATPase is inhibited through a protein kinase which is appears to be PKC (Bertorello *et al.*, 1991). However, Na^+/K^+ ATPase activity has also been demonstrated to fall with reduced $[Na^+]_o$ or removal of available ATP. At this time it remains unclear if Na^+/K^+ ATPase activity

is suppressed allosterically or by effective substrate limitation during metabolic arrest.

An overlooked, but potentially important regulator of ion channel and pump activity is the membrane itself. Since channel and pump proteins are membrane-bound they are subject to regulation by changes in membrane fluidity and composition. In comparison with mammalian cell membranes, reptilian cell membranes are markedly less leaky with lower rates of K^+ and Na^+ exchange (Else & Hubert, 1987). This has been correlated with a markedly different membrane composition in reptiles. In the liver, phospholipid concentration is 35% greater in rat than lizard with a significantly greater proportion (16.2%) of rat fatty acids as polyunsaturates. Total protein content of rat liver is 54% greater than lizard liver and this is reflected in a higher protein:lipid ratio in rat liver mitochondria (Hubert & Else, 1989). Although the proportion of cholesterol was not measured in these studies, the lower proportion of polyunsaturated fatty acids, and the lower protein:lipid ratio of organelle membranes points to an inherently lower fluidity quotient in reptilian membranes (higher microviscosity). Possessing a less leaky membrane does not present any metabolic saving during anoxia in itself since standard metabolic rate is also 4-5 times lower in reptiles than in mammals. However, since membrane-bound proteins can be activated and de-activated depending on the lipid environment (Sweet & Schroeder, 1988), a predisposition to fluidity-related changes in channel and pump conformation in reptiles could be argued.

The plasma membrane is a dynamic 2-dimensional fluid environment in which lipid molecules and proteins continually diffuse, forming regional domains with locally varied composition and biophysical properties according to the relative concentrations of cholesterol, phospholipid and glycosphingolipid (Yechiel & Edidin, 1987; Curtain *et al.*, 1988). Through zonal changes in membrane microviscosity a large number of membrane protein-dependent

processes are affected. From the channel arrest perspective it is significant that diffusion rates of Na^+ and K^+ ions decrease with lowered membrane fluidity (Cooper *et al.*, 1975), as do activities of Na^+/K^+ ATPase (Kimelberg, 1975), Ca^{2+}ATPase (Warren *et al.*, 1975) and ion flux rates through the acetylcholine receptor channel (AchR; McNamee & Tong, 1988). From studies such as these, it appears that functional membrane proteins perform most effectively over an optimal viscosity range, an in association with specific phospholipids, which supports an active protein conformation (Sweet & Schroeder, 1988). The AchR channel has a requirement for cholesterol and negatively charged lipids which appear to interact with the secondary and tertiary structure of the protein subunits. Altering either of these components results in reversible channel deactivation and denaturation (Fong & McNamee, 1985; Sunshine & McNamee, 1994). Hypothetically, if anoxia caused regional changes in the fluidity of cell membranes in turtle brain, the observed decrease in Na^+-channel density could reflect a change in Na^+-channel conformation which is not recognised by the ligand used to measure channel density [^{3}H-brevatoxin (Pérez-Pinzón *et al.*, 1992c)]. Discovering whether the lipid composition of cell membranes from facultative anaerobes predisposes ion channels and pumps to anoxia-induced, but reversible, denaturation constitutes an interesting area for future research.

Anabolic Processes. The third conserved feature of metabolic suppression centres on the requirement to preserve functional viability during the period of metabolic suppression. Anabolic processes such as nucleic acid synthesis, protein synthesis, and membrane synthesis and assembly, are involved in the continuous renewal and replacement of existing cell structures, enzyme pathways and so forth, and are vital in staving off entropic forces. Anabolic processes are energetically expensive, but demonstrate a wide functional scope depending on the growth state of the tissue. For example, in static cultures of rat hepatocytes, protein

synthesis accounts for 13% of total ATP demand (Schneider *et al.*, 1990). However, in more robust, growing cells such as the ascites tumour cell, protein synthesis alone accounts for nearly 30% of total ATP demand (Müller *et al.*, 1986). A cost for RNA synthesis of 4% and 11% has also been measured in these respective cell types bringing the anabolic cost of protein synthesis to 17% and 41% of total ATP demand in each case.

To date, the only observations relating to anoxic anabolic responses during dormancy stem from research on the brine shrimp embryo (*Artemia fransiscana*). The encysted gastrula is capable of suppressing metabolism by 99.6% after one day of entry into anoxia (Hand, 1990) and can persist in this state for up to 18 months when hydrated with little effect on recovery success (Clegg, 1992). During this time, processes involved in sub-cellular differentiation including protein synthesis, mitochondrial maturation and liberation of lipid from yolk platelets are completely inhibited (Kwast & Hand, 1994; Hoffman & Hand, 1990). A major difference between this system and vertebrate anoxia tolerant systems is that the brine shrimp gastrula exhibits relatively poor intracellular buffering capacity. Therefore changes in metabolic, anabolic and catabolic rate processes are governed much more closely by intracellular pH in this system.

The response and control of anabolic cell processes, and in particular protein turnover, during metabolic suppression has received very little attention to date in vertebrate anoxia tolerant systems. Yet, as a cell process, it is vital for ensuring stability within the protein pool and amongst cell structures and is also an important means of implementing phenotypic and metabolic changes within tissues. The next section discusses the response of protein turnover and gene expression to hypoxia in anoxia intolerant systems and uses these examples to draw up a hypothetical picture of the control of these processes in anoxia tolerant systems. This also

brings the literature review to the principal thrust of this thesis: the response, energetic cost, and significance of protein turnover and protein expression in metabolic suppression.

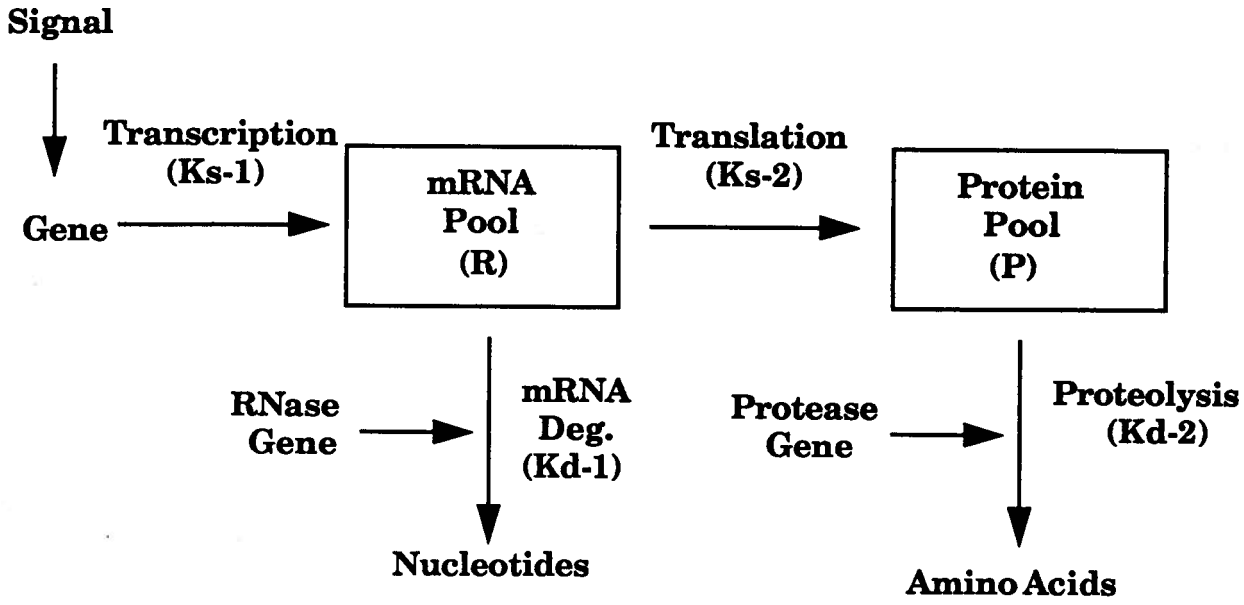
Protein Turnover and Gene Expression in Anoxia Intolerant Systems.

Kinetics of Protein Induction. Protein turnover is a multifaceted cellular process comprised of gene activation, expression of mRNA, translation and protein degradation. Alteration of the pattern of protein expression in response to an external stimulus (anoxia, for instance) requires a balanced interaction between these processes in order to remove inappropriate proteins and synthesise new ones.

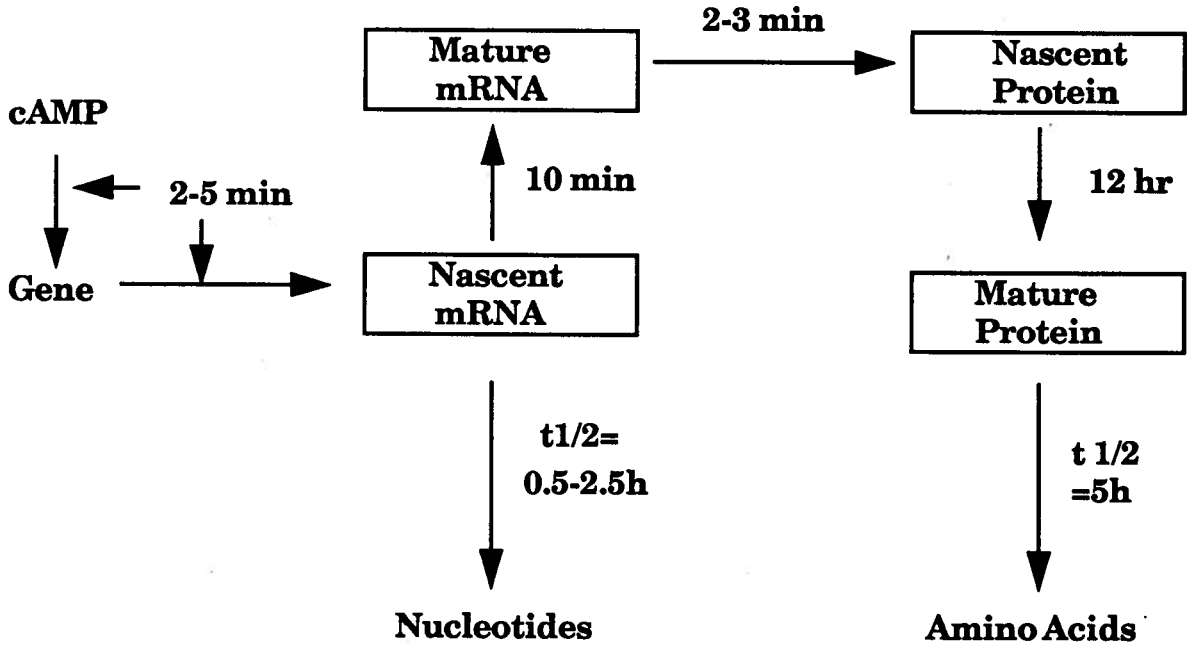
Figure 2 (upper panel) demonstrates the relationship between the turnover of the mRNA pool and the protein pool. From this interaction, Hargrove and Schmidt (1989) have developed a two compartment model that describes the relationship between the stability of a mRNA and the subsequent synthesis of its translate. In this model, rate of formation of both mRNAs and proteins are independent of their own concentrations and as such, exhibit zero order kinetics. Rates of degradation are exponentially linked to the concentration of a mRNA or protein and exhibit first order kinetics. Since rates of degradation are the only concentration-dependent variable, the rate of change in concentration for a given mRNA or protein is governed by its rate of degradation often expressed as the half-life. A further important feature of the two-compartment model is that rates of protein synthesis *are* dependent on the stability and concentration of mRNA. Therefore the induction of a protein to a new steady-state concentration can be described as the following solution (Hargrove & Schmidt, 1989):

$$P_{ss} = \frac{R_{ss}.k_sp}{k_dp} \qquad \text{(Equ. 1)}$$

FIGURE 2. Two compartment model of Protein Expression



Timing of PEPCK Expression.



Compiled from Hargrove & Schmidt, 1989.

where P_{ss} denotes new steady state protein concentration, R_{ss} denotes new steady state mRNA concentration, k_sp denotes the new rate constant for protein synthesis and k_dp is the new protein degradation rate constant.

This model presents overall protein turnover as a function of mRNA and protein half-life. Extending protein half-life, as predicted during metabolic suppression, therefore extends the length of time a protein is present irrespective of its rate of synthesis. Because of the strong dependence of protein synthesis on mRNA concentration, changes in protein concentration to a new steady state can occur either by an alteration in transcription rates or a change in mRNA stability. This dominates over rates of protein degradation since mRNA half-lives for individual proteins are generally much shorter than their active protein translates, and the average translational yield from one mRNA is 10,000 protein translates representing a large amplification effect (Hargrove *et al.* 1991). As a result of the apparent dependence of protein synthesis rates on mRNA concentrations, Hargrove and Schmidt suggest that the most energetically favourable method of suppressing protein synthesis rates is to effectively decrease the concentration of translatable mRNA. Conversely, an increase in protein synthesis rates is best achieved by increasing the rate of translation of existing mRNAs.

A further point of note demonstrated in figure 2 (lower panel) is the timing of gene and protein induction, demonstrated for phosphoenolpyruvate carboxykinase (PEPCK). Signalling, gene induction, transcription, mRNA maturation and translation only require about 2.5% of the time required to produce a fully active, mature protein. Given that the half-life of the mature protein is 5h, and post-translational processing of the nascent protein requires 12h, it appears that further control of rates of protein appearance and removal could be exerted at this rate-limiting step.

Control of translational rates via phosphorylation of initiation and elongation factors appears to be a major mechanism for acute modulation of protein synthesis. About 20 proteins involved in initiation and elongation are known to undergo phosphorylation by protein kinases. Since the synthesis of all proteins begins with the binding of Met-tRNA to the 40S ribosomal subunit, this step represents the rate limiting step for the initiation of protein synthesis and as such, initiation is under tight control. The key enzyme in the binding of Met-tRNA to the 40S ribosomal subunit is eukaryotic initiation factor-2 (eIF-2) whose activity can be suppressed in vivo by phosphorylation of a serine residue on the α-subunit (Davies *et al.*, 1989; Kaufmann, *et al.* 1989). Phosphorylation also occurs amongst several proteins involved in elongation, most significantly, elongation factor-2 (eEF-2) which catalyses the translocation of peptidyl-tRNA from the A to P sites on the ribosome. Phosphorylation by a Ca^{2+}-dependent protein kinase leads to its inactivation (Ryazanov *et al.*, 1988).

The GTP/GDP ratio represents a further level of control in protein synthesis. Increased GDP concentrations results in the competitive inhibition of GTP binding to the eIF-2 initiation complex, together with a number of elongation factors possessing GTPase activity (Walton & Gill, 1976).

Control of protein degradation is much less well understood. Cellular protein degradation is an amalgam of several different pathways, some compartmentalised (eg in the lysosome) and others cytosolic (eg ubiquitin-dependent pathways). Most protein degradation pathways have a requirement for ATP hydrolysis or binding either in their regulation, or the maintenance of a favourable proteolytic environment. For instance, the ubiquitin-dependent pathway requires the binding of ATP in the activation of co-factors involved in binding ubiquitin to proteins, prior to their degradation (Hershko, 1988). In the lysosome, ATP is hydrolysed in the action

of an ATP-dependent proton pump that maintains a low pH within (Schneider, 1981). As such, proteolytic activity ceases if ATP concentrations dissipate (Gronostajski *et al.*, 1985; Plomp *et al.*, 1987)

Phosphorylation appears to play a role in altering the susceptibility of proteins to degradation. For instance, phosphorylation of neurofilaments decreases their susceptibility to degradation by calmodulin (Greenwood *et al.*, 1993) whereas Ca^{2+}-dependent phosphorylation of lipocortins leads to selective degradation by an undescribed membrane-bound protease (Chuah & Pallen, 1989).

Overall then, protein turnover in a steady state system is regulated at numerous points. Gross-level control of the concentration of a protein is manifested through the relative concentration of its mRNA versus its rate of degradation. Generally, acute modulation and fine-tuning of protein synthesis rates occurs via phosphorylation which modulates the catalytic efficiency of initiation and elongation factors. Control of protein degradation is likely much more specific to individual proteins, depending on the pathway employed and mode of substrate recognition. How then, does this framework of control fit with current research on the effects of hypoxia on protein turnover?

Effect of Hypoxia on Protein Turnover. Hypoxia has the general effect of reducing protein turnover rates in mammalian systems. Short-term hypoxia (10% O_2 for 6h) in rats for example, leads to a 20-35% decrease in protein synthesis rates in various tissues with the more characteristically energy demanding tissues (such as liver, kidney and brain) demonstrating the largest inhibition of 25, 29 and 32% respectively (Preedy *et al.*, 1989). Studies on rat hepatocytes have attempted to find the intracellular basis for this inhibition: 120min of anoxic incubation (0mmHg) results in a cessation of protein synthesis within 7 minutes of anoxia whilst

ATP concentrations remain at 80% of normoxic values. Progressively severe hypoxia (80 and 50mmHg) also shows a near complete cessation of protein synthesis rates despite high concentrations of cellular ATP, continued rates of amino acid transport and no apparent increase in rates of protein degradation (Lefebvre *et al.*, 1993). This indicates that the hypoxic inhibition of protein synthesis is rapid and apparently independent of changes in ATP concentrations.

What of the RNA pool? Hypoxia appears to lead to a dissociation of polyribosomal complexes from mRNA resulting in an effective change in translatable mRNA concentration (Surks & Berkowitz, 1970) and in rat hepatocytes, results in a slow fall in transcription rates (Lefebvre *et al.*, 1993). However, as with metabolic changes, neither of these processes occur on the same time-scale as the inhibition of protein synthesis.

The implication from these studies is clear: acute and global changes in protein synthesis rates occur on a time-scale that is different from metabolic changes incurred by hypoxia (evidenced by ATP and AMP concentrations) or changes in RNA concentrations. Protein degradation rates and rates of amino acid incorporation also appear to be negligible in slowing apparent protein synthesis rates (Lefebvre *et al.*, 1993). However, the fall *does* occur directly and proportionately with the loss of O_2, strongly implying a role for molecular oxygen itself in the regulation of protein synthesis.

Oxygen Sensing Processes in Hypoxic Gene Expression. In mammalian systems, hypoxia is associated with a tissue-specific increase in the expression of numerous gene products. The overall response is geared towards improving the capacity for metabolising glucose, increasing the capability of the vascular system to carry and deliver oxygen and substrate, and protecting against protein damage caused by defective post-translational processes. For instance, cultured

skeletal muscle cells demonstrate a 3-5 fold increase in transcription rates for aldolase, triosephosphate isomerase, pyruvate kinase and lactate dehydrogenase over normoxic values over 3 days of incubation at 3% O_2 (Webster, 1987). On a more rapid time-scale, liver and kidney show a several fold increase in the erythropoietic stimulating hormone, erythropoietin (Epo), and endothelial cells lining blood vessels and capillaries express a number of factors including autocoids, vascular epithelial growth factor (VEGF), platelet derived growth factor-β (PDGF-β) and endothelin-1, all involved in stimulating capillary growth or modulating vascular smooth muscle contractility.

Particularly significant is the manner in which some of these genes are activated and transcribed. Studies on the hypoxic induction of the Epo gene product in rat hepatocytes show a close relationship between the amount of Epo mRNA and protein translate expressed, and the severity of hypoxia (Eckhardt *et al.*, 1993). Expression of this gene can be manipulated by factors such as carbon monoxide, cobalt and nickel that affect the oxygen-dependent conformation of heme proteins. Epo gene induction is also blocked by inhibitors of ferro-chetalase activity in protoprophyrin ring synthesis. This kind of evidence has established that Epo gene induction is linked directly to changes in tissue oxygen content by a widely distributed (tissue and species) membrane-bound heme protein (Goldberg *et al.*, 1988; Eckhardt *et al.*, 1993; Maxwell *et al.*, 1993). With increasing hypoxia, the heme group within this protein changes from oxy- to deoxy-conformations with a resulting increase in the expression of one or more Epo transcription factors which subsequently activate the Epo gene (Tsuchiya *et al.*, 1993). The signalling mechanism that transduces the change in heme conformation into a genetic response has not yet been characterised but studies with agonists and second messengers that classically activate protein kinase C did not result in Epo gene expression (Eckhardt *et al.*,

1993).

This kind of O_2-sensing mechanism has also been implicated in the modulation of hormonal control over phosphyoenolpyruvate carboxykinase (PEPCK) activity in the liver. The periportal-perivenous (afferent-efferent) zones of the liver acinus demonstrate a large oxygen gradient from 65mmHg to 35mmHg on the venous side (Jungermann & Katz, 1989). This defines both functional and metabolic characteristics of hepatocytes distributed along this gradient such that periportal cells express higher activities of gluconeogenic enzymes (glucose-6-phosphatase, PEPCK for e.g), and perivenous cells appear preferentially glycolytic (expressing higher activities of glucokinase and pyruvate kinase) (Jungermann and Katz, 1989). Administration of glucagon induces expression of the PEPCK gene via cAMP and increases the amount of active protein present but the magnitude of this response diminishes along the periportal-perivenous oxygen gradient (Keitzmann *et al.*, 1992). Studies using homogenous populations of isolated hepatocytes demonstrate that glucagon-induced synthesis of PEPCK is modulated by oxygen, and the oxygen effect can be diminished with cobalt and stimulated under hypoxia with carbon monoxide (Keitzmann *et al.*, 1993). This strongly suggests that a heme protein oxygen sensing mechanism is involved in the oxygen-dependent modulation of PEPCK expression and possibly accounts for the differential expression of enzymes along the periportal-perivenous O_2 gradient (Jungermann and Katz, 1993). Whether the oxygen-sensitive expression of glycolytic enzymes is also connected with a heme protein oxygen sensing mechanism is not clear at present.

Oxygen-dependent modulation of gene expression appears to be a widespread and evolutionarily conserved phenomenon. In mammals, endothelial cells express vascular epithelial growth factor, interleukin-2, platelet derived growth factor-β, angiotensin converting factor,

endothelin-1, and carotid body tyrosine hydroxylase, in an oxygen-dependent manner (see Fanburg *et al.*, 1990). The involvement of a heme-protein based mechanism in transducing changes in O_2 concentrations also appears to be widespread. The heme-protein mechanism described for Epo expression is present in liver and kidney cell cultures derived from species throughout the class mammalia (Maxwell *et al.*, 1993). Furthermore, the evidence that a heme-protein is involved in hepatic PEPCK expression suggests that this kind of O_2-sensing mechanism is not unique to a single gene system.

Numerous classes of oxygen-sensing genes are also well described in the prokaryotes. In *Rhizobium* for example, the expression of proteins involved in nitrogen fixation is controlled by two proteins, FixL (a heme-protein) and FixJ which are increasingly expressed with decreasing oxygen O_2 tensions. *E. coli* is capable of modulating the expression of high and low affinity O_2 binding cytochrome genes, fumarate reductase and nitrate reductase through the *arc* (aerobic respiration control) and *fnr* (fumarate and nitrate reductases) O_2-sensing systems respectively (Iuchi *et al.*, 1990). The mechanism for O_2-sensing in the *arc* system appears to depend on the redox state of a component in the electron transport system whereas the *fnr* system demonstrated some of the characteristics of a heme-sensing mechanism.

Implications for Anoxia-tolerant Systems. The preceding discussion has established that the turnover of a protein in mammalian systems is regulated by the concentration of its mRNA and the protein's half-life. This in turn is fine-tuned by "acute regulators" of both translation and the various pathways of protein degradation. In anoxia-intolerant systems, progressive hypoxia results in a rapid inhibition of protein synthesis rates that occurs on a different magnitude and time-scale from metabolic changes implying a regulatory role for O_2 itself, over and above other controlling factors. Finally, O_2-sensing via conformation changes in a heme-

protein is a conserved phenomenon that regulates a number of O_2-sensitive gene products. What are the implications for anoxia-tolerant systems?

Since protein turnover is an energetically demanding process it is reasonable to assume protein turnover rates are likely suppressed along with other cellular processes during metabolic suppression. Anoxia-tolerant systems classically conserve ATP concentrations such that the putative fall in protein turnover rates likely occurs with little or no change in metabolic parameters (as observed above in rat hepatocytes). If this is the case then a regulatory role for O_2 at some level is a serious consideration.

A fall in protein turnover rates necessarily means that protein half-lives are extended and the requirement for mRNA transcription reduced. Although the inhibition of protein turnover and transcription rates would result in a large energetic saving, prolonged exposure of proteins to the cellular environment raises a number of other problems. The combined effects of chemical and physical protein modification, coupled with slow rates of protein replacement and removal would result in an accumulation of denatured and dysfunctional proteins over a period of time. For this reason, rates of protein synthesis and degradation are unlikely reduced to zero to avoid overt accumulation of damaged protein. Nevertheless, extended protein half-lives mean reduced rates of removal; if tissues are to regain function rapidly on re-oxygenation, a mechanism for maintaining protein structure and function during anoxia is implied.

The finding that O_2-sensing mechanisms govern the expression of certain hypoxia sensitive genes, and is evolutionarily conserved, has obvious significance in anoxia-tolerant organisms. Depending on the nature of the O_2-sensing mechanism, "tracking" changes in O_2 concentration provides the potential for the expression of a phenotype more suited to anaerobic conditions well before O_2 becomes limiting. The function of differential protein expression under anoxia is

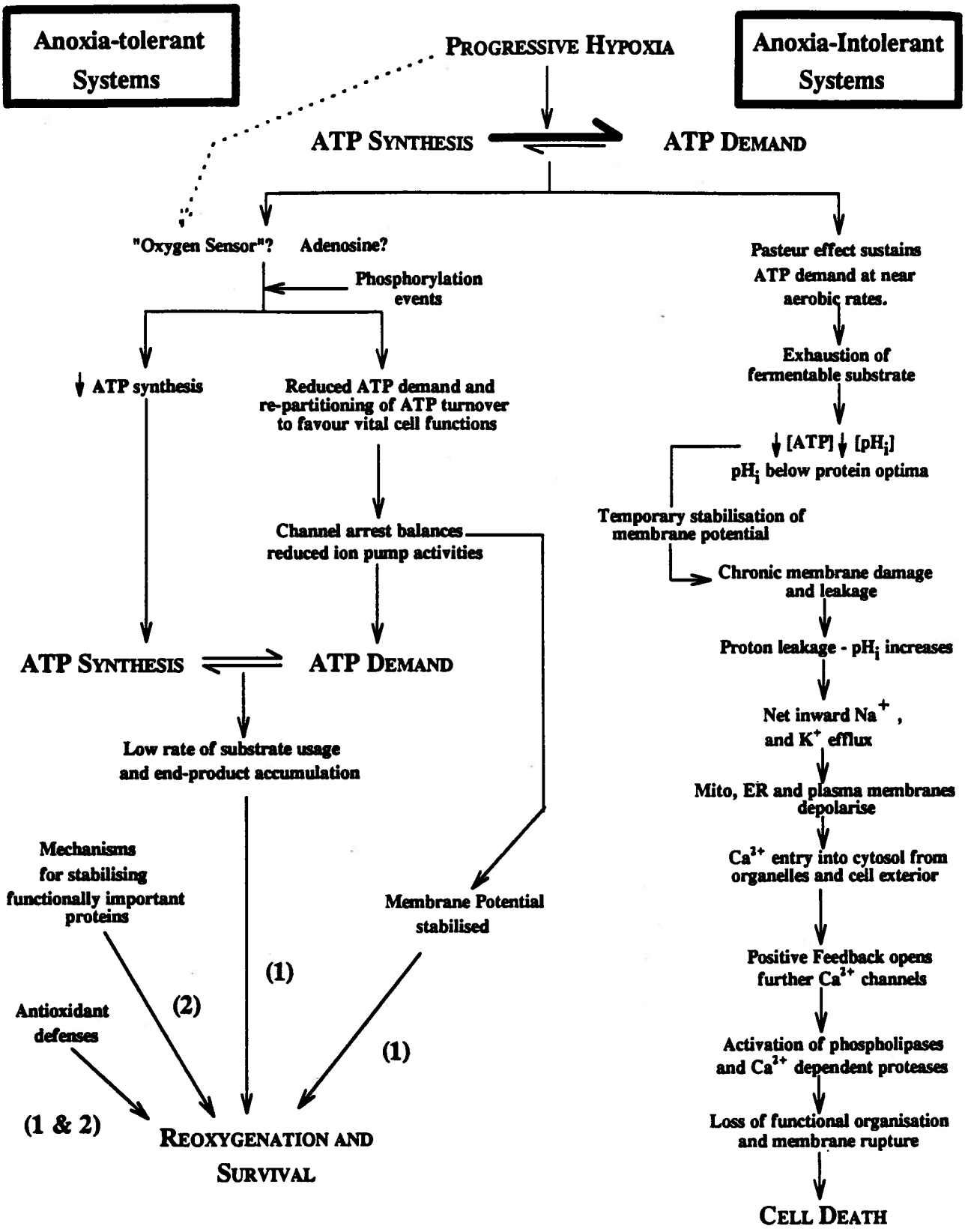
unclear but there exists the possibility that the expression of metabolic enzymes, stress proteins and free radical scavengers represents a part of this response that is associated with preserved cell function in anoxia and neutralising free-radical production on recovery.

Consequences of Suppressing Metabolism: Remaining Viable.

To this point, I have discussed mechanisms for increasing the efficiency of anaerobic ATP yield per mole of substrate, minimising and tolerance to metabolic end-product accumulation, and the coordinated changes required for suppressing metabolism. The final component that is required for the successful survival of a long-term period of anoxia involves maintaining functional stability: how are the metabolic characteristics of tissues preserved so that on recovery, active functions can resume unimpaired? The following discussion demonstrates that anoxia imposes both acute and chronic effects on cellular viability and that in metabolic suppression, these are dealt with in ways which are either novel, or appear as secondary consequences of other metabolic adjustments made in anoxia.

Acute Mechanisms of Cell Death in Anoxia. To understand why remaining viable under metabolic suppression is a problem, it is first important to understand how and why anoxia intolerant cells die as a result of oxygen limitation. Figure 3 demonstrates the sequence of events that result in anoxia-mediated cell death and compares them to the known sequence of events on entry into anoxia in anoxia-tolerant organisms. In anoxia-intolerant systems anaerobic metabolic demand is supported by a high rate of glycolysis which results in the progressive acidification of the cell. As anaerobic glycolysis becomes progressively unable to sustain ATP demand, a large and rapid fall in ATP concentrations occurs which is associated with accumulations of ADP, AMP and the adenosine breakdown products, xanthine and

FIGURE 3. Pathway of acute cell damage (cell death cascade) in anoxia-intolerant tissues, and mechanism of avoidance in anoxia-tolerant tissues. (1) = anoxia-tolerant pathways associated with avoidance of ***acute*** cell damage, (2) = pathways associated with avoidance of ***chronic*** cell damage.


Anoxia-tolerant Systems
PROGRESSIVE HYPOXIA
Anoxia-Intolerant Systems
ATP SYNTHESIS
ATP DEMAND
"Oxygen Sensor"?
Adenosine?
Phosphorylation events
ATP synthesis
Reduced ATP demand and re-partitioning of ATP turnover to favour vital cell functions
Channel arrest balances reduced ion pump activities
ATP SYNTHESIS
ATP DEMAND
Low rate of substrate usage and end-product accumulation
Mechanisms for stabilising functionally important proteins
Membrane Potential stabilised
(1)
(2)
(1)
Antioxidant defenses
(1 & 2)
REOXYGENATION AND SURVIVAL
Pasteur effect sustains ATP demand at near aerobic rates.
Exhaustion of fermentable substrate
↓ [ATP] ↓ [pHi]
pHi below protein optima
Temporary stabilisation of membrane potential
Chronic membrane damage and leakage
Proton leakage - pHi increases
Net inward Na+ , and K+ efflux
Mito, ER and plasma membranes depolarise
Ca2+ entry into cytosol from organelles and cell exterior
Positive Feedback opens further Ca2+ channels
Activation of phospholipases and Ca2+ dependent proteases
Loss of functional organisation and membrane rupture
CELL DEATH

hypoxanthine.

Classically, cell death has been ascribed to a large increase in intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$), resulting from the failure of membrane and mitochondrial potentials maintained by Na^+/K^+ ATPase and Ca^{2+}ATPases, as ATP concentrations fall. The subsequent increase in Ca^{2+} entry via the Na^+/Ca^{2+} exchanger activates phospholipases disrupting the plasma membrane, endoplasmic reticulum and mitochondrial membranes, and activates cytosolic proteases leading to the appearance of dysfunctional proteins. This serves to further increase the cycle of Ca^{2+} entry and cellular disruption. Although this model is appealing, it has recently been established that diminished ATP concentratons ([ATP]) are not associated with increases in $[Ca^{2+}]_i$. Furthermore, increasing $[Ca^{2+}]_i$ does not result in the appearance of overt features of cell disruption such as bleb formation (Lemasters *et al.*, 1987; Nieminen *et al.*, 1990).

The finding that a fall in intracellular pH (pH_i) facilitates cell survival during hypoxia (Bing *et al.*, 1973) lead to a new theory of hypoxic cell injury where buffering of low pH_i towards neutrality served as the initial trigger for the sequence of cell death, and maintenance of a low pH_i actually abrogated events in the cell death cascade (Lemasters *et al.*, 1993). This model of events during anoxic cell death has subsequently been termed the "pH Paradox" and centres on the observation that falling pH_i during anoxia results in a crude inhibition of functional proteins as pH_i falls below their various optima. This inhibition is thought to include a number of processes that regulate Ca^{2+} homeostasis and also some of the processes that play a role in cell disruption. In muscle fibres for example, the chief routes for Ca^{2+} entry [the Na^+/Ca^{2+} exchanger (Kim *et al.*, 1987); Ca^{2+} flux through slow Ca^{2+} channels (Chenais *et al.*, 1975); Ca^{2+} release from the sarcoplasmic reticulum (Nakamaru & Schwartz, 1972)] and phospholipase

A_2 activity (Harrison *et al.*, 1991) are all progressively inhibited as pH_i falls below 6.9. The pH paradox has been well characterised in liver tissue where a fall in pH_i from 7.4 to 6.9 and lower, during periods of anoxia-induced ATP depletion, results in significant increases in cell survival times during both anoxia and normoxic recovery (Currin *et al.*, 1991).

As demonstrated in figure 3, the "pH paradox" model suggests that the onset of cell death is function of pH_i. Slow increases in plasma membrane permeability over time results in the gradual efflux of H^+ and a subsequent increase in pH_i towards neutrality. The speed at which pH approaches neutrality increases as the plasma membrane depolarises. Increased rates of Na^+ entry result in rapid proton entry as Cl^- ions are extruded in exchange for Na^+ and CO_3^{2-} ions via the Na^+-dependent Cl^-/HCO_3^- antiporter. At the point of neutrality, and as the plasma membrane fully depolarises, Ca^{2+}-mediated cell death occurs through the classical pathways described above

Figure 3 also demonstrates how acute cell death is avoided in turtle tissues. Steady ATP concentrations coupled with channel and ion pump arrest avoids plasma and mitochondrial membrane depolarisation and the consequent entry of Ca^{2+} into the cell (cf Nieminen *et al.*, 1994). Furthermore, the proton buffering capacity of turtle tissues results in a gradual fall in pH_i in anoxia as opposed to the rapid and severe fall found in anoxia-intolerant tissues. In the turtle liver for example, pH_i falls linearly from 7.5 to 6.8 over a period of 2h (Wasser *et al.*, 1991) and in the isolated perfused heart, pH_i is maintained at 7.2 during four hours of anoxia (Wasser *et al.*, 1990). Clearly, although the "pH paradox" extends anoxic survival of anoxia-intolerant species, other mechanisms come into play in facultative anaerobes which avoid the necessity for pH-inhibition of processes for acute cell death.

From this analysis it seems that acute effects on cell viability are avoided largely by

metabolic adjustments (coordinated suppression of ATP supply and demand) which also include a reduction in membrane permeability (channel arrest) and a stable membrane potential. However, metabolic suppression as a mechanism for avoiding acute cell death creates a new set of conditions that affect cell survival in anoxia. Periods of suppressed metabolism can last from months to several years in some cases opening the way for long-term deleterious effects on cell viability. The next section discusses the potential for chronic, accumulative cell damage during metabolic suppression.

Chronic Accumulation of Cell Damage. Long-term effects of anoxia and metabolic suppression on cellular viability are poorly characterised in anoxia-tolerant species. However, it is reasonable to assume that if protein turnover is slowed during metabolic suppression, and protein half-lives increase accordingly, then the potential exists for accumulative damage to the protein pool, and subsequent failure of cell function in the long-term.

Extensive increases in protein half-lives for cytochrome c oxidase have been noted during anoxic dormancy in the brine shrimp embryo (*Artemia fransiscana*). Anoxic incubation of *Artemia* gastrulae results in a large depression in protein turnover rates that are associated with a reduced number of polysomes and a static mRNA pool (Hoffman & Hand, 1992). Studies on the anoxic turnover of cytochrome-c oxidase demonstrate a 77 fold increase in half-life for this enzyme from 1.3 to 101 days (Anchordoguy *et al.*, 1993). The significance of increased cellular protein half-life has been noted in cellular pathology studies and in particular, the field of cellular aging. Aging in tissues is associated with a chronic increase in the half-lives of total cell proteins. By extending the time a protein is present within the cell, the probability of post-translational modification resulting in conformational changes increases. With time, and reduced rates of protein removal, this leads to an accumulation of dysfunctional cellular protein

that can have a profound effect on the capability of tissues to remain functional (reviewed by Reff, 1985). If during metabolic suppression, protein turnover was substantially suppressed, the infinite increase in protein half-life could lead to a lethal accumulation of dysfunctional proteins. This clearly does not occur to lethal extents; anoxic incubation of *Artemia* embryos for 1½ years results in minimal reduction in hatch success rates (Clegg, 1992). Similarly in turtles after 6 months of anoxic submergence at 3°C, survivorship was 40%, with a number of deaths associated with fungal disease rather than overt signs of metabolic failure (Ultsch & Jackson, 1982). ***Given the potential for protein damage during long-term metabolic suppression, and the clear ability of anoxia-tolerant animals to regain full aerobic tissue function on recovery, it seems that the implication exists for a mechanism that preferentially stabilises proteins.*** By examining rates of protein synthesis and degradation in turtle hepatocytes, the potential for protein damage during metabolic suppression is assessed in the following research chapters and further discussed in chapter 5.

Cell Damage on Recovery From Anoxia. Recovery from anoxia has the potential to be more lethal than the period of anoxia itself due to re-exposure to O_2. The risk of oxygen superanion ($O_2^{\cdot-}$) production during hypoxia or recovery from anoxia is high, especially if associated with the catabolism of purines to xanthine as is often the case after short periods of hypoxia in anoxia-intolerant systems. Under aerobic conditions xanthine is catabolized by xanthine dehydrogenase (XDH) to uric acid and NADH. However, under hypoxic conditions, depletion of ATP results in the influx of Ca^{2+} ions into the cell activating a Ca^{2+} protease. This protease catalyses the conversion of XDH to its oxidase form, xanthine oxidase (XO), with the resulting catabolism of xanthine to uric acid and $O_2^{\cdot-}$ (McKelvey *et al.*, 1988). Once again, anoxia-tolerant organisms avoid this cascade by maintaining ATP concentrations high and

constant during the period of metabolic suppression thus avoiding changes in $[Ca^{2+}]_i$ and the loss of adenine purine nucleotides beyond IMP (Land *et al.*, 1993). Therefore under steady-state conditions, superoxide formation by this route is likely negligible, with the greatest risk occurring during metabolic adjustment from one condition to another, such as entry into metabolic suppression or recovery. During these times, rapid increases in $O_2^{-\cdot}$ concentrations are likely contained by superoxide dismutases, catalases and peroxidases, as has been demonstrated during anoxia in the garter snake (Hermes-Lima & Storey, 1992).

Overall, this discussion demonstrates that the successful survival of a long-term period of anoxia requires a coordinated metabolic response to negate rapid changes in cell membrane potential and substrate stores. The subsequent hypometabolism creates the potential for a new set of conditions that could foster accumulative damage to cellular macromolecules. Above all, tissues must regain function during the recovery phase and at the same time, reduce the risk of oxygen free radical production and resulting tissue damage.

This literature review has discussed the current views on the mechanisms of metabolic suppression and the proposed significance of protein turnover and protein stability to tissue survival under anoxia. Although the magnitude and fine-tuning of suppressed metabolism are tissue-specific, it is clear that the overall control of events occurs at the cellular level, without exogenous input. To successfully probe the molecular and biochemical events that occur in metabolically suppressed tissues, a model cell culture system is required that demonstrates the ability to survive anoxia by metabolic suppression.

The Turtle Hepatocyte as a Model System for the Study of Anoxia Tolerance.

Over the past 25 years, hepatocyte preparations from numerous vertebrate classes have been widely used to investigate aspects of cellular metabolism and its control. The reason for this large body of interest centres on the ease of hepatocyte preparation from numerous species and the wide metabolic and functional scope of liver tissue in general (Berry *et al.* 1991).

Survival of anoxia clearly requires a coordinated response between organ systems within the animal. Whereas whole animal studies generate highly valuable data regarding the coordination of metabolic changes between organ systems, these preparations are of limited use in examining the cellular energetics and control of anoxic metabolism in facultative anaerobes. Therefore a hepatocyte preparation was developed from the western painted turtle, *Chrysemys picta bellii* and used to further investigate the sub-cellular characteristics and control of metabolism in anoxia (Buck *et al.*, 1993b). Table 1 demonstrates the metabolic features of this cell preparation under normoxia and anoxia, without exogenous substrate. The principal point of note is that these isolated cells demonstrate all of the overt features of anoxia-tolerance discussed in this chapter, *vis:* extended viability in anoxia, high concentrations of fermentable substrate (glycogen), metabolic suppression, constant [ATP] and energy charge, reduced ion-pump activity with a constant membrane potential, and an ability to recover from extended periods of anoxia. The metabolic response to anoxia in rat hepatocytes is very different. Whole liver glycogen concentrations in rat are about 200mM, but much of this is lost during isolation, such that freshly isolated rat hepatocytes possess in the region of 1-10mM glycogen, and are heavily dependent on exogenous glucose (Senglen, 1976; Berry *et al.*, 1991). As a result, a 90% loss of [ATP] occurs during the first 30min of anoxia and a complete loss of membrane potential and viability occurs after a period of 2h, coinciding with dissipation of the

TABLE 1. Metabolic Characteristics of Isolated Turtle Hepatocytes.

Character	Normoxia (10h)	Anoxia (10h)
Metabolic Rate	68.4μmol ATP/g/h	6.5
Heat Flow	1.08mW/g	0.26
[ATP]	2.21μmol/g	2.01
Energy Charge	0.95	0.90
Energy Charge (10mM Iodoacetate)	0.00	0.18
Glycogen	720μmol/g	630
Glucose Release Rate	22.6μmol/g/h	7.5
Lactate Production Rate	n.m.	4.2
Gluconeogenic Rate	1.95μmol glucose/g/h	0.00
Na^+/K^+-ATPase	19.1μmol ATP/g/h	4.8
Membrane Potential	-31.3mV	-30.6

Data Compiled from: Buck, Land & Hochachka, 1993b; Buck *et al.*, 1993a; Buck & Hochachka, 1993; n.m. - not measurable.

mitochondrial membrane potential and exhausted glycogen stores (Andersson *et al.*, 1987; Aw & Jones, 1989).

Based on this data it appears that the turtle hepatocyte preparation represents an excellent model system for studying cellular control and energetics of metabolic suppression. Although the cells are isolated and not subject to systemic signals, they still display the same response to anoxia as whole liver in vivo: metabolic suppression, membrane stabilisation, glycogenolysis and glucose release. Also, since turtle hepatocytes demonstrate an ability to recovery from long periods of anoxia they must also conform to the principal of preservation of cell function.

Aims of the Research and Thesis Overview.

Metabolic suppression requires the coordinated suppression of multiple energy consuming cellular pathways. Each pathway is important to a greater or lesser extent in the continued survival of the cell but none is more important than protein synthesis and protein degradation in governing the phenotypic characteristics of the cell. Clearly, at the most basic level, the balance of protein turnover and gene expression governs cellular function before, during and after acute and chronic periods of dormancy. The aims of this research were therefore:

(1) To calculate the energetic costs of protein synthesis and protein degradation during normoxia and anoxic metabolic suppression (Chapters two and three).

(2) To determine the ATP-dependence of proteolysis and relative change in the stability of short and long lived protein pools as a result of reduced protein turnover (Chapter three).

(3) To examine the expression and suppression of anoxia-specific proteins, and in particular, whether an oxygen sensing mechanism is important in the control of this response

(Chapter four).

(4) To determine the relative energetic costs and significance of protein turnover and gene expression relative to other cellular processes during metabolic suppression (Chapter five)

Within these goals, a picture is presented of how a multifaceted cellular process such as protein turnover (grossly comprised of gene activation, transcription, translation, stabilisation and numerous proteolytic pathways) is suppressed and controlled as part of a larger and highly coordinated molecular, metabolic and physiological response to oxygen lack.

Chapter 2.
Protein Synthesis During Anoxia and Recovery in Anoxia Tolerant Hepatocytes from the Western Painted Turtle *Chrysemys picta bellii*.

Preface.

This chapter appears as a paper published by S.C. Land, L.T. Buck and P.W. Hochachka in the American Journal of Physiology 265(R34): R41-R48, 1993. L.T. Buck developed the method for isolating the hepatocytes. All aspects of the research were performed by myself.

Introduction.

The biosynthesis of proteins is a costly process requiring the hydrolysis of 4 ATP equivalents per peptide bond. In addition, a further cost of 1 ATP has been estimated to be the incidental cost of amino acid transport into the cell (Reeds *et al.*, 1985). Protein synthesis alone accounts for 18-26% of total energy production in various organisms and cell types (reviewed by Hawkins, 1991) and clearly consumes significantly more when the costs of mRNA synthesis and protein degradation are taken into account. In certain differentiating cell types, for example, protein synthesis and degradation (i.e. protein turnover) account for 35% to 41% of the total ATP utilization rate [Ehrlich ascites tumour cells and rabbit reticulocytes (Müller *et al.*, 1986 and Siems *et al.*, 1984 respectively)] with RNA synthesis accounting for a further 11% in the former case. For animals that depress metabolism in order to survive periods of chronic anoxia, sustaining rates of protein turnover would be an expensive process. It was therefore hypothesized that suppression of protein synthesis rates would account for a significant

proportion of the depression in metabolic rate under anoxia.

Tolerance to hypoxia is well developed in the western painted turtle (*Chrysemys picta bellii*). The ability of this animal to withstand anoxic submergence is well characterised with forced dive survival times of up to 2 weeks at 18°C and 4-5 months at 3°C (Robin *et al.*, 1964; Ultsch & Jackson, 1982). A major adaptive strategy enabling survival of such a chronic period of anoxia is the ability to coordinate the suppression of ATP utilizing processes, with slowed ATP synthesis rates sustained by anaerobic glycolysis. The result is that overall ATP turnover is suppressed while ATP concentrations remain steady (Hochachka, 1986; Kelly & Storey, 1988). Hepatocytes isolated from this species show a similar response when exposed to anoxia and have been demonstrated to maintain viability and energy charge for at least 10 hours under anoxia and cyanide administration at 25°C (Buck *et al.*, 1993b). By comparison, rat hepatocytes under cyanide lose up to 90% of the intracellular ATP pool after 30 mins of anoxia and display a complete loss in viability after 2 hours (Anderson *et al.*, 1987; Aw & Jones, 1989). As such, hepatocytes isolated from *C. picta* provide an excellent model for studies in anoxia tolerance and metabolic suppression.

In order to assess the role of protein synthesis in this metabolic suppression, fractional rates of protein synthesis during normoxia, anoxia and recovery were measured in painted turtle hepatocytes. From this an energy cost for protein synthesis was calculated under normoxic and anoxic conditions. Since protein synthesis rates are closely correlated with energy state through alterations in the GTP:GDP ratio (Walton & Gill, 1976; Garcia-Esteller *et al.*, 1983), the response of the purine nucleotide pool to anoxia was also investigated. Finally, rates of urea production were measured to provide a window into the costs of amino acid and nucleotide catabolism under normoxia and anoxia.

Materials and Methods.

Chemicals. L-[2,6-^{3}H]phenylalanine (specific activity (SA)= 56.0Ci.mmol^{-1}), L-[U^{14}C]serine (SA = 150mCi.mmol^{-1}) and L-[U ^{14}C]leucine (SA = 147Ci.mmol^{-1}) were purchased from Amersham Canada Ltd (Oakville, ON). HPLC grade K_2HPO_4 was from BDH Chemicals (Vancouver, BC). Minimum essential medium (MEM) amino acids were purchased from Gibco Canada (Burlington, ON). Electrophoresis reagents were purchased from Bio-Rad (Mississaugua, ON). All other chemical were purchased from Sigma Chemical Co (St Louis, MO).

Animals. Adult painted turtles were obtained from Lemberger Co., Inc. (Oshkosh WI) and housed in an outdoor tank equipped with a basking platform and heat lamps. Water temperature was maintained at an average of 22°C. Animals were fed chopped beef heart and liver *ad lib* and exhibited excess visceral fat and stomach contents prior to hepatocyte isolation. All animals used were females in the early stages of seasonal sexual maturation and were sampled over a five week period in mid summer.

Solutions. Isolation media were as previously described (Buck *et al.*, 1993b). Briefly they were as follows (in mM): Solution A: 78.5 NaCl, 34.5 $NaHCO_3$, 2.2 KCl, 0.9 Na_2HPO_4 and 10 Na^+-HEPES, pH7.5. Solution B: Solution A with 5.8 $CaCl_2$, 3.8 $MgCl_2$, 0.1 $MgSO_4$ and 2% BSA. Solution C: Solution A with 2U/ml Sigma protease XXIV. All isolation solutions were saturated with 95%O_2:5%CO_2.

Primary culturing media were: i) Storage Medium: Solution B with 2% BSA (dialysed against water), MEM amino acids and 200U/ml penicillin. MEM amino acids were reconstituted according to the manufacturers recommendations and diluted 100 fold into the storage medium. ii) Incubation Medium: Solution B with 2% dialysed BSA, MEM amino

acids (without phenylalanine), plus 0.6mM phenylalanine (phe) and 200U/ml penicillin. iii) Labelling medium: As for incubation medium but with 0.6mM non-radioactive phe and 0.6μCi.mg cells^{-1} L[2,6-^{3}H] phe added separately, during the experiment. Each primary culturing medium was saturated with 95%air:5%CO_2 unless otherwise indicated. All culturing media were sterile filtered and treated aseptically.

Hepatocyte Preparation. Hepatocytes were isolated by protease disaggregation as previously described (Buck *et al.*, 1993b). Immediately after isolation, cells were placed into storage medium, plated and kept overnight at 5°C. Contamination by non parenchymal cells was always <2%. Adenylate energy charge (EC) was 0.9 1hr post isolation and increased to 0.94 overnight. Prior to the experiment, cells were warmed to 25°C for 1hr before being placed in the appropriate experimental incubation medium. Cell viability throughout the experiment was assessed in terms of the adenylate energy charge as determined from parallel experiments.

Experimental Design. The experiments were conducted in two parts. In part A., each hepatocyte preparation was divided into three distinct pools for the parallel assessment of protein synthesis parameters, rates of oxygen consumption and purine nucleotidephosphate profiles. Cells from the protein synthesis pool were further subdivided into two groups for technique validation and the measurement of fractional rates of protein synthesis. Part B. describes separate experiments, conducted to investigate rates of urea production and labelling of proteins during recovery from anoxia for 1 dimensional SDS polyacrylamide gel electrophoresis (1-D SDS-PAGE). All determinations were made at 25°C.

Part A. - Rate of Protein Synthesis.

Validation. To validate the conditions used to measure fractional rates of protein

synthesis, cells were incubated in normoxic incubation medium with the following alterations: i) 0.6mM non-radioactive phe + 30μCi.$^{-1}$ml L[2,6-^{3}H]phe. ii) 2 μCi.$^{-1}$ml L-[U-^{14}C]serine (ser), iii) 2μCi.ml^{-1}L-[U-^{14}C]ser + 1.2mM non-radioactive phe. Incorporation of amino acids into protein was assessed over the course of an hour by removing 50μl samples at 5, 20, 40 and 60 minutes onto Whatman #3 filter discs. These were washed in 10%(w/v) TCA (5ml/filter) for 20 minutes before being placed in fresh 5% TCA and heated to 100°C for 5min. Finally, filters were washed in 10% TCA at 20°C, then 95% ethanol and air dried. The radioactivity of the intracellular free pool for condition i) was determined by centrifuging a 100μl aliquot of cells through a mix of dimethyl phthalate and dioctyl phthalate (1:5 v:v) into 10% TCA. The intracellular protein was allowed to precipitate on ice for 30min before being removed by centrifugation. The resulting TCA soluble radioactivity was measured to give intracellular phe free pool activity. [^{14}C]-ser and [^{3}H]-phe counts were determined on a LKB Rack-beta 1214 scintillation counter using ACSII (Amersham) scintillation cocktail with a counting efficiency of 43.5% for [^{3}H] and 95% efficiency for [^{14}C]. Condition i) above, represents the incorporation behaviour of cells under the labelling conditions used to measure fractional rates of protein synthesis (see below) and groups ii) and iii) represent the effects of double the dosage of non-radiolabelled phe on rates of ser incorporation into protein.

Incorporation of Isotope into Protein. Cells apportioned to the measurement of fractional rates of protein synthesis were divided into the following experimental groups: Normoxic (NOR, 12h aerobic), anoxic (ANX, 12h anoxic), cycloheximide (CYC, 12h aerobic; 10μmol cycloheximide.mg packed cells^{-1}, administered 2hr prior to, and during, addition of isotope), 1st recovery period (R-1, 12hr anoxic, 1hr normoxic), 2nd recovery period (R-2, 12hr anoxic, 2hr normoxic). For each experimental group, 1ml of cells at 50mg.ml^{-1}, in duplicate, were

suspended in either air (95%air;5%CO_2 -aerobic) or nitrogen (95%N_2;5%CO_2 - anoxic) saturated incubation media as appropriate and placed in sterile culture tubes. Each culture tube was set up to maintain a pressure head of the appropriate, humidified gas mixture for the duration of the experiment as previously described (Buck *et al.*, 1993b). Cells were kept continuously suspended by rotation ($\sim$1.5 cycles.sec^{-1}) using an IKA-Vibrax-VXR rotator (Janke & Kunkel Co., W. Germany).

30min before the end of the 12hr experimental incubation period, cells were removed from incubation medium by gentle centrifugation (30xg for 2 minutes) and resuspended in the same volume of labelling medium without isotope, saturated with the appropriate gas mixture. Labelling medium for the CYC group contained the same dosage of cycloheximide as during the incubation. At this time, recovery groups were also resuspended in normoxic labelling medium. Cells were then allowed to recover from handling for 30min before the addition of isotope.

At 12hr, 0.6 μCi,L-[2,6 ^{3}H] phe.mg cells^{-1} was administered by injection though the chamber cap. Cells were allowed to incorporate isotope into protein under the appropriate experimental condition for 1hr. For the R-2 group, label was administered 1hr post anoxic recovery. Incorporation was stopped by centrifuging cells out of labelling medium (30xg, 2min) and washing the cell pellet 3 times with BSA-free solution B to remove all extracellular label and BSA (1-D SDS-PAGE confirmed that 3 washes in BSA-free solution B were required to remove all of the BSA from the cells). A relatively high dose of isotope was used in order to maximise the amount of label incorporated into cycloheximide and anoxia treated cells. An excess of non-radioactive phe in the labelling medium was used to give linear rates of radiolabel uptake with a constant intracellular free phe pool activity (fig 4).

Total cellular protein was precipitated by adding 1ml 5% perchloric acid (PCA) to cell pellets. Precipitates were allowed to form on ice for 30min before centrifugation and removal of acid soluble radioactivity (intracellular free pool). The protein pellet was washed a further 3 times in 2ml 5% PCA, then resuspended in 2.5ml 0.5M NaOH and incubated for 1hr at 37°C to re-solubilise the protein and hydrolyse RNA. The resulting solution was used to determine protein content (BioRad protein assay kit) and total acid insoluble radioactivity. All determinations of radioactivity were carried out by liquid scintillation spectrophotometry as described above.

In order to determine total RNA content and the specific activity of protein-incorporated [^{3}H]-phe, protein dissolved in the remaining NaOH solution was re-precipitated with 0.444ml 20% PCA and pelleted by centrifugation. The supernatant was removed to measure RNA content using the dual wavelength procedure of Munro & Fleck (1969). The extinction coefficient for RNA was A_{260}=32.9 with contaminating protein corrected as A_{232}=6.11 (Ashford & Pain, 1986). The remaining protein pellet was washed a further 3x with 2ml 5% PCA and then hydrolysed in 6ml 6M HCl for 48hr at 110°C. The HCl was removed by evaporation and the resulting amino acids were washed with water and again evaporated to dryness. The hydrolysate was then dissolved in 2ml 0.5M Na^+-citrate (pH6.3).

Determination of the specific activity of [^{3}H]-phe in the intracellular free pool and hydrolysate fractions was measured by the enzymatic conversion of phe to β-phenylethylamine (β-PEA) as previously described (Garlick *et al.*, 1980). We found that the formed β-PEA product was light sensitive and as a result this step and all ensuing steps (including organic extraction) were performed in the dark. To 1ml sample, 3ml 3M NaOH and 8ml n-heptane:chloroform (3:1v/v) were added and the sample mixed thoroughly. The NaOH was

removed by freezing and the β-PEA was extracted from the organic layer by mixing thoroughly with 0.01M H_2SO_4. After extraction, 0.1ml of hydrolysates and 1ml of free pools were assayed for [β-PEA] using the method of Suzuki & Yagi (1976). Briefly, this involved incubation of the samples with 10mM ninhydrin, 0.2mM L-leucyl-L-alanine and 0.5M potassium phosphate (pH8.0) at 60°C for 1 hour. After incubation, samples were cooled to room temperature and fluorescence measured at 495nm (excitation 390nm) on a Farrand Ratio Fluorometer-2 (Farrand Optical Co, NY). Since the light from the fluorometer caused a constant rate of decay of the fluorescent product, readings were standardized to 10s after exposing the sample to the light source.

The fractional rate of protein synthesis (k_s, $\%.hr^{-1}$) was calculated as follows:

$$k_s = \frac{S_B}{S_A} \times \frac{60}{t} \times 100 \qquad \text{(Equ. 2)}$$

where S_B is the protein bound specific activity of phenylalanine ($disintegrations.min^{-1}$ $(DPM)nmol\ phe^{-1}$), S_A is the radioactivity of the intracellular free pool ($DPM.nmol\ phe^{-1}$) and t is the length of time the cells were incubated with label (in minutes).

Absolute rates of protein synthesis (mg protein synthesized.g $cells^{-1}.hour^{-1}$), used to compute ATP turnover rates, were calculated as the fractional rate of protein synthesis multiplied by the total cellular protein content. Finally, the RNA:protein ratio ($\mu gRNA.mg\ protein^{-1}$) was used to express rates of protein synthesis relative to the tissue RNA content (k_{RNA}, $gProtein.mgRNA^{-1}.hour^{-1}$) as follows:

$$k_{RNA} = \frac{10k_s}{\text{RNA:protein}} \qquad \text{(Equ. 3)}$$

(Millward *et al.*, 1973; Preedy *et al.*, 1988).

Oxygen Consumption. Rates of oxygen consumption were measured polarographically

using Clark type electrodes (Yellow Springs, Ohio) in a 2ml thermostatted Gilson oxycell at 25°C (Middleton, WI). Electrodes were calibrated using air equilibrated super-pure H_2O at 25°C, assuming an O_2 solubility of 258.3μmol $O_2.dm^{-3}$ at 1 atmosphere (Forstner & Gnaiger, 1983). Cultures in storage medium were equilibrated to 25°C and continually gassed with 95% air;5% CO_2 for 2hr prior to being placed in the oxycell at approximately 6mg.ml^{-1}. To assess the proportion of cycloheximide inhibitable oxygen consumption, 10μmol cycloheximide.mg^{-1} cells was administered at a point after a linear rate of oxygen consumption had been obtained under control conditions. The concentration of cycloheximide used was based on the dosage at which a sustained maximal effect could be obtained immediately. All experiments were carried out in duplicate.

Extraction and Measurement of Purine Nucleotide Phosphates. Purine nucleotide phosphate profiles were measured in normoxic, anoxic and cycloheximide treated cultures from experiments conducted parallel to the protein synthesis group. The gassing and cycloheximide administration procedure was the same with the exception that the cells were incubated in 25ml erlenmeyer flasks at an initial volume of 4ml at 50mg.ml^{-1}. PCA extracts were taken at 0,5 and 10hr for normoxic and anoxic cultures and at 2hr post administration for the cycloheximide treated culture; each culture was sampled in duplicate. The procedure used to extract for purine nucleotides was as described (Buck *et al.*, 1993b). After extraction, samples were immediately frozen in liquid N_2 and stored at -80°C until required.

ATP, ADP, AMP and IMP were measured using an LKB 2152 HPLC with a 7μm Aquapore AX300 ion exchange column (Brownlee Columns, Applied Biosystems Inc, CA) as previously described (Schulte *et al.*, 1992). GTP,GDP and GMP were measured simultaneously with the adenylates by extending the run time to 36min allowing the elution of GTP.

Part B. - Urea Production. PCA extracts were taken from experiments conducted separately from the studies in part A. Hepatocytes suspended in solution B. were incubated in 25ml erlenmeyer flasks under normoxia and anoxia as previously described (Buck *et al.*, 1993b). PCA extracts of the cell suspension were taken in duplicate at 0 2.5,5,7.5 and 10hr of incubation. Urea was measured in K_2CO_3 neutralised extracts using the glutamate dehydrogenase linked assay (Bergmeyer, 1974). A rate of urea production was calculated from the steady state rate of urea accumulation over 5, 7.5 and 10hr under normoxic and anoxic conditions.

In Vivo *Labelling of Proteins During Recovery for Electrophoresis and Autoradiography.* A separate series of experiments was conducted to investigate the 1 dimensional electrophoretic pattern of total cellular proteins exposed to 12hr normoxia and 1-3hr recovery from 12h anoxia. Incubation procedures, prior to labelling, were as described above for the protein synthesis rate study with the exception that a third hour recovery group was also included. After 12hr incubation under the appropriate gassing regime, the normoxic and first hour recovery groups were resuspended in normoxic storage medium without leucine (leu) but with 50μCi L-[U^{14}C]leu.ml^{-1}). At this time, second and third hour recovery groups were resuspended in non-radioactive normoxic storage medium. At 2 and 3hr into the recovery period, these groups were suspended in medium containing L-[U^{14}C]leu as described above. Each culture was allowed to incorporate label for 1hr. At the end of the labelling period, each experimental group was washed in BSA-free solution C x3 and the final cell pellet lysed by boiling for 10 minutes in 2x loading buffer (100mM Tris base (pH6.8), 200mM dithiothreitol, 4% SDS, 0.2% bromophenol blue, 20% glycerol and 1mM phenylmethyonylsulfonyl fluoride (PMSF)). The resulting slurry was sonicated for 2x 15s and centrifuged at 10000xg for 10 minutes. L-

[U^{14}C]leu was used as the labelling amino acid as it labelled the protein pool with the highest specific activity when compared to [^{14}C]-ser, [^{3}H]-phe and [^{35}S]-methionine.

[^{14}C]-leu labelled proteins were electrophoresed on a one dimensional gel using the discontinuous buffer system of Laemmi (1970). Briefly, this employed a 5% acrylamide stacking gel and a 11.5% resolving gel. The running buffer consisted of 26mM Tris base (pH to 8.8 using HCl), 200mM glycine and 0.1% SDS. $1x10^5$ trichloroacetic acid insoluble counts per minute, per sample, were loaded and the gel run overnight at 4°C using a constant amperage of 10mA. At the end of the run, gels were soaked for 30min in Amplify fluor (Amersham), rinsed in distilled water and dried using a Bio-Rad gel drier. Flourographs were exposed for 72hr on pre-flashed Kodak OMat X-AR film at -70°C. Molecular weights were determined using Bio-Rad low molecular weight range standards.

Statistical Analysis. All data are presented as mean $\pm$ standard error with the number of observations indicated in each case. The value of n is defined as the number of independent hepatocyte preparations used in each series of experiments. Student's t-test was used to compare control and experimental groups with multiple groups compared using ANOVA with Tukey's HSD. Statistical significance is at $p < 0.05$.

Results.

Validation for the Measurement of Fractional Rates of Protein Synthesis. In order to investigate whether the conditions used to measure fractional rates of protein synthesis reflected an accurate rate of amino acid incorporation into cellular protein, a series of validation experiments were carried out. For this to be an accurate technique, the intracellular free pool activity should remain high and constant with rates of phe incorporation into protein linear over

the sampling period. In addition, administration of a flooding dose of phe should not artefactually affect rates of protein synthesis. Figure 4 demonstrates that the rate of L-[U-^{14}C]ser incorporation into protein was not affected by a two fold excess of non-radioactive phe. This suggests that the use of a high, flooding dose of phe did not affect the overall rate of amino acid incorporation into protein. Under the conditions used to measure fractional rates of protein synthesis, the rate of incorporation of L-[2,6-^{3}H]phe into cellular protein remained linear over the 1hr sampling period with the activity of the intracellular free pool remaining high and constant. Therefore, the conditions employed provide an accurate indication of the rates of protein synthesis in turtle hepatocytes.

Rates of Protein Synthesis. Anoxia induced a fall in the fractional rate of protein synthesis (the percentage of the protein pool that turns over per hour) by 92% of control values which was indistinguishable from the rate obtained with the inhibitor, cycloheximide (Fig. 5A). During normoxic recovery, k_s increased to 160% of control values and returned to normal after 2hr. These changes in protein synthesis rates were not accompanied by any change in the RNA:protein ratio such that k_{RNA} reflected the same pattern as the k_s results from normoxia to anoxia and under cycloheximide administration (Figs. 5B and C). However, first hour recovery k_{RNA} values were statistically insignificant from control groups.

Specific ATP Requirement for Protein Synthesis. In order to investigate rates of ATP turnover for protein synthesis under normoxia and anoxia, it is necessary to calculate a specific ATP requirement for the synthesis of each peptide bond. Reeds *et al.* (1985) have suggested that the minimum theoretical cost for protein synthesis is 5 ATP equivalents per peptide bond. From this, a theoretical value of 46mmol ATP.g protein synthesized^{-1} is arrived at, assuming an average amino acid molecular weight of 110da. Table 2 demonstrates the route taken to

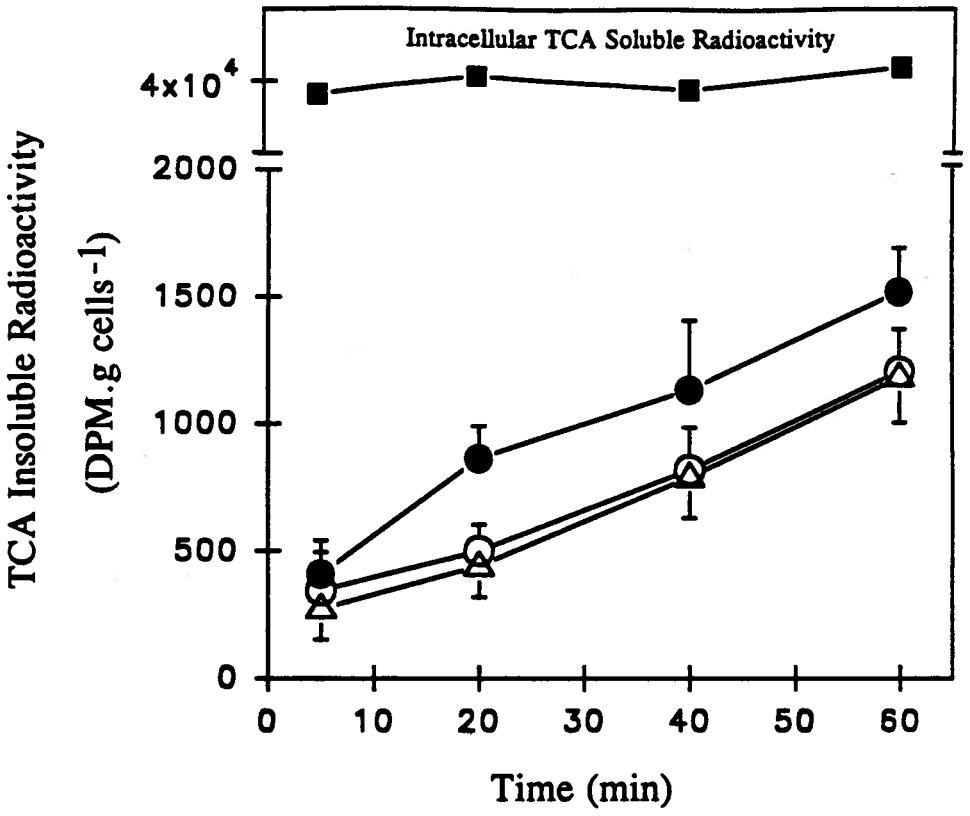


FIGURE 4. Validation of experimental conditions used to measure fractional rates of protein synthesis. 0.6mM non-radioactive phe + 30μCi.$^{-1}$ml L[2,6-^{3}H]phe (Closed circles) and the resulting intracellular TCA soluble radioactivity (closed squares, error bars within symbols), 2 μCi.$^{-1}$ml L-[U-^{14}C]ser (open circles), 2μCi.ml^{-1}L-[U-^{14}C]ser + 1.2mM non-radioactive phe (open triangles). N=3. *Abbreviations*: DPM, Disintegrations per minute; TCA, Trichloroacetic acid.

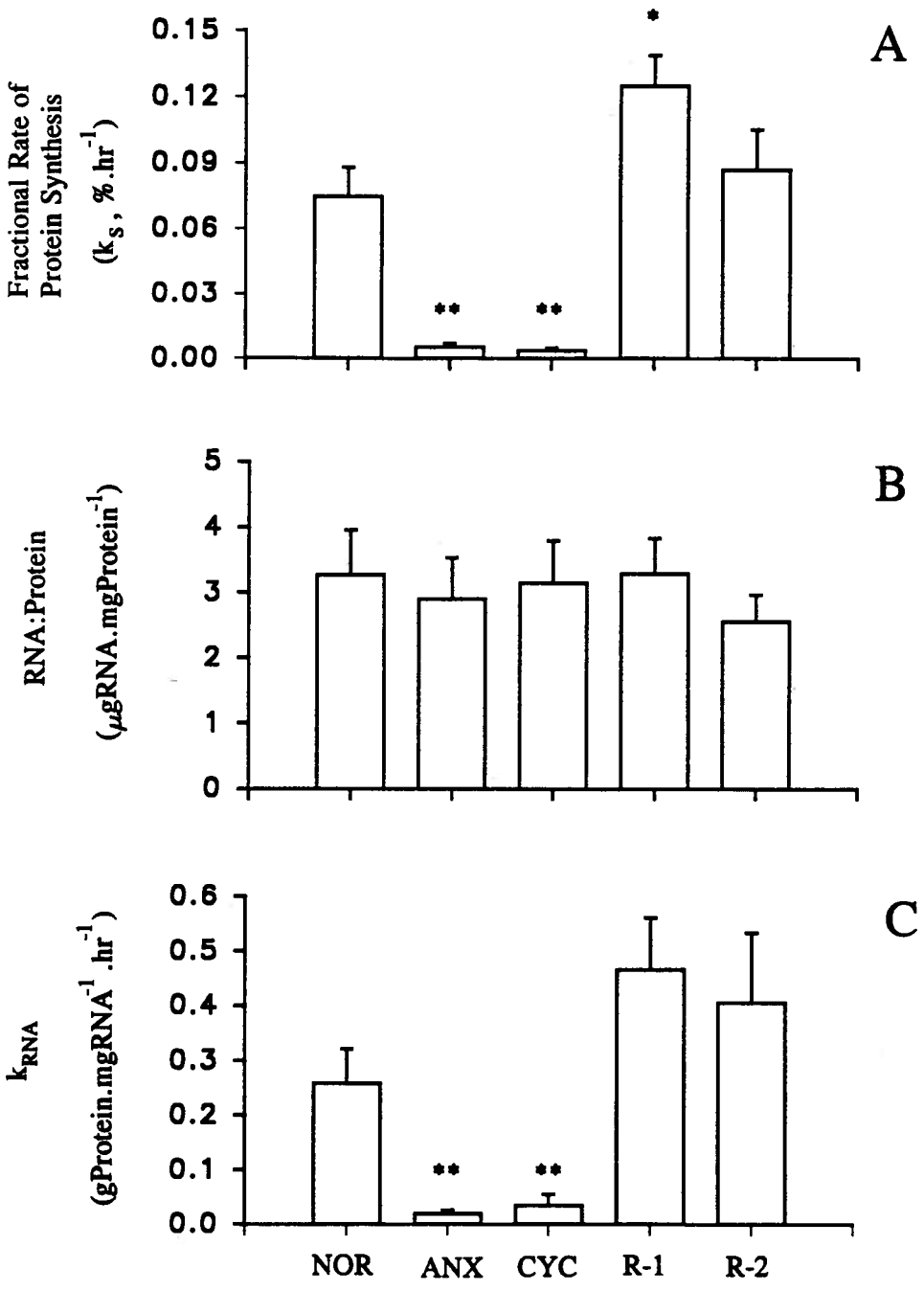


FIGURE 5. Response of translation to anoxia and recovery. (A.) Fractional rates of protein synthesis, (B.) RNA:Protein ratio and (C.) K_{RNA} for 12hrs normoxia (NOR), 12hr anoxia (ANX), 12hr normoxic - final 2hr with 10μmol.mg^{-1} cells cycloheximide (CYC), 1hr post anoxic recovery (R-1) and 2hr post anoxic recovery (R-2). N=5. **P<0.01, *P<0.05.

empirically determine the specific ATP requirement for protein synthesis from the present data. By using cycloheximide inhibitable O_2 consumption to calculate a cellular rate of ATP turnover for protein synthesis and dividing this by the change in absolute rates of protein synthesis with cycloheximide, we were able to calculate a specific ATP requirement for protein synthesis of 47.6 $\pm$ 6.8mmols ATP.g Protein^{-1}. Assuming an average amino acid molecular weight of 110da, we arrived at 5.2 $\pm$ 0.9 ATP per peptide bond, close to the predicted theoretical value.

Proportion of Metabolism Utilized by Protein Synthesis. Using the specific ATP requirement for protein synthesis, we calculated protein synthesis ATP turnover rates from absolute rates of protein synthesis measured under normoxia and anoxia (Table 3). These were expressed as percentages of total ATP turnover, calculated from the rate of O_2 consumption (normoxic total rate) and from the rate of lactate production [anoxic total rate; data from Buck *et al.*, (1993b)]. In addition, the extent of cycloheximide inhibitable O_2 consumption was used to provide an independent measurement of ATP turnover required for protein synthesis under normoxic conditions. ATP turnover rates for protein synthesis fell 92% under anoxia, as expected from the k_s values presented above. Despite the reduction in ATP turnover, the proportion of total metabolism required by protein synthesis remained statistically insignificant between normoxia and anoxia. The normoxic rate of ATP turnover calculated from cycloheximide inhibitable O_2 consumption was similar to that from the absolute protein synthesis rate (19.6 and 24.4μmolATP.g cells^{-1}.hr^{-1}, respectively). Under normoxic conditions, the proportion of ATP turnover for protein synthesis calculated from these routes was 28.1 to 36.2%.

Proportion of Metabolism Utilized by Urea Production. Rates of urea production fell 72% from 0.51 $\pm$ 0.13 to 0.16 $\pm$ 0.09μmol urea.g cells^{-1}.hr^{-1} under anoxia. Assuming a cost of 4

TABLE 2. Calculation of a Specific ATP Requirement for Protein Synthesis from Cycloheximide Inhibitable Oxygen Consumption and Absolute Rates of Protein Synthesis.

Change in ATP turnover with cycloheximide.	
Control rate of O_2 consumption	$12.0 \pm 1.3 \mu molO_2.g\ cells^{-1}.hr^{-1}$
Cycloheximide rate of O_2 consumption	$8.7 \pm 1.2 \mu molO_2.g\ cells^{-1}.hr^{-1}$
Δ O_2 consumption	$3.3 \pm 0.3 \mu molO_2.g\ cells^{-1}.hr^{-1}$
Assuming a P:O ratio of 3, then:	
ΔATP turnover with cycloheximide	$19.6 \pm 1.7 \mu molATP.g^{-1}.hr^{-1}$
Change in absolute rates of protein synthesis with cycloheximide.	
Normoxic rate	$0.53 \pm 0.08 mgP.g\ cells^{-1}.hr^{-1}$
Cycloheximide rate	$0.06 \pm 0.04 mgP.g\ cells^{-1}.hr^{-1}$
Δ Absolute rate of protein synthesis	$0.47 \pm 0.08 mgP.g\ cells^{-1}.hr^{-1}$

†ATP Requirement = 47.6 ± 6.8 mmolATP.g Protein^{-1}

Assuming average amino acid molecular weight as 110da then:

Specific ATP Requirement = 5.2 ± 0.9 ATP.Peptide Bond^{-1}

Values are mean ± S.E., n=5. All determinations made under normoxic conditions.

$$\dagger \text{ ATP Requirement} = \frac{\Delta\text{ATP turnover with cycloheximide}}{\Delta\text{Absolute rate of protein synthesis with cycloheximide}}$$

TABLE 3. Percentage of Normoxic and Anoxic ATP Turnover Utilized by Protein Synthesis.

Conditions	Total ATP Turnover (μmol.g cells^{-1}.hr^{-1})	Absolute Rate of Protein Synthesis (mgP.g cells^{-1}.hr^{-1})	Protein Synthesis ATP Turnover (μmol.g cells^{-1}.hr^{-1})	% of Total
Normoxia	67.0 ± 4.3[†]	0.53 ± 0.08	24.4 ± 3.9[§] (19.6 ± 1.7[†])	36.2 ± 5.8 (28.1 ± 2.1)
Anoxia	6.3 ± 0.4[‡]	0.03 ± 0.01[*]	1.6 ± 0.5[*§]	25.0 ± 7.3

Values are mean ± S.E., n=5. [*]$P<0.01$ relative to Normoxic group.

[†] Calculated from rate of oxygen consumption assuming P:O ratio of 3. Numbers in parentheses for the normoxic group indicate protein synthesis ATP turnover calculated from cycloheximide inhibition of O_2 consumption (Control and inhibited O_2 consumption values given in Table 2).

[‡] Calculated from rate of lactate accumulation; data from Buck *et al.*,(1993b).

[§] Calculated from absolute rate of protein synthesis values assuming 46mmol ATP.gprotein^{-1}.

ATP per urea produced (Atkinson, 1992), an ATP turnover rate for urea synthesis was calculated (Table 4). Under normoxic conditions, the proportion of metabolism utilized by urea synthesis was 3% of the total ATP turnover rate. In anoxia, this proportion remained statistically insignificant from the normoxic rate.

Purine Nucleotide Phosphate Profiles. In order to investigate the relationship between the rate of protein synthesis and energy state, adenine and guanine nucleotide phosphate profiles were measured during anoxia and under cycloheximide administration (Table 5). Neither [ATP] nor [GTP] changed significantly, however both [ADP] and [GDP] increased 2 fold up to 5hr anoxia and remained constant to 10hr. As a result of the rise in [GDP] the GTP:GDP ratio fell 3 fold by 5hr anoxia and remained constant to 10hr. The control nucleotide diphosphokinase mass action ratio (MAR) was calculated at 1.5 and this did not change significantly between normoxia and anoxia.

To investigate whether there was any loss of purine nucleotides by deamination, inosine monophosphate (IMP) concentrations were measured. [IMP] increased 4 fold over controls by 5hr of anoxia, decreasing to 2 fold by 10hr. The elevation in [IMP] was not large enough to reflect a loss of adenylates or guanylates however; despite the [IMP] change the total purine nucleotide phosphate pool remained statistically similar in all groups.

Administration of cycloheximide did not alter purine nucleotide phosphate concentrations with the exception of AMP and IMP, both being significantly elevated relative to control.

Patterns of Protein Synthesis During Recovery. Figure 6 demonstrates the pattern of radiolabel incorporation into normoxic and first, second and third hour recovery proteins separated by 1 dimensional SDS-PAGE. Two minor protein bands were detected during recovery at 44.5 and 36.3 kDa. However, expression of these protein bands alone could not

TABLE 4. Percentage of Normoxic and Anoxic ATP Turnover Utilized by Urea Synthesis.

Conditions.	Total ATP Turnover (μmol.g cells^{-1}.hr^{-1})	Urea Synthesis ATP Turnover (μmol.g cells^{-1}.hr^{-1})	% of Total
Normoxic	67.0 ± 4.3	2.0 ± 0.5	3.0 ± 0.7
Anoxic	6.3 ± 0.4	0.6 ± 0.3*	10.3 ± 5.5

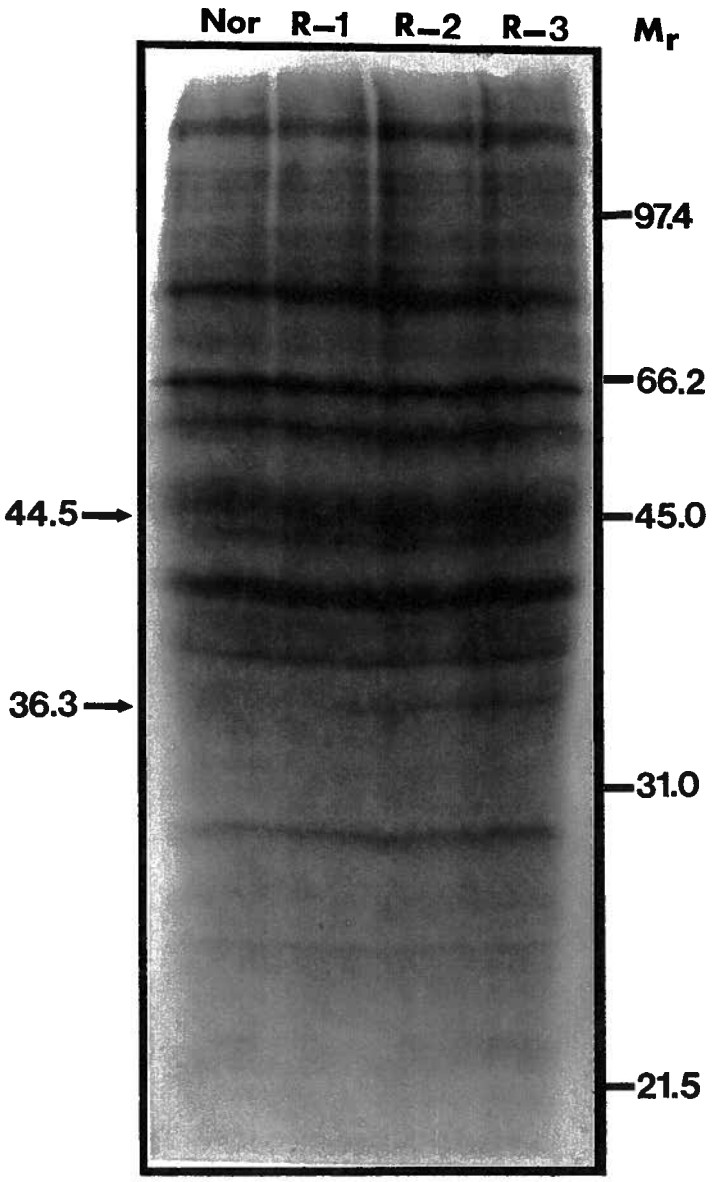
Values are mean ± SE, n=6. *$P<0.05$ relative to normoxic group. ATP- turnover for rates of urea synthesis were calculated assuming a cost of 4 moles of ATP per mole of urea (Atkinson, 1992). Rates were calculated from slopes between 5, 7.5 and 10hr time points. Total ATP turnover rates are as defined in Table 3.

TABLE 5. Purine nucleotide phosphate profiles during normoxia, anoxia and cycloheximide administration.

Condition	ATP	ADP	AMP	GTP	GDP	GMP	IMP	NDPKase MAR	GTP:GDP
Control.									
0 hr	2.208 ± 0.031	0.268 ± 0.013	0.036 ± 0.010	0.237 ± 0.009	0.020 ± 0.003	0.015 ± 0.007	0.016 ± 0.003	1.51 ± 0.22	18.57 ± 2.59
Normoxia.									
5 hr	2.250 ± 0.134	0.235 ± 0.077	0.040 ± 0.013	0.193 ± 0.028	0.015 ± 0.003	0.012 ± 0.003	0.017 ± 0.003	1.68 ± 0.50	17.52 ± 3.46
10	2.314 ± 0.164	0.261 ± 0.052	0.034 ± 0.010	0.246 ± 0.023	0.018 ± 0.004	0.014 ± 0.002	0.016 ± 0.004	2.16 ± 0.83	20.05 ± 2.23
Anoxia.									
5 hr	1.740 ± 0.078	0.475 ± 0.040*	0.096 ± 0.046	0.193 ± 0.026	0.041 ± 0.006*	0.015 ± 0.002	0.067 ± 0.007*	1.63 ± 0.41	6.33 ± 1.79*
10	2.008 ± 0.304	0.517 ± 0.052*	0.065 ± 0.023	0.281 ± 0.024	0.045 ± 0.012*	0.010 ± 0.002	0.034 ± 0.002*	4.01 ± 1.74	6.62 ± 1.86*
Cyclo-heximide.	2.136 ± 0.032	0.214 ± 0.032	0.117 ± 0.032*	0.289 ± 0.038	0.013 ± 0.001	0.012 ± 0.002	0.027 ± 0.006	2.45 ± 0.80	16.72 ± 1.71

Values are means ± SE in $\mu mol.g^{-1}$ cell wet weight. Value of *n* in each case is 7. *P < 0.05 relative to 0hr control point. ATP, ADP and AMP data are expanded from results previously published in Buck *et al.*, (7). *Abbreviations:* GTP, Guanosine 5' triphosphate; GDP, Guanosine 5' diphosphate; GMP, Guanosine 5' monophosphate; IMP, Inosine 5' monophosphate; NDPKase, Nucleotide diphosphate kinase; MAR, mass action ratio. NDPKase MAR= [ATP].[GDP]/[ADP].[GTP].

FIGURE 6. Protein profile of recovery from 12h anoxia in whole turtle hepatocyte lysates. 1-D SDS-PAGE autoradiogram of proteins labelled with [^{14}C]-leu under normoxia (NOR) and first (R-1), second (R-2) and third (R-3) hour aerobic recovery from anoxia. Positions and weights of the Bio-Rad molecular weight markers are shown on the right.


Nor
R–1
R–2
R–3
M_r
97.4
66.2
44.5
45.0
36.3
31.0
21.5

account for the full extent of the recovery elevation in protein synthesis rates. It is therefore likely that this is a generalised increase in protein expression of the total protein pool response.

Discussion.

A previous study reported a fall of 90% in turtle hepatocyte metabolic rate on entry into anoxia (Buck *et al.*, 1993b) which is similar to the 83% reduction in metabolism reported for the whole animal during an anoxic dive (Herbert & Jackson, 1985). In this study, protein synthesis and urea production were investigated as single aspects of metabolism and it was found that the extent of anoxic depression (92 and 72%, respectively) was similar in magnitude to the fall in total cellular metabolism. Furthermore, energy requirements for both protein synthesis and urea production remained at constant proportions of total ATP turnover from normoxia to anoxia. From this, it is concluded that anoxia results in a suppression of these pathways that is coordinated with the fall in total cellular metabolism.

The question remains as to how the depression in protein synthesis might be controlled. A number of studies have noted a correlation between basal k_s and the RNA:protein ratio (Houlihan *et al.*, 1990 and refs. therein) and this has been explained in terms of a high proportion of the RNA pool being ribosomal (Young, 1970). If this is the case, the RNA:protein ratio serves as a gross measure of tissue ribosomal content. In this study, the observed changes in k_s occurred independently of any alteration in the RNA:protein ratio suggesting that acute, environmentally induced changes in k_s are not the result of changing relative concentrations of the macromolecules required for protein synthesis. However, this does not preclude a redistribution of the protein synthesis components. Previous studies have indicated that hypoxia induces a breakdown of polysomes in rat liver (Surks & Berkowitz, 1971)

and a recent study on dormant *Artemia* cysts suggests that anoxia induces polysomal disaggregation there also (Hoffman & Hand, 1992).

A regulator of the state of polysomal aggregation is the GTP:GDP ratio. GDP is a competitive inhibitor of GTP binding to several protein synthesis factors and has been demonstrated to inhibit formation of the Met-tRNA$_f$.GTP.eIF-2 initiation complex (Walton & Gill, 1976) and several elongation factors possessing GTPase activity (reviewed by Pall, 1985). The process of protein synthesis is therefore under sensitive feedback control with a fall in the GTP:GDP ratio resulting in an inhibition of polyribosome formation and peptide elongation in existing polyribosomes. Furthermore, the guanylate pool is in equilibrium with the adenylate pool through the enzyme nucleoside diphosphate kinase (NDPKase). In this study, the fall in rates of protein synthesis under anoxia was accompanied by a 3 fold decrease in the GTP:GDP ratio despite the maintenance of the ATP and GTP pools. The rise in nucleotide diphosphates led to the speculation that changes in this ratio may be linked to inhibition of NDPKase as previous studies in *Artemia* have suggested that rates of protein synthesis might be altered under anoxia by acidotic inhibition of NDPKase (Hoffman & Hand, 1992). However, this does not appear likely for turtle hepatocytes since i) the MAR for NDPKase (1.5) did not change significantly between normoxic, anoxic or cycloheximide groups, ii) NDPKase from rat liver has a pH optimum between 6.5 and 9.5 (Kimura & Shimada, 1988) suggesting that this enzyme is active over a wide range of pH values and iii) pH_i is reported to fall gradually from 7.5 to 6.8 at 20°C in whole turtle liver over 2hr of anoxic submergence (Wasser *et al.*, 1991).

To explain the NDP accumulation within the cell, it is tentatively suggested that ATP and GTP are hydrolysed to new, lower steady state values during the first 5hr of anoxia. Although NTP concentrations appear to remain steady during the anoxic incubation the standard deviation

is large enough to mask the small perturbation in [NTP] that would be required to account for the observed increase in [NDP]. This may be coupled with an inhibition of the pathway for purine catabolism. The total purine nucleotide pool remains constant with accumulating IMP suggesting that purine catabolism is inhibited past 5'-nucleotidase. This is supported by the observed decrease in rates of urea production, the final end-product of amino acid and purine catabolism, which is largely inhibited by anoxia (Table 3).

During recovery from anoxia, fractional rates of protein synthesis rose to 160% of normoxic values before returning to normal after 2hr. Figure 6 shows that, other than the synthesis of two minor protein bands, there were no major differences in normoxic and recovery 1-dimensional SDS-PAGE patterns suggesting that this increase is not attributable to the synthesis of specific sets of proteins but is a generalised response of total cell protein. Hypoxic recovery treatment in mammalian cell lines induces the synthesis of a set of stress proteins known as oxygen regulated proteins (ORP) or hypoxia associated proteins (HAP) (Heacock & Sutherland, 1986; Zimmermann *et al.*, 1991). Synthesis of these proteins is thought to confer tolerance to repeated exposure to hypoxia and recovery in these cell types. In turtle hepatocytes there was no apparent up-regulation of proteins of similar molecular weight on recovery from anoxia suggesting that either these cells may constitutively express ORP/HAP's or are not subject to the same stresses as mammalian cells during recovery from anoxia.

Exactly what leads to the increase in protein synthesis rates during recovery is unclear, however it is possible that this is part of an elevation in total cellular metabolism. Such an event has been characterised during recovery from anoxia in marine intertidal organisms where total oxygen consumption and metabolic heat production are elevated relative to pre-anoxic

levels ["oxygen debt repayment" (Shick *et al.*, 1988)]. Although this response is partially explained in terms of the removal of anaerobic end-products, it is possible that there could be an anabolic component to this also.

Empirical determinations of the number of ATP's required per peptide bond tend to range widely in the literature with the closest determination to the theoretical estimate (5 ATP per peptide bond) put at 7.5 ATP per peptide bond (68mmol ATP.gProtein synthesized^{-1}) (Aoyagi *et al.*, 1988). In this study it was assumed that cycloheximide does not affect aspects of energy metabolism aside from the inhibition of protein synthesis, that attenuation of protein synthesis is not accompanied by an increased rate of protein degradation and that the mitochondrial genome contributes negligibly to whole cell protein synthesis (not inhibited by cycloheximide). Calculation of energy charge values from table 4 reveals that, with the exception of increased [AMP], cycloheximide administration did not perturb [ATP], [ADP] or the GTP:GDP ratio. Furthermore, based on low state 3 rates of mitochondrial oxidation and from qualitative electron microscopy, it appears that mitochondrial volume density is low in turtle liver tissue (Alamedia-Val and Buck, unpubl. obs). An ATP requirement of 5.2 $\pm$ 0.9 ATP per peptide bond was calculated which is close to the theoretical value of 5. From this, it was determined that protein synthesis constitutes 28-36% of the normoxic and 25% of the anoxic ATP utilization rate in this type of tissue.

The explanation as to why protein synthesis should account for such a high proportion of ATP turnover may lie in the state of sexual maturation of the animals from which the hepatocyte preparations are obtained. During seasonal gonadal development in oviparous vertebrates, the liver becomes increasingly involved in the *de novo* synthesis of vitellogenin. In fish, the result is that hepatocyte protein synthesis rates double over the course of

gonadogenesis (Haschmeyer & Mathews, 1983). In turtles therefore, it is likely that sexual maturation in females is also accompanied by elevated hepatic protein synthesis rates.

To conclude, this chapter has shown that metabolic suppression in turtle hepatocytes is associated with the coordinated down-regulation of the pathways for both protein synthesis and urea production. The reduction in the GTP:GDP ratio, an event that is likely independent of changes in pH_i, may play a role in the inhibition of protein synthesis through inhibiting the formation of polyribosomal complexes. Recovery from anoxia is associated with a transient increased protein synthesis rates and this does not appear to be related to major changes in expressed protein profiles. Such post-anoxic increases in protein synthesis rates may be aimed at clearing and replacing dysfunctional proteins formed during the anoxic bout or on recovery.

Chapter 3.
Protein Turnover During Metabolic Suppression: Role and Energy Dependence of Proteolysis.

Preface.

The data presented in this chapter were published in a paper by S.C. Land and P.W. Hochachka (American Journal of Physiology (Cell Physiology 35): C1028-C1036, 1994 . All aspects of this work were performed by myself.

Introduction.

Entrance into a hypometabolic state in response to adverse environmental conditions requires two key conditions to be met: *i)* the reduction in rates of ATP synthesis must be balanced and coordinated with reduced ATP demand such that ATP concentrations remain constant (Hochachka, 1986), and *ii)* the functional capability of tissues must be maintained during the period of metabolic arrest so that on recovery, the tissue may resume active metabolism unimpaired.

Although there has been much interest placed on the mechanisms behind the coordinated reduction in ATP turnover during metabolic arrest, little is known about the status of cellular functions that are responsible for preserving the functional stability of tissues. Of particular importance to this question is the stability of the protein pool. As noted in cellular aging studies, suppression of protein turnover rates increases the exposure of proteins to chemical post-translational modifications resulting in conformational changes and inactivation (reviewed by Reff, 1985). With reduced rates of removal by proteolysis, damaged proteins accumulate

eventually leading to the death of the tissue. Since dormancies can last from months to years in some cases, avoiding the occurrence or accumulation of damaged proteins is vital to sustaining tissue functional stability during chronic metabolic arrest. Therefore it was hypothesised that there would be a significant component of remaining ATP turnover concerned with the continued replacement and renewal of cellular protein.

The experiments presented in chapter two demonstrated that during such a metabolic suppression, turtle hepatocyte protein synthesis, and the ATP-turnover specific to this process, was reduced by 92% under anoxia. This occurred in a manner that was proportional to, and coordinated with, the suppression of whole cell metabolic rate. The arrest of protein synthesis was not complete, however, with remaining protein synthesis rates accounting for the same proportion of total anoxic metabolism as under normoxic conditions (about 30%).

If protein synthesis is sustained under anoxia, despite a large down regulation from normoxic rates, what is the role and cost of sustaining proteolysis during this time? Although peptide bond hydrolysis is a thermodynamically favoured process, most proteolytic pathways have a functional requirement for metabolic energy either for maintenance of the proteolytic environment [eg, lysosomal ATP-dependent proton pump, (Schneider, 1981)] or activation of co-factors [as in ATP-dependent ubiquitin conjugating enzymes, (Hershko, 1988)]. It is difficult to assess the relative costs incurred by each proteolytic pathway *in vivo*; however, previous studies indicate that the total energy dependence of proteolysis may account for about 1-14% of total ATP consumption depending on the synthetic status of the tissue (Siems *et al.*, 1984; Müller *et al.*, 1986). When considered together with protein synthesis, the costs for turning over total cell protein can amount to as much as 40% of total normoxic ATP turnover rates in the various differentiating cell types studied so far (Siems *et al.*, 1984; Müller *et al.*, 1986;

Schneider *et al.*, 1990).

Because of the costs involved in sustaining protein turnover, a balance must be reached between the conflicting demands of energy conservation and the renewal of cellular protein. To investigate the role and energy dependence of protein degradation during anoxic metabolic suppression, this study aims to 1) measure changes in protein degradation rates during exposure to anoxia and recovery for fast- and slow-turnover proteins, 2) calculate an energy budget for proteolysis and total protein turnover under normoxic and anoxic conditions and 3) measure differences in the proportion of coupling between labile and stable protein pools and ATP turnover.

Materials and Methods.

Chemicals. L[U-^{14}C]phenylalanine ([^{14}C]phe; specific activity (SA)= 479mCi.mmol^{-1}), L[2,6-^{3}H]phenylalanine ([^{3}H]phe; SA= 53Ci.mmol^{-1}) and L[4,5-^{3}H]leucine ([^{3}H]leu; SA=147Ci.mmol^{-1}) were purchased from Amersham (Oakville, Ontario, Canada). Minimum essential medium (MEM) amino acids were from GIBCO (Burlington, Ontario, Canada). α_2-Macroglobulin was purchased from Boehringer Mannheim (Laval, Quebec, Canada). Cycloheximide, emetine dihydrochloride, leupeptin, pepstatin-A, penicillin-streptomycin, iodoacetate, antimycin-A and all other bench chemicals were purchased from Sigma Chemical Co, (St. Louis, MO).

Primary Culture. Adult female western painted turtles weighing 300-500g were obtained from Lemberger (Oshkosh, WI). Hepatocytes were isolated from females in the early stages of sexual maturation as described in chapter two. After several cleaning stages, cells were suspended in storage medium containing 4% bovine serum albumin (BSA) and 100U/ml

penicillin:10μg/ml streptomycin as modified from Land *et al.* (1993). 3ml of cells, at a density of 35mg.ml^{-1}, were then plated in sterile 35x10mm Corning tissue culture dishes and then maintained at 25°C in a 5% CO_2 humidified incubator. Under these conditions cells had firmly adhered to the base of the culture dish within 12h of plating and LDH leakage remained less than 4% to 72h post-plating. Procedures for measuring cell viability through lactate dehydrogenase (LDH) leakage, glycogen content and adenylate concentrations between 0 and 55h post-plating are previously described (Buck *et al.*, 1993b).

Measurement of Protein Degradation.

Protein degradation rates were measured by following the rate of liberation of radioactive phenylalanine from prelabelled proteins in plated, static cultures of hepatocytes as described by Bradley (1977). The method required prelabelling incubations with two radiolabels ([^{14}C]phe and [^{3}H]phe) to discern if there existed differences between protein degradation rates in cultures pre-labelled to favour slow or rapidly turning over proteins (Vandenburgh & Kaufmann, 1980).

Assessment of Conditions for Measuring Protein Degradation. Appropriate conditions for the conduct of the protein degradation study were determined in the following pilot experiments. The minimum concentration of non-radioactive phe required to minimize isotope re-incorporation into protein during the study was assessed in cultures labelled with 2μCi [^{14}C]phe for 22h at 25°C. After washing cultures free of excess label, the release of [^{14}C]phe into the medium was measured at 6h post-labelling in the presence of various concentrations of non-radioactive phe. From this, 1.1mM non-radioactive phe was determined as the optimal minimum concentration required to prevent the reincorporation of isotope into cellular protein. To ensure that the use of 1.1mM phe did not affect rates of cellular protein degradation, cultures were pre-labelled for 22h with 5μCi [^{3}H]leu and 2μCi [^{14}C]phe. The rates of liberation

for each isotope were followed in the presence of 0.1 and 1.1mM non-radioactive phe. Intracellular TCA-soluble radioactivity for [^{14}C]phe labelled cultures was also followed in the presence of 1.1mM phe to confirm that there was no accumulation of radioactivity within cells.

To ensure that there was no appreciable loss of [^{14}C]phe label to [^{14}C]O_2, oxidation rates were measured as described by French *et al.* (1981). Briefly, cells were incubated at 25°C for 1-3h in the presence of 1μCi [^{14}C]phe. [^{14}C]O_2 that evolved during the incubation period was collected on glass-fibre filters impregnated with 1M benzethonium hydroxide (hyamine hydroxide). The resulting radioactivity was quantified by liquid scintillation spectroscopy as described below. Finally, radioactivity eluting with phenylalanine was assessed by chromatographic separation of amino acids on a Bio-Rad Rosil C-18 HL column (250 x 4.6mm, 5μm particle size) using a Waters 625 LC system (Toronto, Ontario, Canada) equipped with a Waters 995 photodiode array detector. Data were analyzed using Millennium v1.1 software (Waters).

Pre-labelling of Proteins. 3ml storage medium without phe but with 2μCi.ml^{-1} [^{14}C]phe was added to each culture at 20h post-plating to label slower turning over proteins. Cells were allowed to incorporate label for a further 22h at which point the [^{14}C]phe labelling medium was removed and each culture washed 5 times with 3ml, non-radioactive storage medium containing 1.1mM excess phe (degradation medium). [^{14}C]phe was removed from the more labile protein pool by incubating the cultures for 2h in degradation medium at 25°C after which, each culture was again washed as described above. At this point, rapidly turning over proteins were labelled by incubating cultures in 3ml storage medium without phe, containing 10μCi.ml^{-1} [^{3}H]phe for 30min at 25°C. Cultures were then rinsed 5 times with 3ml degradation medium reducing [^{14}C] and [^{3}H] radioactivity in the supernatant to a constant minimum number of counts.

Experimental Procedure. After the proteins had been pre-labelled, independent sets of experiments were conducted with cultures maintained under the following conditions: 1) Four experimental groups incubated under normoxia, anoxia, anoxia+1mM NaCN, 5h anoxia+5h normoxic recovery. 2) Six experimental groups incubated under normoxic conditions with additions of 0.5mM leupeptin, 0.1mM pepstatin-A, 1U.ml^{-1} $\alpha_2$macroglobulin, leupeptin+pepstatin-A+$\alpha_2$macroglobulin together (same concentrations), 0.1mM cycloheximide and 0.5mM emetine. Titers of proteolytic inhibitors, cycloheximide and emetine, were maintained by replacing medium after each sampling point with degradation medium containing the appropriate concentration of each inhibitor; 3) Two experimental groups incubated under normoxia+1mM antimycin-A and anoxia + 10mM iodoacetate. All cultures to be incubated under normoxic (95% air; 5% CO_2) or anoxic (95% N_2;5% CO_2) conditions were placed in sealable plexiglass chambers with a positive pressure of the appropriate atmosphere maintained throughout the experiment. All cultures were incubated at 25°C on a rotary shaker (50 rpm) for the duration of each experiment.

At 0.5, 1, 1.5, 2, 3, 5, 7 and 10h into the experiment, the entire 3ml of degradation medium was removed and replaced with 3ml freshly gassed, and temperature equilibrated, medium containing the appropriate concentration of proteolytic or metabolic inhibitor as required. Total radioactivity was measured directly in a 100μl aliquot of the removed supernatant. To 0.9ml of the remaining supernatant, 0.1ml 100% (vol/vol) trichloroacetic acid (TCA) was added, the sample vortexed and the precipitate allowed to form for 20h at 4°C. TCA insoluble material was removed by centrifugation for 30min at 10,000xg and the TCA soluble radioactivity determined in a 100μl aliquot. After sampling the medium at the final time point, each culture was washed three times in degradation medium before being resuspended

in 2ml 10% (vol/vol) TCA. Cells were then sonicated on ice for 2x15s and the resulting mixture was also incubated for 20h at 4°C. After centrifugation, an aliquot was removed from the supernatant for the determination of intracellular TCA soluble radioactivity. The remaining protein pellet was then washed a further two times in 10% TCA and finally dissolved in 1ml 1M NaOH by vigorous shaking on a IKA-Vibrax-VXR rotator (Janke & Kunkel, Bremen, Germany) at 25°C for 2h. Radioactivity in each sample was determined by scintillation counting in ACSII aqueous scintillation cocktail (Amersham, ON) using an LKB Rack-beta 1214 scintillation counter set to dual mode for [^{14}C] and [^{3}H] spectroscopy (efficiency = 95 and 43.5%, respectively).

Total initial radioactivity incorporated into protein was determined as the sum of the total intracellular radioactivity and the total radioactivity released into the medium. Percent radioactivity remaining at each time point was calculated as:

$$\frac{\text{Total Initial Radioactivity - Released TCA Soluble Radioactivity}}{\text{Total Initial Radioactivity}} \times 100 \qquad \text{(Equ. 4)}$$

Half-lives of [^{3}H]phe- and[^{14}C]phe-labelled protein pools were calculated as:

$$T½ = \ln 2/k \qquad \text{(Equ. 5)}$$

Where k= the logarithmic rate constant for the protein fraction degraded per hour (taken from the slopes between 0.5-3h for [^{3}H]phe-labelled proteins and 5-10h for [^{14}C]phe-labelled proteins).

Measurement of Normoxic and Anoxic Proteolytic ATP Turnover. The method used to measure ATP requirement for proteolysis was essentially as described by Seims *et al.* (1984). Normoxic ATP requirement was assessed by the polarographic measurement of oxygen

consumption rates. Cells suspended in degradation medium at a density of 8mg.ml^{-1} were placed into 2ml Gilson oxycell chambers (Middleton, WI) equipped with Clark-type electrodes and thermostatted to 25°C. Data were collected and analyzed using Datacan Acquisition System software (Sable Systems, UT) and criteria for electrode calibration were as described in chapter 2. After obtaining normoxic control rates of oxygen consumption, 0.1mM cycloheximide was administered to inhibit protein synthesis followed by 0.1mM cycloheximide with 0.5mM emetine-HCl to inhibit both protein synthesis and proteolysis (Siems *et al.*, 1984). The change in oxygen consumption rates was used to calculate the ATP-turnover associated with protein synthesis and protein degradation by assuming a P:O ratio of 3.

The anoxic ATP requirement for proteolysis was determined in cell suspensions from the same preparations by incubation under a humidified atmosphere of 95%N_2;5%CO_2 for 6h in the presence of anoxia only, anoxia + cycloheximide and anoxia + cycloheximide + emetine-HCl in the same concentrations as above. Duplicate samples were removed at 0,2h,4h and 6h and extracted in a 7% (v/v) final concentration of perchloric acid (PCA) and finally neutralised as previously described (Buck *et al.*, 1993b). Lactate was measured on a Perkin-Elmer Lambda 2 UV-vis spectrophotometer using the lactate dehydrogenase and glutamate-pyruvate transaminase linked assay described by Bergmeyer (1974). Conversion of lactate production rates to ATP turnover assumed P:lactate ratio of 1.5 (from glycogen).

Data Handling and Statistics. Statistical manipulations were performed using Systat version 5 (Evanston, IL) software. The value of *n* indicates the number of individual hepatocyte preparations used in each experiment and all percentage values were arcsin transformed. Confidence limits for significance are at 95% with the statistical test for each data set outlined in the figure or table legend. All data are expressed as mean ± standard error.

Results.

Culture Conditions. Table 6 lists cell viability criteria up to 55h postplating in cultures held under normoxic experimental conditions. LDH leakage remained at about 2% throughout and remained less than 4% to 72h. Metabolic competency based on adenylate concentrations from 0 to 55h was judged to be excellent and glycogen content remained high, although there was some loss from glucose mobilization and release (Buck *et al.*, 1993b). Nevertheless, remaining glycogen at 55h would have been enough to fuel a further 17.8h of glycolytically supported metabolism at 25°C [assuming 2 lactates/glucosyl unit, a rate of lactate accumulation of 6.6μmol.g^{-1}.hr^{-1} (this study) and a rate of glucose release of 17.1μmol.g^{-1}.hr^{-1} (Buck *et al.*, 1993b)].

Validity of the Technique. Since the liver is a major site of amino acid catabolism, it was of concern to investigate whether phe was metabolised significantly by turtle hepatocytes. [^{14}C]O_2 liberation rates from [^{14}C]phe were 23.7nCi [^{14}C]O_2.g wet weight^{-1}.hr^{-1} (n=3) representing approximately 1.8% of the rate of lactate oxidation in the same cell type (Buck *et al.*, 1993). However, it should be noted that this method provides only a qualitative estimate of rates of decarboxylation and oxidation of phe *in vivo* due to randomisation of label. When analyzed by HPLC, 80.3% of [^{14}C] radioactivity eluted with phe with a further 3.4% and 6.1% appearing at elution times corresponding to tyrosine (tyr) and tryptophan (trp) respectively (Fig 7A). Therefore, despite some loss of label to [^{14}C]O_2 and unidentified metabolic intermediates, about 90% of label could be accounted for as amino acids.

Criteria for deciding the appropriate conditions to be used for measuring protein breakdown are shown in figures 7B and C. Figure 7B demonstrates that re-incorporation of released isotope into proteins was minimal at concentrations of non-radioactive phe in excess of 1mM in the

degradation medium. In each degradation experiment, an excess of non-radioactive phe to 1.1mM was therefore used to minimise isotope reutilisation. Figure 7C demonstrates that the use of an excess of 1.1mM phe did not alter the rate of liberation of [^{3}H]leu indicating that protein degradation rates were not affected by the use of excess phe. Furthermore, there was no intracellular accumulation of label indicating that label liberated from proteins equilibrated rapidly to the outside of the cell when in the presence of 1.1mM phe. Values for slopes regressed through each line are given in the figure legend.

Protein Degradation Rates in [^{3}H]phe- and [^{14}C]phe-labelled Proteins. The intermittent perfusion technique has previously been shown to be an effective method for the measurement of protein degradation in labile and stable protein pools from several cell types (Bradley, 1977; Vandenburgh & Kauffman, 1980) including rat hepatocytes (Hopgood, 1977). In this study, we were able to confirm the presence of both fast-turnover ([^{3}H]phe-labelled) and slow-turnover [^{14}C]phe-labelled) protein pools. Figure 8 demonstrates the rate of [^{3}H]phe and [^{14}C]phe liberation from prelabelled proteins as a function of time for cells held under normoxic conditions. Slopes for the two curves from 0.5 to 3h were significantly different by analysis of covariance ($p<0.05$, $n=9$) at -2.519 and -1.582 $\%.h^{-1}$ for [^{3}H]phe and [^{14}C]phe liberation respectively. However, from 3-10h, [^{3}H]phe liberation rates had slowed and matched [^{14}C]phe liberation rates during this time. Rates of [^{14}C]phe liberation did not differ significantly between 0.5-3h and 3-10h.

Effect of Anoxia and Recovery on Protein Half-life. By measuring the rate of isotope liberation from pre-labelled protein (i.e. protein degradation rate) it was possible to calculate protein half-lives for both [^{3}H] and [^{14}C]phe-labelled proteins. The protein half-lives reported reflect the time for 50% of protein to be degraded at the degradation rate measured over the

TABLE 6. Viability data for plated cells under primary culture conditions at 25°C form 0 to 55 hours post-plating.

Criterion	Hours Post Plating	
	0	55
LDH Leakage[1]	2.12 ± 0.61(5)	1.87 ± 0.54(5) (72h = 2.53 (3))
Glycogen[2]	664.0 ± 57.7(6)	540.1 ± 44.6(6)
ATP[3]	1.77 ± 0.17(5)	2.16 ± 0.23(5)
ADP[3]	0.30 ± 0.02(5)	0.16 ± 0.007(5)
AMP[3]	ND	0.055 ± 0.005(2)

Values are mean ± SEM, with the value of n for each group given in parentheses.

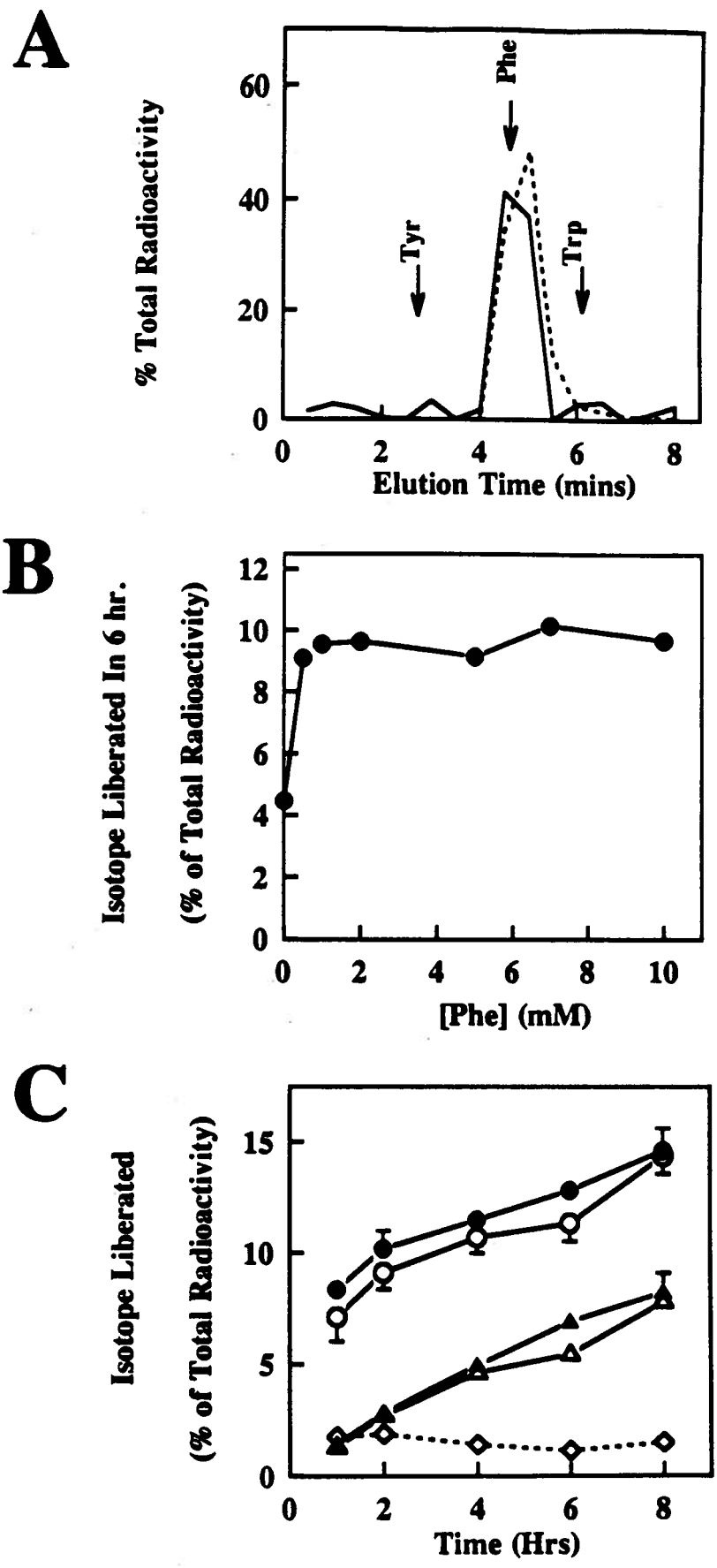
[1] % of total cellular LDH activity in the extracellular medium.

[2] Values expressed as μmol glucosyl units.g wet weight^{-1}.

[3] Values expressed as μmol.g wet weight^{-1}.

ND, not detectable.

FIGURE 7. Validation of experimental conditions used to measure proteolytic rates from radiolabel release. A) Separation of radioactivity eluting with phe by HLPC. *Broken lines*: elution of [^{14}C] with [^{14}C]phe radioactive standard; *Solid lines:* elution of [^{14}C] radioactivity from deproteinised extracellular medium. Elution times for phe, tyrosine (Tyr) and tryptophan (trp) non-radioactive standards are shown. Results are the means of 3 samples. B) Release of TCA soluble [^{14}C]phe from prelabelled protein at 6h in the presence of increasing concentrations of non-radioactive phe (means only, n=3). C) Release of TCA soluble radioactivity expressed as a percentage of total radioactivity. *Closed triangles:* cells pre-labelled with [^{14}C]phe, release of [^{14}C]phe followed in the presence of 0.1mM phe (Slope=0.863$\pm$0.05, r^2=0.964); *Open triangles:* as above but in the presence of 1.1mM phe (Slope=0.971$\pm$0.08, r^2=0.927); *Closed circles*, cells incubated in the presence of [^{3}H]leu, release of [^{3}H]leu followed in the presence of 0.1mM phe (Slope=0.916$\pm$0.14, r^2=0.761); *Open circles:* as above but release of label followed in the presence of 1.1mM phe (Slope=0.827$\pm$0.10, r^2=0.835); *Open diamonds:* cells pre-labelled with [^{14}C]phe; intracellular TCA soluble radioactivity followed in the presence of 1.1mM phe. Values are means $\pm$ SEM, n=4 and slopes were compared using analysis of covariance.


A
% Total Radioactivity
60
40
20
0
Phe
Tyr
Trp
0
2
4
6
8
Elution Time (mins)
B
Isotope Liberated In 6 hr.
(% of Total Radioactivity)
12
10
8
6
4
2
0
0
2
4
6
8
10
[Phe] (mM)
C
Isotope Liberated
(% of Total Radioactivity)
15
10
5
0
0
2
4
6
8
Time (Hrs)

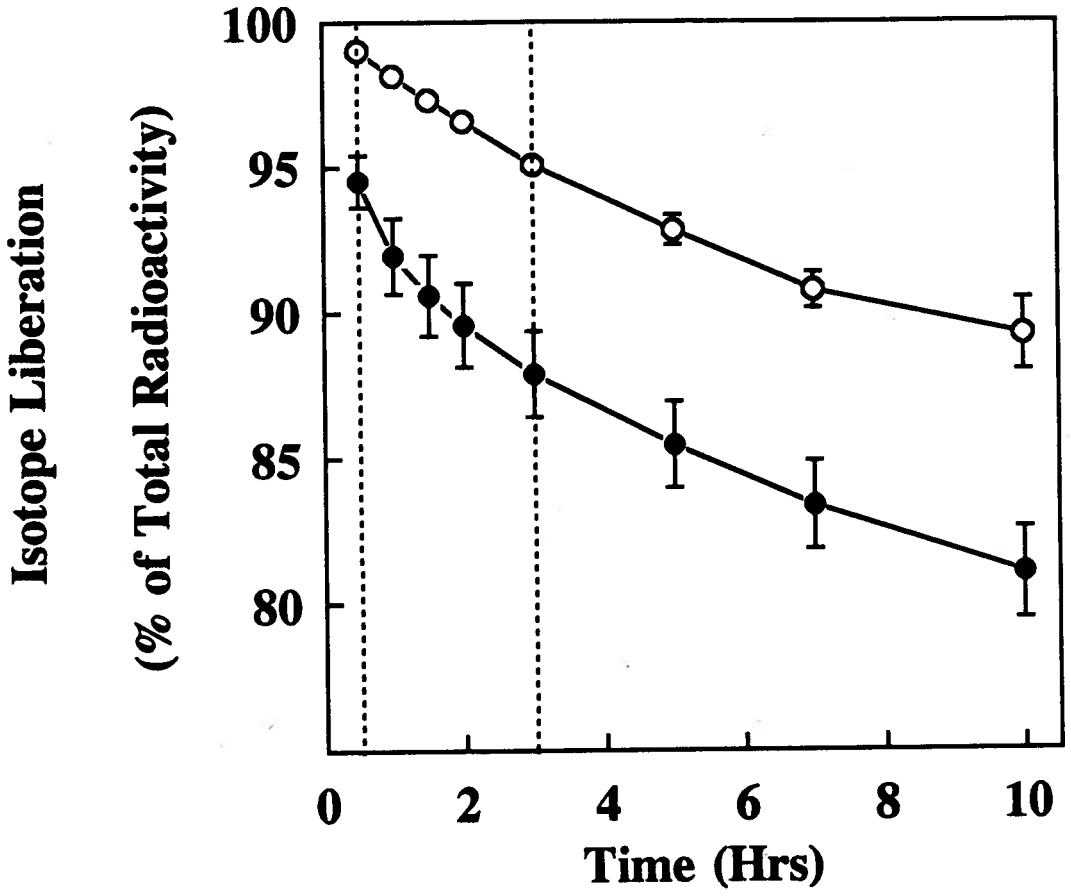


FIGURE 8. Isotope liberation from [^{3}H]phe-labelled (closed circles) and [^{14}C]phe-labelled (open circles) protein pools expressed as percent of total radioactivity. Dotted transect lines indicate the 0.5-3h sampling period used for [^{3}H]phe-labelled proteins. Values are mean ± SEM, n=9. Data are plotted in an arithmetic relationship to emphasise differences between rates of fast- and slow-turnover protein pool radiolabel washout.

sampling period. This assumes that the rates of isotope liberation accurately reflect rates of protein degradation, *in vivo* which we deem reasonable based on the technique validation data above.

Rate constants, half-lives and percentage suppression in response to anoxia and recovery are shown in Table 7. In all treatments with the exception of recovery, the rate of isotope liberation from [^{3}H]phe-labelled protein was significantly different from the corresponding [^{14}C]phe-labelled protein degradation rate ($P < 0.01$ by analysis of covariance) confirming the presence of labile and stable proteins under normoxia and anoxia. Anoxia and cyanide administration resulted in an approximate doubling of protein half-lives for both the [^{3}H]phe- and [^{14}C]phe-labelled protein pools (Table 7). The percentage suppression of proteolytic rate (calculated from the difference between normoxic and anoxic rates of isotope release) was 30-39% for [^{3}H]phe-labelled proteins but was more pronounced in [^{14}C]phe-labelled proteins at values approaching 50%. The total mean suppression of proteolytic rate (the summed suppression for both [^{3}H]phe- and [^{14}C]phe-labelled protein pools) was 35-41%.

To compare values for recovery half-lives in the [^{3}H]phe-labelled protein pool, recovery [^{3}H]phe release rates were compared to control release rates during the 5-10h sampling period. [^{14}C]phe-labelled protein recovery rates were compared as before. Protein half-lives in [^{3}H]phe-labelled protein pool were significantly extended relative to control with proteolytic rates suppressed by 43%. However, in the [^{14}C]phe-labelled protein pool, there were no significant changes in half-life or proteolytic activity during recovery from anoxia.

Proportion of ATP-dependent and ATP-independent proteolysis. For the purposes of this study, ATP-dependent proteolysis is defined as the part of protein degradation that requires ATP hydrolysis for continued activity. ATP-independent proteolysis is defined as that portion

TABLE 7. Logarithmic rate constants, half-lives and suppression of proteolytic activity in [^{3}H]phe and [^{14}C]phe-labelled proteins as influenced by anoxia and recovery.

Condition	[^{3}H]Phe Labelled Proteins			[^{14}C]Phe Labelled Proteins			Total
	k[1] ($\times 10^{-2}$)	Half-Life (hr)	%Suppression[2]	k[1] ($\times 10^{-2}$)	Half-life (hr)	%Suppression[2]	%Suppression[3]
Normoxia	3.07±0.53	24.7±3.3	—	1.27±0.08	55.6±3.5	—	—
Anoxia	2.10±0.20	34.4±3.7*	30.8±5.2	0.64±0.04	109.6±7.4*	44.2±2.2	35.7±3.8
Anoxia +NaCN	1.92±0.42	45.2±11.8*	38.6±4.7	0.59±0.10	131.7±22.7**	47.5±3.2	41.0±4.1
Recovery	1.80±0.30	41.7±5.2***	43.4±3.5	1.30±0.16	58.1±8.7	-10.3±8.7	39.8±4.8

Values are mean ± SEM, n=5 in each case. Significance was assessed relative to control using ANOVA with Tukey's HSD.

$^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.01$ as determined by Student's t-test relative to control degradation slopes from the 5-10h period.

[1] k = Logarithmic rate constant (Log %.hr^{-1})

[2] Calculated as the % difference compared to normoxic degradation slopes.

[3] Calculated as % difference between summed [^{3}H]phe-labelled and [^{14}C]phe-labelled protein slopes for each group versus normoxic control.

of protein degradation that does not require the hydrolysis of ATP and demonstrates continued activity under administration of metabolic inhibitors. Figures 9A and B demonstrate the extent of proteolytic inhibition by administration of inhibitors of energy metabolism. For [^{3}H]phe-labelled proteins, antimycin-A and iodoacetate produced no statistical differences in rates of isotope liberation from normoxia to anoxia. However, for [^{14}C]phe-labelled proteins addition of antimycin-A under normoxic conditions resulted in a 57.6±5.6% inhibition of proteolysis relative to the control slope. Administration of iodoacetate under anoxic conditions resulted in a further 24.0± 12.0% inhibition of proteolysis. From this it appears that about 82% of proteolysis in turtle hepatocytes is ATP-dependent.

Energetic Cost of Proteolysis. Figure 10 demonstrates lactate accumulation rates under cycloheximide alone, and cycloheximide with emetine-HCl administration over a 6 hour time course. Incubation with each inhibitor produced a statistically significant reduction in the rate of lactate production compared to that under anoxia alone. From this, anoxic ATP turnover rates for protein synthesis, protein degradation and protein turnover were calculated and are shown, together with normoxic ATP turnover rates (calculated from rates of oxygen consumption) in Table 8. Justification for the use of cycloheximide and emetine-HCl to achieve differential inhibition of protein synthesis and protein degradation are discussed below.

Total ATP turnover was suppressed by 87% on exposure to anoxia with ATP turnover specific to protein synthesis (cycloheximide inhibitable metabolism) and proteolysis (cycloheximide + emetine inhibitable metabolism) falling by similar proportions of 88 and 93% respectively. Under both normoxic and anoxic conditions, ATP turnover for protein synthesis remained at the same proportion of total metabolic rate (~33%), supporting the results from chapter 2. However, the proportion of total ATP turnover utilised by ATP-

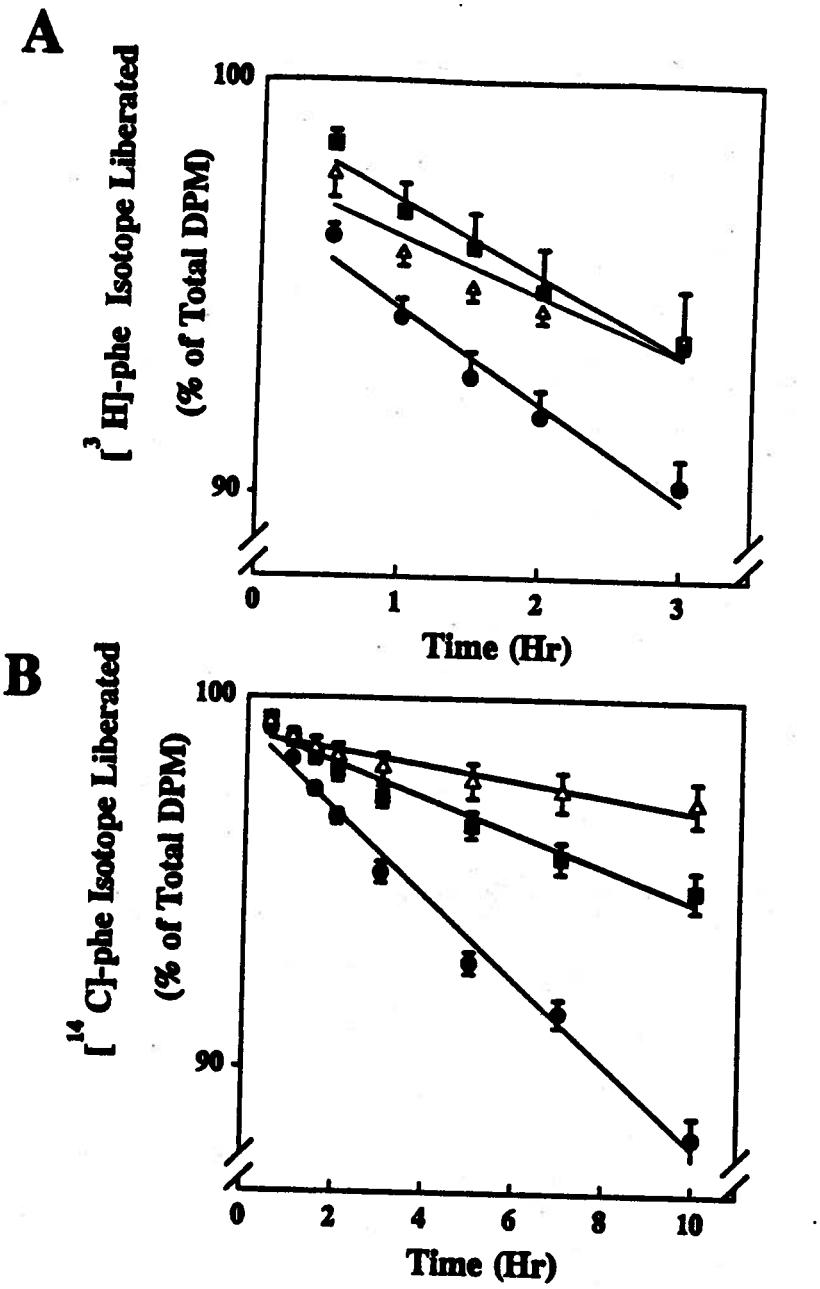


FIGURE 9. Response of proteolysis to various metabolic inhibitors. A) [^{3}H]phe-labelled protein pool. Release of TCA soluble [^{3}H]phe was followed in the presence of normoxia (closed circles), normoxia + 1mM antimycin A (closed squares), anoxia + 10mM iodoacetate (open diamonds). B) [^{14}C]Phe-labelled protein pool. Release of TCA soluble [^{14}C]phe was followed as detailed for figure 2A). Values are mean ± SEM, and are shown plotted as a semi-logarithmic relationship (n=5 in each case). Comparisons between slopes were by analysis of covariance.

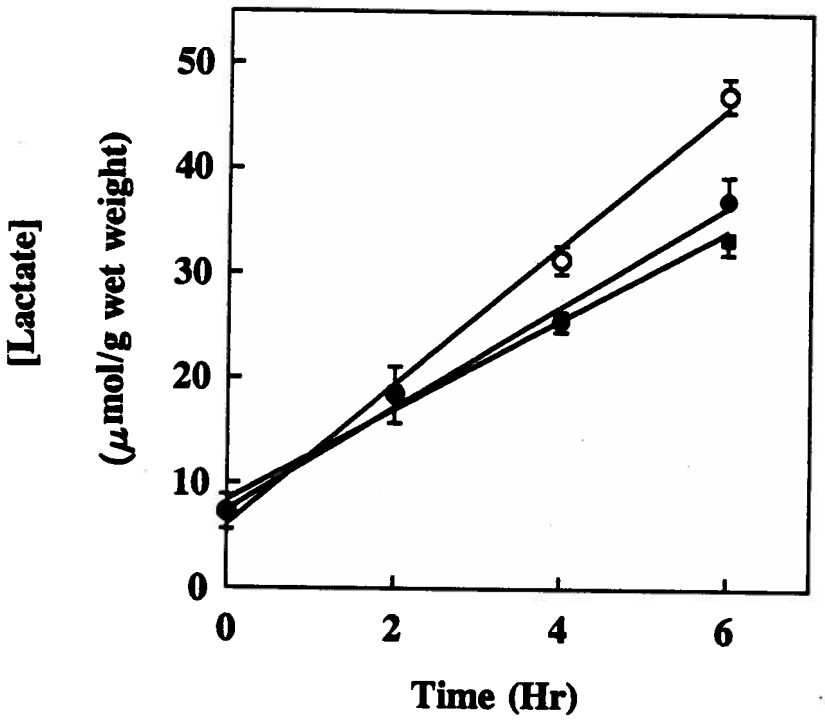


FIGURE 10. Rates of lactate production under anoxia (open circles), anoxia + 0.1mM cycloheximide (closed circles, $P<0.01$) and anoxia + 0.1mM cycloheximide + 0.5mM emetine-HCl (closed squares, $P<0.01$). Values are mean ± SEM, n=5. Slopes of experimental plots compared to control (anoxia only) by analysis of covariance.

TABLE 8. Cost of protein synthesis and ATP-dependent proteolysis under normoxic and anoxic conditions as determined by stepwise inhibition of protein turnover using cycloheximide and emetine.

Condition	ATP Turnover					% of Total	
	Total	Cyc Inhibitable	Cyc+Eme	Protein Synthesis[3]	Proteolysis[4]	Protein Synthesis	Proteolysis
Normoxia[1]	80.3±9.4	56.9±10.6*	45.8±9.5**	23.4±5.3	11.1±1.7	33.5±5.9	21.8±1.4
Anoxia[2]	9.95±0.31	7.36±0.93	7.08±0.99	2.79±0.79	0.73±0.43	32.9±4.8	12.4±7.8
% Reduction	87.6			88.1	93.4		

ATP turnover values are expressed as μmol ATP.g^{-1}.hr^{-1} and are means ± SEM, n=5. Significance was assessed relative to control using ANOVA with Tukey's HSD, *$P<0.05$ **$P<0.01$. [1] Calculated from cycloheximide and cycloheximide + emetine inhibitable oxygen consumption assuming a P:O ratio of 3. [2] Calculated from the rate of lactate accumulation (fig. 4) under cycloheximide and cycloheximide + emetine administration assuming 1.5 moles of ATP per mole of lactate produced. [3] Calculated as the difference between total ATP turnover and cycloheximide inhibitable ATP turnover. [4] Calculated as: Total ATP turnover - (cycloheximide ATP turnover - cycloheximide+ emetine ATP turnover).

dependent proteolysis was disproportionately reduced from 22 to 12% under anoxia.

Effect of Cycloheximide, Emetine-HCl and Protease Inhibitors. The translation inhibitors, cycloheximide and emetine-HCl, have been previously used to achieve a concentration-dependent inhibition of protein synthesis and protein turnover (Siems *et al.*, 1984; Müller *et al.*, 1986). It was of concern here to ensure the conditions employed resulted in a total inhibition of protein synthesis rates and also ATP-dependent proteolysis to obtain protein turnover ATP requirements. As demonstrated in Figure 11, cycloheximide had no significant effect on protein half-lives in either protein pool at the concentrations employed (0.1mM) but was found to produce a total inhibition of protein synthesis rates (data not shown). Emetine-HCl administered at 0.5mM resulted in a maximal extension of protein half-lives for [^{14}C]phe-labelled proteins which was similar in magnitude to the extension of half-life produced by the inhibitors leupeptin, pepstatin and $\alpha_2$macroglobulin summed together. No effect of emetine-HCl was evident on [^{3}H]phe-labelled proteins. Since most of the ATP-dependent proteolytic activity appears amongst [^{14}C]phe-labelled proteins (Fig 9), we conclude that the administration of 0.5mM emetine-HCl was sufficient to inhibit all ATP-dependent proteolysis.

The proteolysis inhibitors leupeptin and pepstatin-A have previously been shown to be effective inhibitors of protein degradation rates in rat hepatocytes (Dean, 1975; Hopgood, 1977). At the concentrations employed in this study it was found that these inhibitors produced a rapid and maximal, sustained inhibition of proteolysis for the duration of the experiment. Figure 11 demonstrates the effects of various proteolytic inhibitors on both [^{3}H]phe- and [^{14}C]phe-labelled protein pools under normoxic conditions. The serine protease inhibitor, leupeptin, and acid protease inhibitor, pepstatin-A increased the turnover time for [^{14}C]phe-labelled proteins significantly although leupeptin had little effect on the turnover of

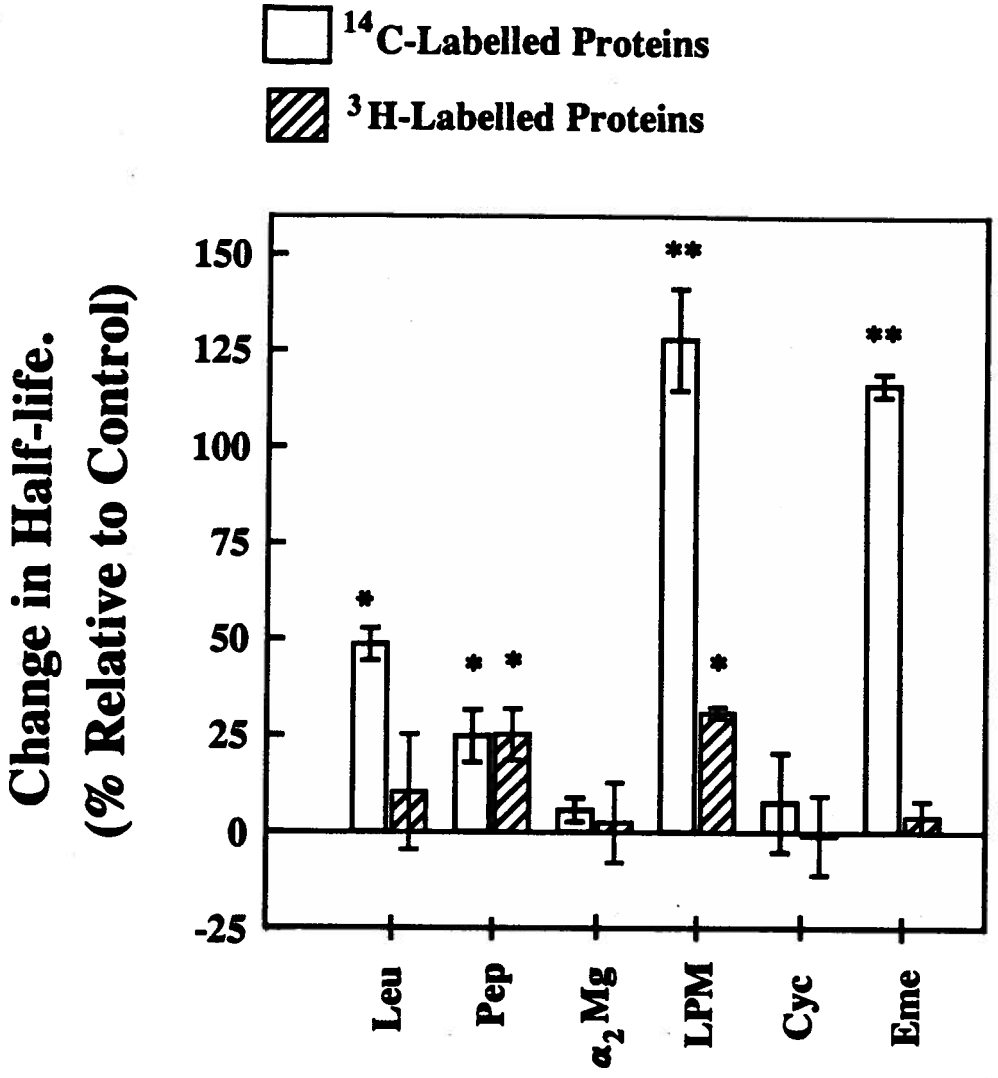


FIGURE 11. Effect of various protease inhibitors and protein synthesis inhibitors on [^{3}H]phe- and [^{14}C]phe-labelled protein half-lives. Leu: 0.1mM leupeptin, Pep: 0.1mM Pepstatin-A, α_2Mg: 0.5U.ml^{-1} α_2Macroglobulin, LPM: Leupeptin+Pepstatin+ α_2Macroglobulin (same concentrations), Cyc: 0.1mM Cycloheximide, Eme: 0.5mM Emetine hydrochloride. Values are Means ± SEM, n=5. Values were compared using ANOVA with Tukey's HSD. $^{*}P<0.05$, $^{**}P<0.001$.

[^{3}H]phe-labelled proteins. The endoprotease, $\alpha_2$macroglobulin, had no significant effect on either pool. Addition of leupeptin, pepstatin-A and $\alpha_2$macroglobulin together more than doubled the turnover time for [^{14}C]phe-labelled proteins, but turnover times for [^{3}H]phe-labelled proteins was affected no more than for pepstatin-A administration alone.

Discussion.

Anoxic submersion in *Chrysemys picta* is accompanied by a 83% suppression in metabolic rate (Herbert & Jackson, 1985). This reduction in metabolism has been well characterised in isolated hepatocytes where ATP turnover rates fall on the order of 88-90% during anoxia (Buck *et al.*, 1993a&b, chapter 2 and this chapter) with coordinate and proportional reduction in turnover rates measured for Na^+/K^+ATPase (Buck & Hochachka, 1993), protein synthesis and urea synthesis (Chapter 2). A significant finding from these parallel studies is that Na^+/K^+ATPase activity accounts for the principal proportion of remaining metabolic rate under anoxia (Buck & Hochachka, 1993).

The current study set out to determine the energy requirements of protein degradation and from this, estimate the proportion of metabolism that is required to sustain remaining protein turnover during anoxic metabolic arrest. From the present data it is clear that protein degradation is an energetically expensive process that accounts for 22% and 12% of total ATP turnover under normoxic and anoxic conditions respectively. When the anoxic ATP turnover rates for protein synthesis and protein degradation are summed together, we can account for 45% of total ATP turnover supporting protein turnover during anoxic metabolic arrest. Given that protein turnover accounts for 45%, and Na^+/K^+ ATPase, 74% (Buck & Hochachka, 1993), of total anoxic metabolic rate in turtle hepatocytes, it appears that we can account for over

100% of anoxic ATP metabolism. Some of this overestimation can be attributed to the use of different culture conditions and seasonal effects on protein turnover (Chapter 2). Nevertheless, these data indicate that during anoxic metabolic arrest, the primary energy expenditure is directed towards the maintenance of a constant membrane potential with a substantial secondary expenditure concerned with the turnover of cellular protein.

Normoxic protein half-lives for [^{3}H]phe- and [^{14}C]phe-labelled proteins were comparable to those previously measured for mammalian cell lines (Bradley, 1977; Vandenburgh & Kaufmann, 1980). However, there was a doubling of total protein half-life within the first 10h of anoxic metabolic arrest. This is in agreement with previous studies on the brine shrimp embryo (*Artemia fransiscana*) where chronic anoxic metabolic arrest resulted in a 77 fold extension of half-life for cytochrome c oxidase (Anchordoguy *et al.*, 1993). In terms of increasing survival time, extending protein half-life appears paradoxical however. As noted in cellular aging studies, the probability of protein structural damage by post-translational and chemical modifications increases significantly with time (reviewed by Reff, 1985). Therefore, if tissues are to retain overall functional stability during long-term metabolic arrest, the presence of a stabilising mechanism is implied. As a means of achieving protein replacement and renewal, the maintenance of reduced protein turnover during anoxic metabolic arrest demonstrated in this study possibly represents an initial line of defense. It would be of interest to know if this is complemented by other innate mechanisms that stabilise proteins such as amino acid sequence features that do not predispose proteins to degradation (eg. Bachmair *et al.*, 1986; Rogers *et al.*, 1986) or the synthesis of stress proteins such as glucose regulated protein 78 (GRP78 or Binding Protein) that have been noted to stabilise nascent protein subunits during transport to the golgi body in other cell types (Hendershot *et al.*, 1988).

Although limited protein synthesis and degradation are maintained under anoxia, the current data suggest that they are not evenly matched. Here and in chapter 2, it was reported that protein synthesis rates were reduced by 88-92% during exposure to anoxia. Here, the total mean proteolytic suppression for [^{3}H]phe- and [^{14}C]phe-labelled protein pools under anoxia was 36-41% of control values with both protein pools exhibiting different individual magnitudes of suppression. The smaller suppression of total protein degradation rates indicates that under anoxia, a negative protein balance exists within the cell. Whether this negative protein balance plays a part in setting the limits for maximum survival at 25°C remains unclear at this point.

An imbalance between protein synthesis and degradation is also observed during recovery. In chapter 2, it was noted that normoxic recovery from anoxia resulted in a 160% increase in protein synthesis rates compared to control values. However, proteolysis did not appear to show the same response since proteolytic rates in the[^{3}H]phe-labelled protein pool were lower in recovery compared to controls by nearly 50%. The previously observed exaggeration in recovery protein synthesis rates may partly be the result of inhibited proteolysis in the more labile protein pool. Nevertheless, it appears that normoxic recovery results in a positive protein balance that may, in part, compensate for the negative protein balance that occurs upon entry into anoxia.

The basis for the observed imbalance in protein turnover during anoxia may be related to the degree of coupling between proteolysis and ATP hydrolysis. A particularly striking observation from the data is the magnitude of the depression of ATP-dependent proteolysis (93%; Table 8) which closely matches the proportional suppression found for whole cell metabolism and protein synthesis. Since total proteolytic suppression was on the order of 36-41%, it appears that remaining proteolysis under anoxia is largely comprised of ATP-

independent pathways which may be less responsive to alterations in cellular metabolism and therefore could persist during the metabolic suppression. This implies that there is a shift in the control of proteolysis away from ATP-coupled metabolism during the transition from normoxia to anoxia. With the majority of ATP-dependent proteolysis residing in the stable [^{14}C]phe-labelled protein pool (fig 9), it appears that the negative protein balance under anoxia is largely attributable to loss of protein from more labile proteins, an important observation since the majority of regulatory proteins possess short half-lives (Hargrove & Schmidt, 1989).

Mechanisms resulting in the suppression of energy-dependent proteolysis are likely to be many-fold depending on the dominant proteolytic pathway involved. However, a key feature of anoxic metabolic suppression in both turtle hepatocytes and the whole animal is that ATP demand is coordinately down-regulated with ATP supply resulting in high and constant intracellular ATP concentrations (Chapter 2; Kelly & Storey, 1988; Buck *et al.*, 1993b; chapter 2). Therefore, despite observations in rat hepatocytes that have demonstrated a direct positive relationship between ATP concentrations and proteolytic rates (Gronostajski *et al.*, 1985; Plomp *et al.*, 1987) it appears that regulation by absolute changes in [ATP] alone is unlikely in this case. Other factors that may be involved in the suppression of energy-dependent proteolysis may include metabolites such as 2,3-bisphosphoglycerate [inhibits ATP dependent lysosomal proteolysis (Roche *et al.*, 1987)], increased expression of protease inhibitors, as has been shown for mammalian hibernators (Srere *et al.*, 1992) and availability and successful targeting of proteolytic substrates. Of particular significance to this final point is the observation that phosphorylation-mediated changes in protein conformation alters protein susceptibility to Ca^{2+}-activated proteases (Chuah & Pallen, 1989; Greenwood *et al.*, 1993). With the current evidence in turtle tissues for phosphorylation control of enzyme activities (reviewed by Storey & Storey,

1990) and the postulated role for phosphorylation in modulating ion channel and pump activities (Buck *et al.*, 1993), control of protein stability or protease activity by such a mechanism should not be ignored.

In order to understand the nature of proteolytic suppression and activation it is important to know which pathways are active and when. Although a number of studies have suggested that the bulk of cellular protein, including regulatory, denatured, short, and some long lived proteins are degraded by cytosolic ATP and ubiquitin dependent processes (Goldberg & Rock, 1992), in liver, protein degradation by autophagic vacuole formation appears to be the predominant proteolytic pathway (Mortimore & Kurana, 1990). The protease inhibitor pepstatin-A inhibits lysosomal acid proteases such as cathepsin D with little or no effect on serine and thiol proteases. As is clear in figure 11, under pepstatin-A administration, protein half-lives for both the [^{3}H]phe- and [^{14}C]phe-labelled protein pools were extended by about 25%, a relatively small proportion of total cell proteolysis. Leupeptin, which inhibits a broad range of thiol and serine proteases, resulted in a 50% increase in half-life of the [^{14}C]phe-labelled protein pool and, when summed together with pepstatin-A and $\alpha_2$macroglobulin, doubled slow-turnover normoxic proteolytic rates with minor, but significant effects on the [^{3}H]phe-labelled protein pool. This provides some initial evidence that serine, thiol and acid proteases constitute a large proportion of total cell proteolytic activity in this type of cell.

A final note concerns the fate of amino acids that are liberated by proteolysis during anoxia. Previous studies have noted that hepatic phospho*enol*pyruvate carboxykinase is exclusively mitochondrial in this, and other turtle species (Buck *et al.*, 1993b; Land & Hochachka, 1993). It therefore appears unlikely that liberated amino acids play a major role in the repletion of hepatic glycogen stores on recovery. Instead, it seems probable that the bulk

of liberated amino acids are either oxidised or reincorporated into protein as part of the observed exaggeration in protein synthesis rates during re-oxygenation.

In conclusion, evidence is presented that, despite an increase in total cellular protein half-life, a limited turnover of protein is maintained during anoxic metabolic arrest in painted turtle hepatocytes. The energetic costs to sustain this amount to approximately 45% of remaining ATP turnover. The data presented on protein degradation support the coordinate suppression of ATP-dependent proteolysis together with cellular energy metabolism but expose continued energy-independent proteolysis amongst the more labile [^{3}H]phe-labelled proteins.

Chapter 4.
A Heme-protein Based Oxygen Sensing Mechanism Controls the Expression and Suppression of Multiple Proteins in Anoxia-tolerant Turtle Hepatocytes.

Preface.

The data presented in this chapter are in a manuscript by S.C. Land and P.W. Hochachka. which is currently in submission stages to The Proceedings of the National Academy of Sciences, U.S.A.. All aspects of the work were performed by myself.

Introduction.

In the absence of oxygen, numerous species of facultative anaerobes suppress their metabolism to survive prolonged periods without oxygen. Intrinsic to this response is a coordinated re-organisation of ATP supply and demand that enables tissues to retain viability and function, in hand with slow rates of flux through all cell processes.

The partitioning of energy demand and re-organisation of metabolism during metabolic suppression has been examined in hepatocytes isolated from a successful vertebrate anaerobe, the western painted turtle [(*Chrysemys picta bellii* (Buck *et al.*, 1993b)]. On exposure to anoxia, these cells mount a coordinated physiological response, similar to that characterised in whole liver, that is directed towards: *i)* a 10-fold reduction in rates of ATP synthesis and ATP demand, *ii)* conservation of the cellular membrane potential by concurrent reductions in ion channel flux rates and ion pump ATPase activity, *iii)* reduced ATP demand by protein turnover,

urea synthesis and gluconeogenesis and, *iv)* maintenance of functional viability during the period of metabolic suppression (Buck *et al.*, 1993a&b, Buck & Hochachka, 1993, chapters 2 and 3).

A notable feature of this metabolic re-organisation is that it occurs independently of change in ATP concentrations or cellular energy charge. Yet the changes in ATP demand for individual cellular processes are clearly coordinated with one-another and exhibit rate reductions in proportion to the depression of total ATP turnover (Buck *et al.*, 1993a&b, chapter 2). This is put further into perspective in both anoxia-tolerant and -intolerant systems, where numerous studies report that the transition to anaerobic metabolism occurs in ranges where oxygen is still saturating at cytochrome c oxidase (cf figures 1 and 20). Taken together, these observations suggest that during progressive hypoxia, alterations of cell function can occur independently of metabolic changes associated with oxygen limitation at the mitochondrion. Therefore, in the absence of large-scale metabolite concentration changes and exogenous effectors, by process of elimination, the oxygen molecule itself appears as the primary signalling agent responsible for coordinating metabolic changes in progressive hypoxia.

The present study examines the relationship between the oxygen signal, its transduction mechanism, and changes in protein expression during anoxic metabolic suppression in turtle hepatocytes. Because protein expression is composed of multiple ATP-demanding pathways and exhibits control from gene induction to protein degradation, this cell process tests the ability of the oxygen signal to invoke and coordinate a hypoxic response between numerous inter-related pathways. Furthermore, protein turnover in turtle hepatocytes conforms well to an ATP-independent pattern of control. During metabolic suppression protein synthesis and ATP-dependent protein turnover are suppressed by 90% but these processes still account for about 40% of remaining anaerobic ATP-turnover, suggesting that changes in protein expression are

important in metabolic suppression. However, the suppression of protein turnover occurs without change in ATP concentrations or cellular energy charge, is synchronous with the suppression of whole cell ATP supply and demand, and therefore appears independent of oxygen limitation at the mitochondrion.

Transduction of an oxygen signal over broad ranges of oxygen concentration requires a mechanism that possesses appropriate sensitivity (a high K_m) for oxygen. Particularly significant is the finding that in numerous cell types, hypoxia associated gene expression can be regulated through oxygen-dependent conformational changes in a ferro-heme protein. In the liver, this kind of mechanism appears to be at the basis for O_2-dependent expression of erythropoietin (Goldberg *et al.*, 1988) and modulates expression of phosphoenolpyruvate carboxykinase (PEPCK) expression along the liver periportal-perivenous O_2 gradient (Keitzmann *et al.*, 1992, 1993).

Based on evidence for metabolism-independent control of protein turnover in turtle hepatocytes, and the emergence of control of hypoxia-associated gene expression via a heme-protein oxygen receptor, the specific aims of this study were 1) to establish whether the control of protein expression under normoxia and anoxia was specifically oxygen responsive in turtle hepatocytes, 2) to determine whether a conformational change in a heme-protein group was likely involved as a mechanism for transducing oxygen concentration changes to a cellular-level response and 3) to compare oxygen-responsive protein expression to the turtle hepatocyte heat-shock response.

Materials and Methods.

Chemicals.

L-[U-^{14}C]Leucine ([^{14}C]Leu, sp. act. 311 mCi/mmol) was purchased from New England Nuclear, Dupont (Quebec, Canada) and [^{14}C]-methylated protein molecular weight markers (high range) from Amersham (Oakville , Ontario, Canada). Minimum essential medium (MEM) amino acids were from GIBCO (Burlington, Ontario, Canada). All other chemicals were purchased from Sigma Chemical (St. Louis, Mo).

Culture Preparation.

Adult female western painted turtles (300-500g) were purchased from Lemberger (Oshkosh, WI). Hepatocytes were prepared as previously described (chapter 2). Once the post-isolation cleaning stages were complete, cells were suspended in culture medium containing (in mM): 78.5 NaCl, 34.5 $NaHCO_3$, 10 Na^+-N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES, pH7.5), 5.8 $CaCl_2$, 3.8 $MgCl_2$, 2.2 KCl, 0.9 Na_2HPO_4, 0.1 $MgSO_4$, 4% bovine serum albumin (BSA), 10% MEM amino acids and 100U/ml penicillin-10μg/ml streptomycin and stored at 4°C until needed. Prior to each experiment, cells were warmed to the experimental temperature (25°C) for a minimum of 3h.

Cell viability during the course of each experiment described below was assessed by measuring rates of lactate dehydrogenase (LDH) leakage (Buck *et al.*, 1993b). The expression of a full molecular weight range of proteins in each experimental group was also taken as a convenient post-hoc measure of overall cell competence.

Experimental Design.

The experiments were conducted in three parts. In Part A, cells were divided into parallel groups for the assessment of the oxygen-responsiveness of protein expression, and Part B was

designed to investigate whether the oxygen-responsive protein expression observed in Part A, could be manipulated through factors that affect the conformation of ferro-heme proteins. Previous studies, especially in the EPO field, have developed strategies for probing this system. Aerobic incubation with NaCN provides a "physiological mimic" of anoxic cell metabolism whilst oxygen is still present. When compared to true anoxic incubation, this provides a means for determining oxygen-dependent effects on protein expression. The involvement of a heme-protein may be determined by substitution of Co^{2+} or Ni^{2+} ions into the central Fe^{2+} in protoporphyrins, inducing the deoxygenated conformational state regardless of oxygen concentrations. Conversely, an oxygenated conformation can be induced by incubation with CO, a potent ligand of ferro-heme proteins (Goldberg *et al.*, 1988, Eckhardt *et al.*, 1993).

Finally, Part C examines whether the observed oxygen-responsive protein expression was related to the heat shock response. Except for heat-shocked groups, all experiments were conducted at 25°C.

Part A: Oxygen-responsive Protein Expression. Hepatocyte suspensions were adjusted to a density of 30mg/ml and split into two groups. Cells were then allowed to settle out of suspension, the supernatant removed, and replaced with an appropriate quantity of either air-equilibrated (95% air/5% CO_2, humidified) or nitrogen-equilibrated (95% N_2/5% CO_2, humidified) culture medium. After resuspension, cells were aliquotted into four experimental groups (3ml volume at 30 mg/ml) for the parallel determination of *de novo* protein expression (group 1) and cell viability criteria (group 2; 30mg/ml, 10ml volume) under the following conditions: normoxic (6h aerobic), normoxic+2mM NaCN (6h aerobic with 2mM NaCN added at 2h intervals), anoxia (6h anaerobic) and anoxia+2mM NaCN (6h anaerobic with 2mM NaCN added as above). Because NaCN slowly oxidised in the normoxic-cyanide group, 2mM NaCN

was added at two-hourly intervals to ensure complete inhibition of oxidative phosphorylation. Rates of oxygen consumption were measured in cells from group 2. At two-hour intervals, 2ml of each cell suspension was removed and placed in 2ml thermostatted oxycell chambers held at 25°C. To reduce the risk of air diffusing into anoxic groups, samples were pipetted and analyzed under a meniscus of paraffin oil. Oxygen consumption was then measured polarographically using Clarke-type electrodes as described in chapters 2 and 3.

To determine the pattern of *de novo* protein synthesis under each condition, cells in group 1 were washed free of culture medium 4h into the incubation and were resuspended in the same volume of NaCN and gas-equilibrated culture medium lacking leucine, but with 12.5μCi [^{14}C]Leu/ml. After a 2h labelling period under each experimental condition, cells were washed three times in BSA-free culture medium at 3°C by centrifuging at 50xg for 2min. The final washed cell pellets were frozen in liquid N_2 and stored at -80°C for later analysis.

Part B: Heme-group conformation experiments. This series of experiments tested for the involvement of a heme-group in the regulation of oxygen-responsive protein expression. To incorporate cobalt or nickel into the central iron position of heme proteins, 18h incubations were constructed in appropriate experimental volumes held at a density of 30mg/ml, as follows: 200μM $CoCl_2$, 300μM $NiCl_2$, 200μM Co^{2+}+2mM 2,4-dioxoheptanoic acid (DHA) and remaining cells incubated as a control group. Throughout the incubation period, cells were held under a humidified, 95%air/5%CO_2 atmosphere at 25°C. After incubating for 18-20h, cells were washed once then aliquotted into groups 1 and 2 as indicated under Part A. Further separation of groups 1 and 2 into the experimental treatments (same cell density in 3ml volumes) was as follows: normoxic (6h aerobic), Co^{2+} (6h aerobic), Ni^{2+} (6h aerobic), Co^{2+}+DHA (6h aerobic), Co^{2+}+95%N_2/5%CO_2 (6h anaerobic), 10% Carbon monoxide/5%

CO_2/85% N_2 (6h anaerobic), Co^{2+}+10% Carbon monoxide/5% CO_2/85% N_2 (6h anaerobic). Concentrations of Co^{2+}, Ni^{2+} and DHA were held constant as above. Labelling of group 1 cells from 4 to 6h into the incubation with [^{14}C]Leu, and collection of final cell pellets, were conducted as described in Part A.

Part C. Heat Shock Response. A period of heat shock was used to induce stress proteins groups. The profile of stress protein expression under these conditions was then compared to the oxygen-sensitive protein response observed in parts A and B. Control and heat-shock groups were held at 25°C for 4h under normoxic conditions. At this time, cells apportioned to the heat-shock group were transferred to a waterbath pre-heated to 40°C and incubated with shaking for 1h. Because of the small volume of cells and media in each group (3ml at 30mgcells/ml) the incubation media equilibrated to 40°C in <5min. Once the heat-shock period was over, controls and heat-shock groups were washed once and resuspended in leucine free-media with 12.5μCi [^{14}C]Leu/ml, previously equilibrated to 25°C, and incubated for a further 1h. Cells were then washed and stored as described above.

Detection of Radiolabelled Proteins.

[^{14}C]Leu-labelled cells were lysed in 4 vols of lysis buffer [In mM: 100 NaCl, 10 tris(hydroxymethyl)aminomethane-HCl (Tris-HCl, pH7.6), 1 EDTA (pH 8.0), 1 Phenylmethyonyl-sulfonylfluoride (PMSF) and 1μg.ml aprotinin] and sonicated in an ice-salt slush bath twice for 10s. The crude lysate was then placed in 2vols, 2x Laemmi sample buffer [100mM Tris base (pH6.8), 200mM dithiothreitol, 4% sodium dodecyl sulfate (SDS), 0.2% bromophenol blue, 20% glycerol and 1mM PMSF] and heated to 100°C for 10min. Samples were then sonicated again for 2x10s and then centrifuged at 10,000xg for 20 min. The

supernatants were saved and the amount of protein-bound radioactivity was determined by trichloroacetic acid (TCA) precipitation. This was done by adding 3μl of each supernate to 147μl of distilled water in duplicate. A 50μl aliquot of this mixture was then added to 500μl of a solution containing 1M NaOH and 0.5%(vol) H_2O_2 and incubated for 15min at 40°C. Protein within each sample was precipitated by the addition of 400μl 50%(vol) TCA containing 0.02% (w:v) casein hydrolysate and stored on ice for a minimum of 30min. Protein precipitates were collected on Whatman GF/C filter disks, pre-soaked in 10% TCA, using a vacuum manifold apparatus and then washed with 6x10ml 5% TCA containing 10mM $Na_2P_4O_7$. The washed filter disks were then dried at 40°C for 20min and the radioactivity incorporated into protein determined by scintillation counting in ACSII scintillation cocktail (Amersham), using an LKB Rack-beta 1214 scintillation counter set to an efficiency of 95% for [^{14}C] activity.

2.5x10^4 TCA insoluble degradations per minute (DPM) per sample were loaded onto sodium dodecylsulfate (SDS)-polyacrylamide gels consisting of 5% stacking and 12% resolving gels for 1-dimensional SDS-polyacrylamide gel electrophoresis (PAGE). Proteins were electrophoresed at 30mA for approximately 4h using a running buffer consisting of 26mM Tris base (pH8.8), 200mM glycine and 0.1%SDS. Once the dye front had reached the bottom of the gel, gels were removed and fixed for 3h with 3 washes of glacial acetic acid:methanol:water (0.5vol:0.1vol:1vol), rinsed in distilled water for 5min and then washed in Amplify fluor (Amersham) for 30min. Gels were then dried and the resulting fluorograph exposed to pre-flashed Kodak O-Mat XAR5 autoradiography film for 24-48h at -80°C. Molecular weights were determined using high range (200-14.4 kDa) [^{14}C]-methylated molecular weight markers (Amersham). Autoradiographs were quantified using a Molecular Dynamics laser scanning densitometer operated with ImageQuant version 3.15 software (Molecular Dynamics, NY).

Data Presentation and Statistics.

Statistical manipulations were performed using Systat version 5 (Evanston IL) software. Except where noted, significance was determined using analysis of variance with Dunnett's t-test. Confidence limits for significance are at 95% and all data are expressed as means ± S.E.M. The value of *n* indicates the number of individual hepatocyte preparations used in each experiment.

Results.

Cell Viability. Throughout each experiment, the percentage of extracellular LDH remained less than 6% for all groups except carbon monoxide/anoxia where LDH leakage rose to 10.4±3.8% over the last 2 hours. All groups demonstrated the incorporation of [^{14}C]leu into a wide molecular weight range of proteins (figs 12,14,16,18). Taken together, the low LDH leakage and pattern of protein expression suggests that the cell preparations remained healthy throughout the experimental period.

Oxygen-sensitive Protein Expression. The experiments in *Part A* were designed to test whether the presence of molecular O_2 was required for expression of anoxia associated protein. Cyanide was used to mimic physiological anoxia under normoxic conditions and resulted in a 89% inhibition of oxygen consumption over the experimental period (fig 12). Remaining oxygen consumption is presumably comprised of oxygen requiring processes that are not involved in the aerobic production of ATP (eg. peroxisome oxidation). Based on this, and previous studies noting that in turtle hepatocytes, aerobic incubation with cyanide does not appear to perturb cellular metabolism differently from anoxia (see discussion), the use of 2mM NaCN under aerobic conditions was considered to be an adequate mimic of physiological anoxia.

In response to anoxia, turtle hepatocytes express or suppress certain proteins in an oxygen-sensitive manner. This was judged on the basis of whether the presence of molecular oxygen negated the response despite mimicked physiological anoxia in the presence of O_2, using NaCN. Figure 1 demonstrates the change in the distribution of protein expression from normoxia and normoxic physiological anoxia, to complete anoxia. The expression of five proteins of 83, 70.4, 42.5, 35.3, and 16.1 kDa was consistently increased in anoxia resulting in a rise in the proportional contribution of each protein to the total protein pool (table 9). Similarly, expression of a distinct set of five proteins of 63.7, 48.2, 36.9, 29.5 and 17.7 kDa were down-regulated from normoxia to anoxia also demonstrating a decrease in the proportional contribution to the total protein pool (Table 9). The mean molecular weights for expressed and suppressed proteins in anoxia are given in table 9.

Figure 13 demonstrates the densitometric change in protein-band peak absorbance area for the changes in protein expression identified in figure 12. Panel A demonstrates the pattern of oxygen-sensitivity for proteins that increase expression in anoxia. In each case, environmental anoxia resulted in a significant increase in protein band peak absorbance area relative to normoxic controls suggesting that the increased proportion of each protein in the anoxic protein pool was not simply due to a change in the overall size of the protein pool. In addition, anoxic expression of 83,42.5, 35.3 and 16.1 kDa proteins was significantly greater than under normoxic treatment with cyanide suggesting that mimicked physiological anoxia was not sufficient to cause an increase in the expression of these proteins by itself. Since the principal difference between environmental anoxia and normoxic physiological anoxia is the presence or absence of oxygen (see discussion), we describe the expression of these proteins as being oxygen-sensitive. Expression of the 70.4 kDa protein was distinct from this group in that it

FIGURE 12. Representative autoradiograph demonstrating the distribution of anoxia-associated protein expression and suppression in the following conditions: normoxia (Nor), normoxia+2mM NaCN (Nor+NaCN), anoxia (Ax) and anoxia+2mM NaCN (Ax+NaCN). Molecular weights of protein up-regulated in anoxia are shown on the right side of the figure and those for down-regulated proteins, on the left. Positions of Amersham [^{14}C]-methylated protein molecular weight markers (M_r) are shown on the right of the figure and all molecular weights are given in kilodaltons with positions. The rate of oxygen consumption (VO_2) under each treatment is given at the base of each lane (n=5). *Abbreviations*: n.d.- not detectable).

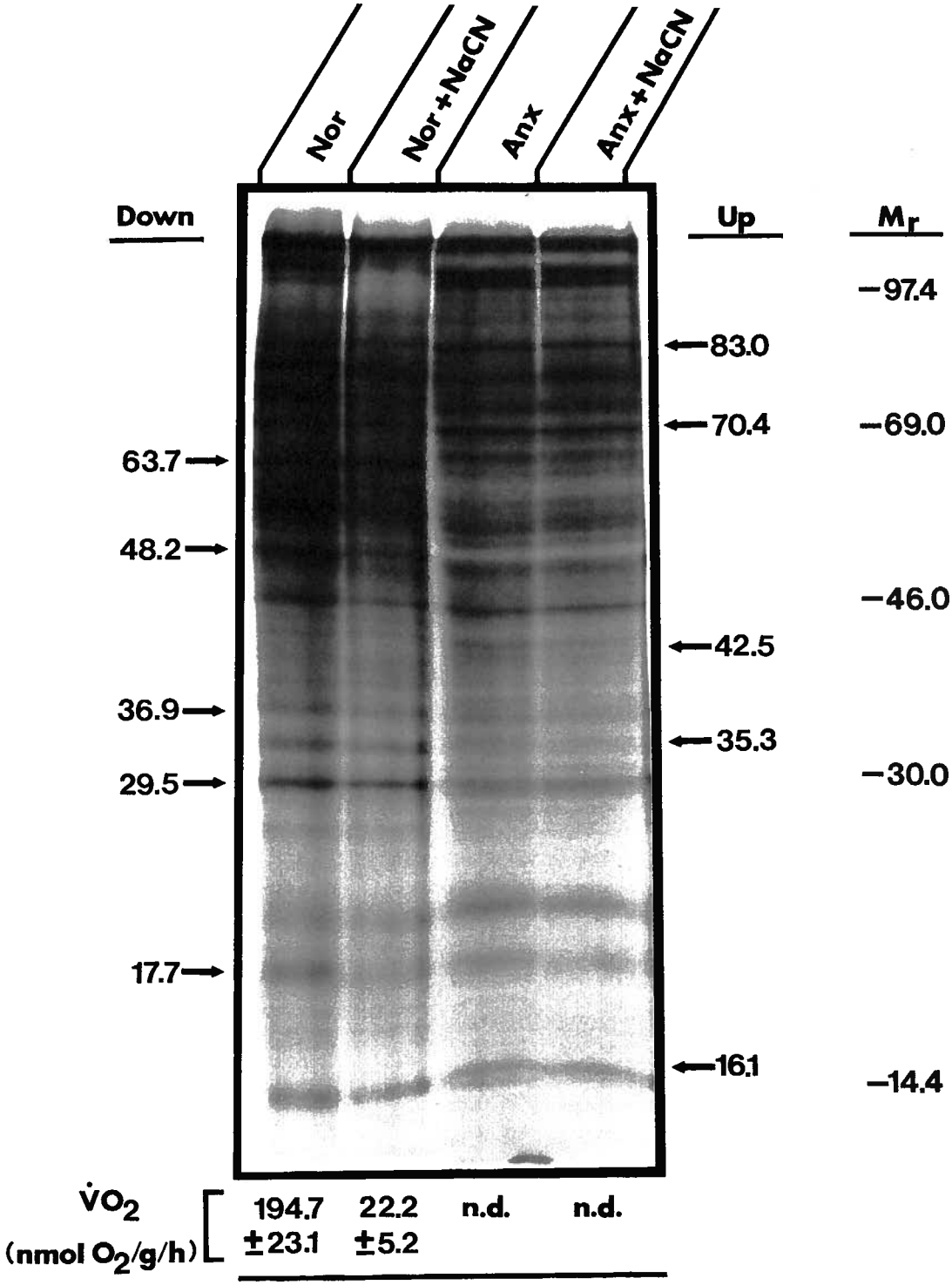

Nor
Nor+NaCN
Anx
Anx+NaCN
Down
Up
Mr
97.4
83.0
70.4
69.0
63.7
48.2
46.0
42.5
36.9
35.3
29.5
30.0
17.7
16.1
14.4
V̇O2
(nmol O2/g/h)
194.7 ±23.1
22.2 ±5.2
n.d.
n.d.

TABLE 9. Oxygen-sensitive and Insensitive Protein Expression in Anoxia.

Proteins Expressed in Anoxia			Proteins Suppressed in Anoxia		
Molecular Weight (kDa)	% of Total Protein Pool[3] Normoxia	Anoxia	Molecular Weight (kDa)	% of Total Protein Pool[3] Normoxia	Anoxia
Oxygen Sensitive[1]			Oxygen Sensitive[1]		
83.0± 0.8	3.01 ± 1.14	10.33 ± 2.89*	63.7± 0.5	9.82 ± 2.42	4.96 ± 3.13
42.5± 0.8	0.49 ± 0.49	7.12 ± 1.51*	48.2± 0.5	9.00 ± 2.06	1.69 ± 0.79*
35.3 ± 0.2	0.27 ± 0.27	4.17 ± 1.40*	36.9 ± 0.5	4.84 ± 1.30	2.06 ± 1.12*
16.1± 0.8	0.34 ± 0.26	2.03 ± 0.70*	29.5± 0.2	4.85 ± 1.64	1.44 ± 1.15▲
			17.7± 0.3	1.91 ± 0.51	0.32 ± 0.24*
Oxygen Insensitive[2]					
70.4 ± 0.2	7.12 ± 3.39	19.34 ± 2.30*			

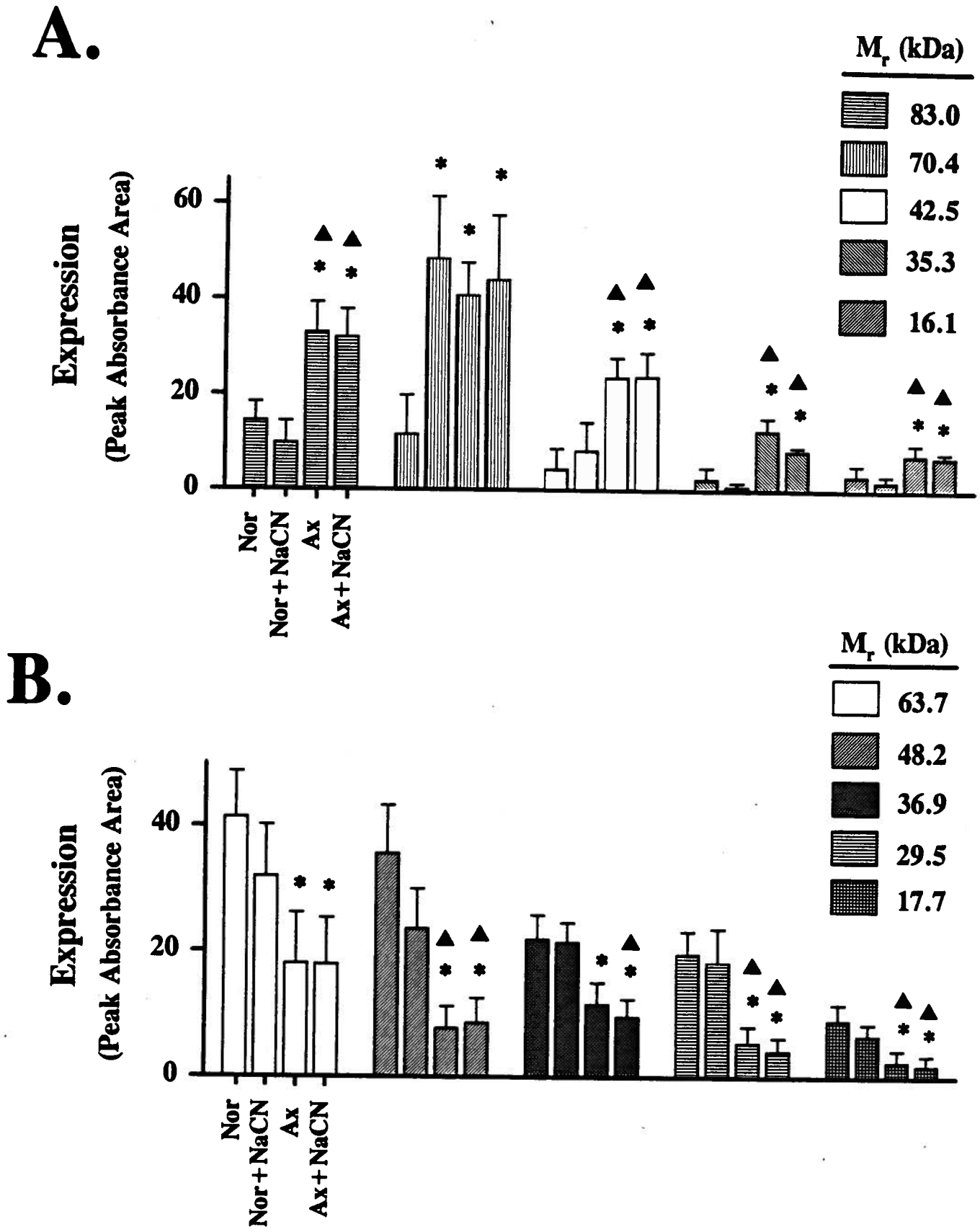
Values are means ± SE, n=5 in each case. *P<0.05; ▲P=0.08 (students paired t-test)

[1]Defined as a change in protein expression only in the absence of oxygen (response does not occur in normoxia + NaCN group).

[2]Defined as a change in protein expression in all anoxic groups, including normoxia+NaCN.

[3]Calculated as a percentage of total densitometric absorbance area.

FIGURE 13. Oxygen-sensitivity of protein expression during physiological anoxia (O_2 still present in medium) and environmental anoxia. A) Anoxia-induced increases in specific protein-band peak absorbance area determined by densitometry; B) Anoxia induced decreases in specific protein-band peak absorbance area. Error bars depict standard error and n=5 in each case; $^{*}P<0.05$ relative to normoxic group; $^{\blacktriangle}P<0.05$ relative to normoxia+NaCN group. *Abbreviations*: normoxia (nor), normoxia+10mM NaCN (nor+NaCN), anoxia (ax) and anoxia+10mM NaCN (ax+NaCN).


A.
M$_r$ (kDa)
83.0
70.4
42.5
35.3
16.1
Expression
(Peak Absorbance Area)
60
40
20
0
Nor
Nor+NaCN
Ax
Ax+NaCN
B.
M$_r$ (kDa)
63.7
48.2
36.9
29.5
17.7
Expression
(Peak Absorbance Area)
40
20
0
Nor
Nor+NaCN
Ax
Ax+NaCN

exhibited an increase in expression in all anoxic groups regardless of the presence of oxygen (oxygen-insensitive).

Panel 13B demonstrates the oxygen-sensitivity of proteins suppressed in anoxia. All proteins demonstrated decreases in expression relative to normoxia and with the exception of a protein at 63.7 kDa, this overall trend was also statistically significant for all protein bands relative to normoxia+cyanide.

Oxygen-Sensing Mechanism. The substitution of cobalt or nickel into the central iron position of protoporphyrin, changes the state of the heme group from oxy- to deoxy-conformations (Goldberg *et al.*, 1988). If a heme-protein oxygen receptor is active in regulating the expression of the oxygen-sensitive proteins observed in part A then normoxic incubation with Co^{2+} or Ni^{2+} should result in increased expression of proteins described in fig. 13A (except 70.4 kDa), conversely decreased expression would be expected for proteins described in fig. 13B. In addition, incubation of cells with DHA, an inhibitor of heme-protein synthesis, should abrogate the response with cobalt.

Figure 14 shows the positions of the oxygen-sensitive proteins described above, demonstrating the predicted pattern of responsiveness to Co^{2+}, Ni^{2+} and Co^{2+}+DHA. The compiled densitometric data for specific protein-band peak absorbance areas is shown in figure 15. Panel A demonstrates statistically significant increases in the expression of 42.5, 35.3 and 16.1 kDa with cobalt or nickel treatment. The oxygen-sensitive protein at 83kDa did not show responsiveness to any treatment group. For each protein band, incubation with Co^{2+}+DHA resulted in a return of protein expression to normoxic values for 42.5 and 16.1kDa proteins but did not abrogate the increase in expression with Co^{2+} for the 35.3 kDa protein. Panel B demonstrates the same overall pattern for oxygen-sensitive proteins that were found to be

FIGURE 14. Response of oxygen-sensitive proteins to normoxia (nor) cobalt, nickel and cobalt+dioxoheptanoic acid (Co^{2+}+DHA) treatment. All treatments were conducted under normoxic conditions. Positions of oxygen sensitive protein bands and molecular weight markers are given as for figure 13.

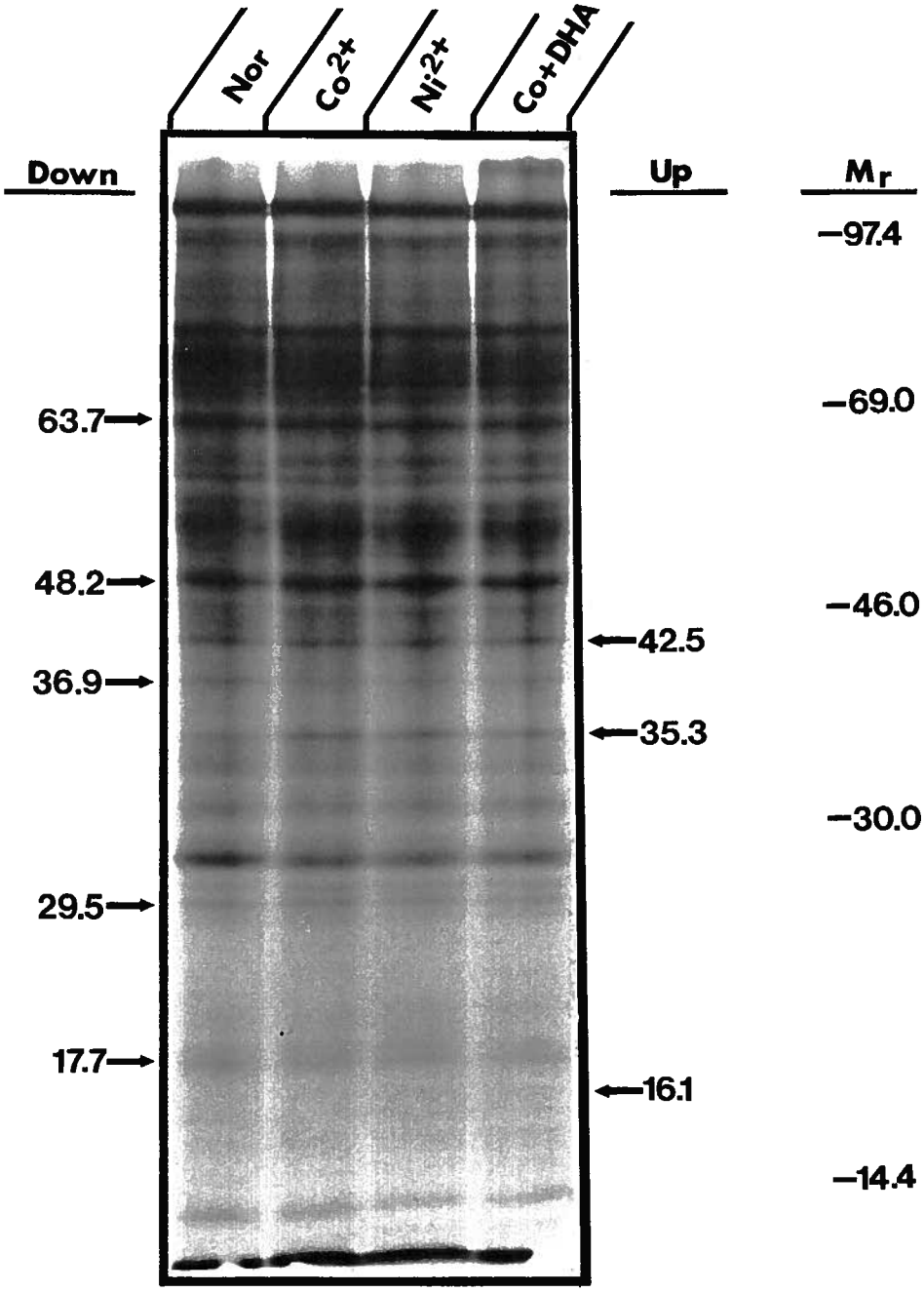
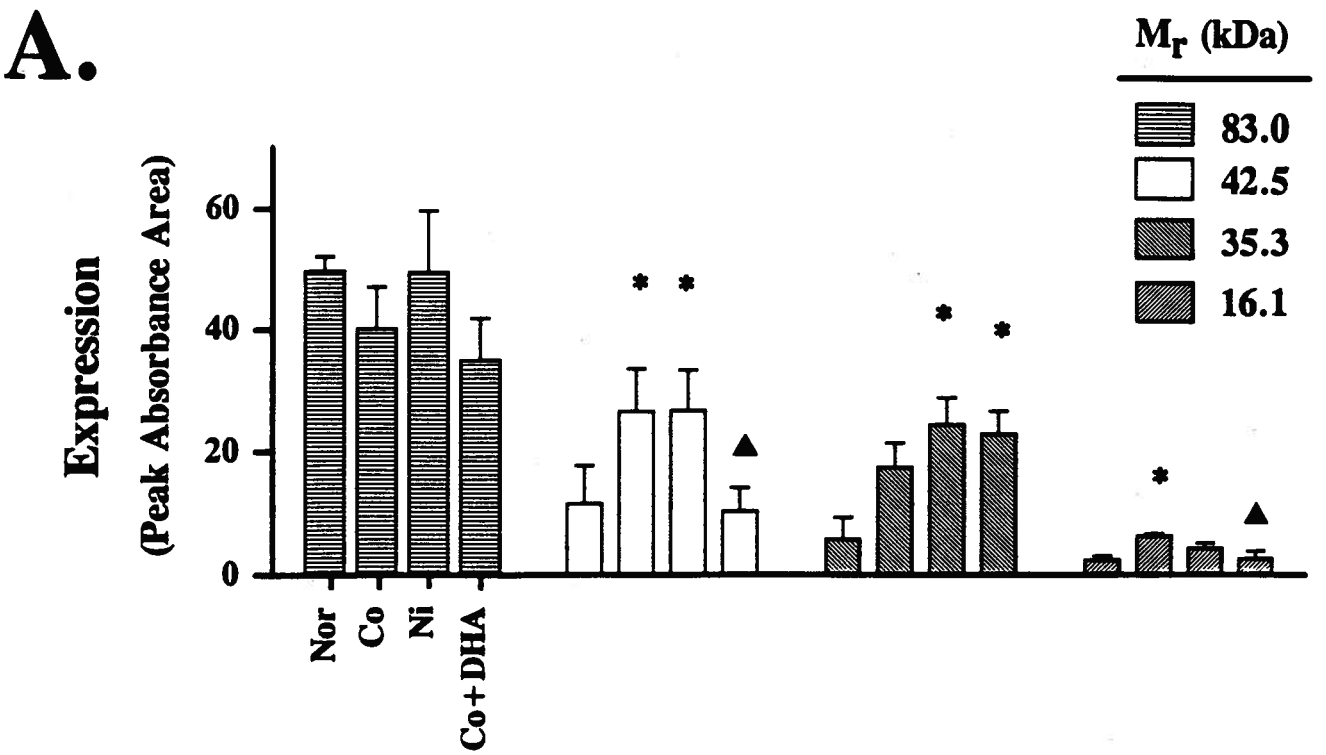
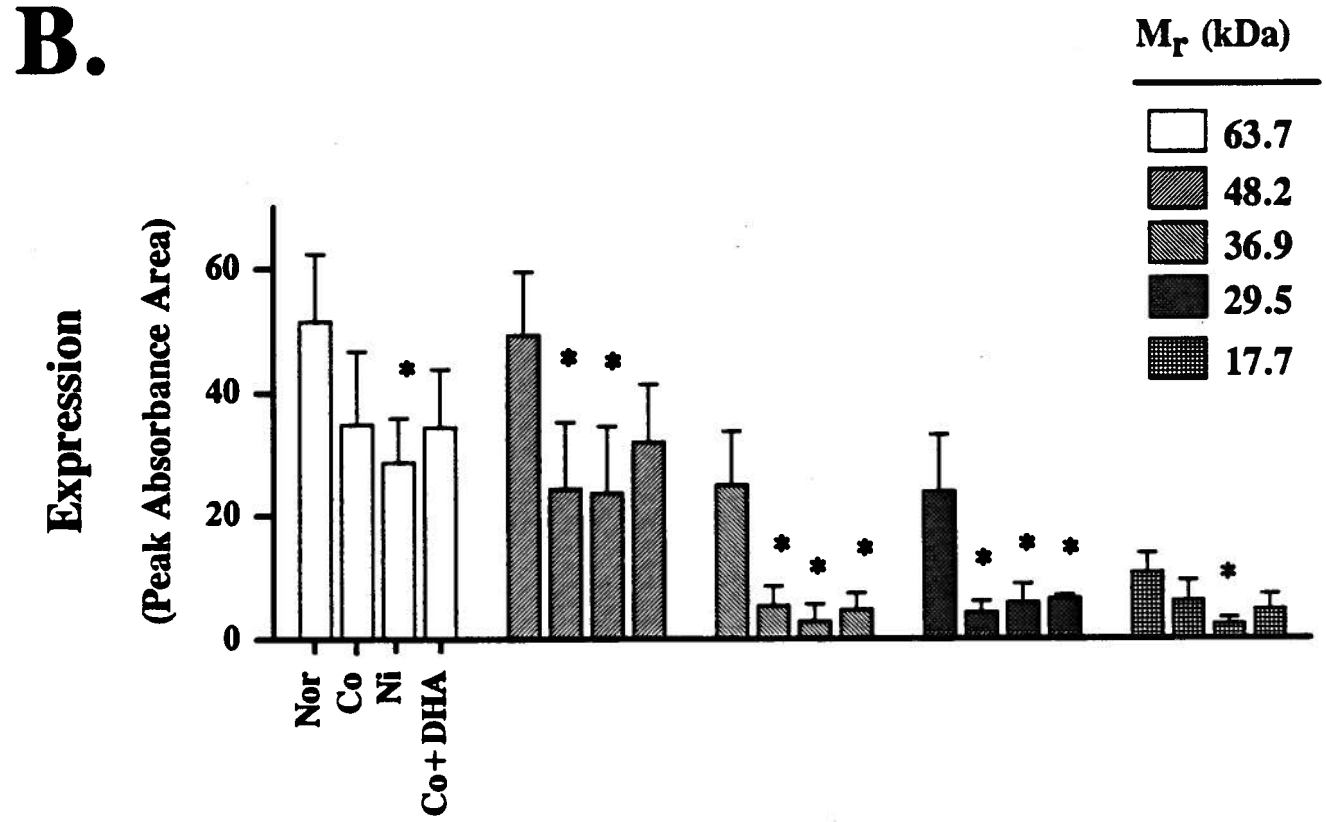

Nor
Co2+
Ni2+
Co+DHA
Down
Up
Mr
97.4
69.0
46.0
30.0
14.4
63.7
48.2
36.9
29.5
17.7
42.5
35.3
16.1

FIGURE 15. Changes in protein expression for oxygen sensitive protein bands during treatment with cobalt, nickel and cobalt+dioxoheptanoic acid. A) Increased protein expression with cobalt and nickel. B) Decreased protein expression with cobalt and nickel. Statistical significance was determined as described in figure 14; $^{*}P<0.05$ relative to nor, $^{\blacktriangle}P<0.05$ relative to Co, n=4 in each case.


A.
Mr (kDa)
83.0
42.5
35.3
16.1
Expression
(Peak Absorbance Area)
60
40
20
0
Nor
Co
Ni
Co+DHA


B.
Mr (kDa)
63.7
48.2
36.9
29.5
17.7
Expression
(Peak Absorbance Area)
60
40
20
0
Nor
Co
Ni
Co+DHA

suppressed in anoxia in Part A. Incubation with cobalt or nickel resulted in clear differences in the amount of protein present. Interestingly, the protein at 63.7kDa which did not show a statistical difference relative to mimicked physiological anoxia in figure 13B, demonstrated a significant suppression with nickel treatment. This supports the possibility that this protein may indeed be oxygen-sensitive. Incubation with Co^{2+}+DHA abrogated the cobalt or nickel induced suppression for 63.7, 48.2 and 17.7 kDa proteins but had no effect on the suppression of 36.9 and 29.5 kDa proteins.

To confirm that the responses observed with cobalt or nickel were indeed brought about by changes in heme-protein conformation, carbon monoxide was used to alter heme protein conformation to the "oxygenated state" whilst in the presence of anoxia and anoxia+cobalt. Figure 16 demonstrates the positions of oxygen and Co^{2+}/Ni^{2+}-sensitive proteins on a representative autoradiograph and the change in protein band absorbance area is given in figure 17. Panel A demonstrates that incubation with CO significantly reduced the anoxia, and Co^{2+}+anoxia-induced expression from normoxic control groups for 42.5, 35.3 and 16.1 kDa proteins. Panel B demonstrates the response of suppressed oxygen and Co^{2+}/Ni^{2+}-sensitive proteins to CO. The most profound effect of CO was found in the 17.7 kDa protein band which demonstrated a complete abrogation of the anoxia-induced suppression by CO treatment. However, this did not occur if Co^{2+} was included in the incubation. CO also lead to a modest return towards normoxia from a significantly suppressed point for 63.7 and 48.2 kDa proteins. However, CO treatment did not abrogate the anoxia and Co^{2+}/Ni^{2+} suppression in the 36.9 and 29.5 kDa protein bands.

Heat-Shock Response. To determine if any of the oxygen-sensitive proteins expressed in anoxia were part of an overall stress response, hepatocytes were heat-shocked to 40°C for 1h.

FIGURE 16. Response of oxygen and cobalt-sensitive protein expression to treatment with 10% carbon monoxide+anoxia. Positions of oxygen and cobalt sensitive protein bands and molecular weight markers are given as for figure 13.

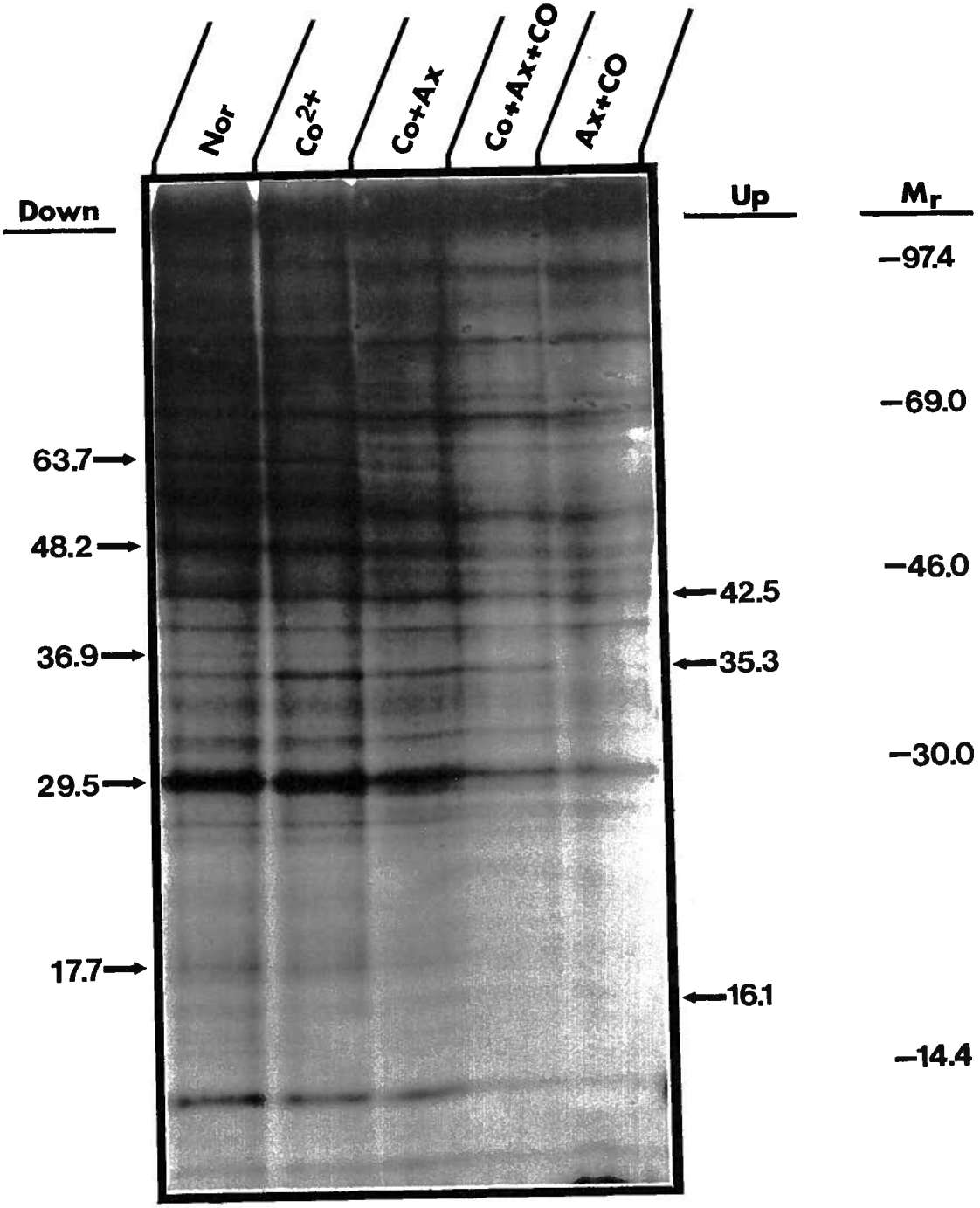
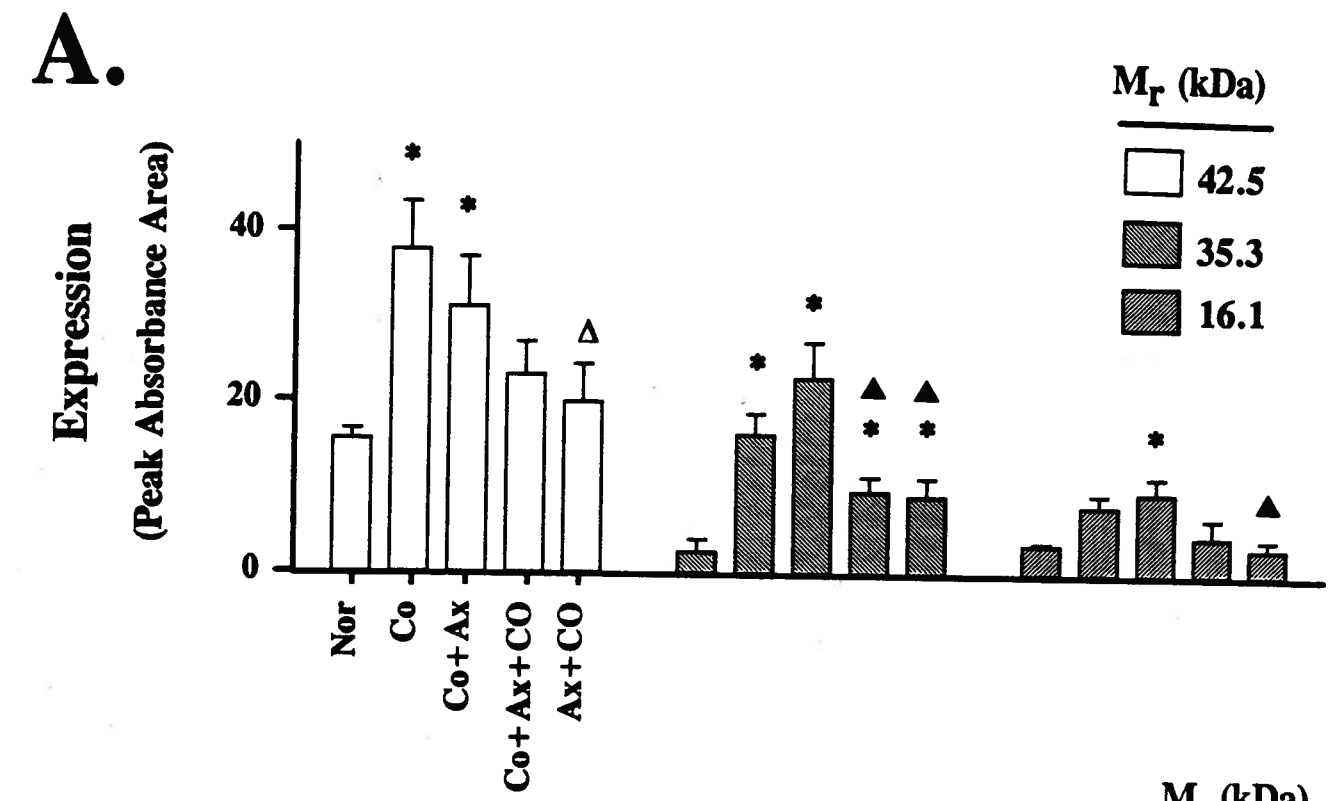

Nor
Co2+
Co+Ax
Co+Ax+CO
Ax+CO
Down
Up
Mr
97.4
69.0
63.7
48.2
46.0
42.5
36.9
35.3
29.5
30.0
17.7
16.1
14.4

FIGURE 17. Changes in oxygen and cobalt-sensitive protein expression as a function of carbon monoxide+anoxia treatment. A)Effect on proteins that increase expression with cobalt and anoxia. B) Effect on proteins that decrease expression during treatment with cobalt and anoxia. Significance was determined as described in fig 14; $^{*}P<0.05$ relative to nor, $^{\blacktriangle}P<0.05$ relative to Co^{2+}+Ax, $^{\Delta}P<0.05$ relative to Co^{2+}, n=4 in each case.


A.
Expression
(Peak Absorbance Area)
40
20
0
Nor
Co
Co+Ax
Co+Ax+CO
Ax+CO
Mr (kDa)
42.5
35.3
16.1

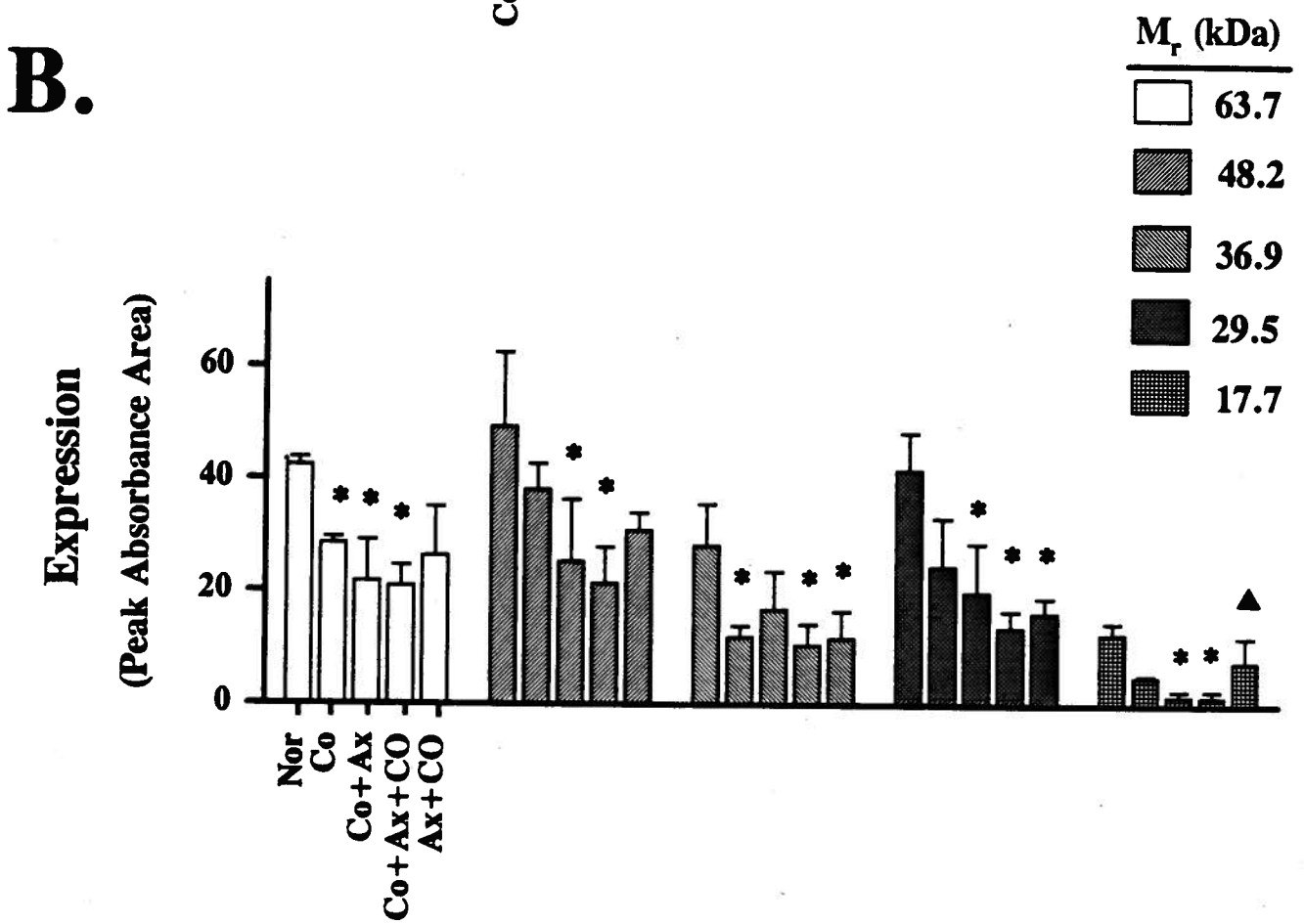

B.
Expression
(Peak Absorbance Area)
60
40
20
0
Nor
Co
Co+Ax
Co+Ax+CO
Ax+CO
Mr (kDa)
63.7
48.2
36.9
29.5
17.7

FIGURE 18. Response of turtle hepatocyte protein expression to heat shock at 40°C for 1h. Positions of heat shock proteins are given on the right of the figure and position of molecular weight markers on the left. *Abbreviations*: C, control; HS, heat shock; Hsp, heat shock proteins.

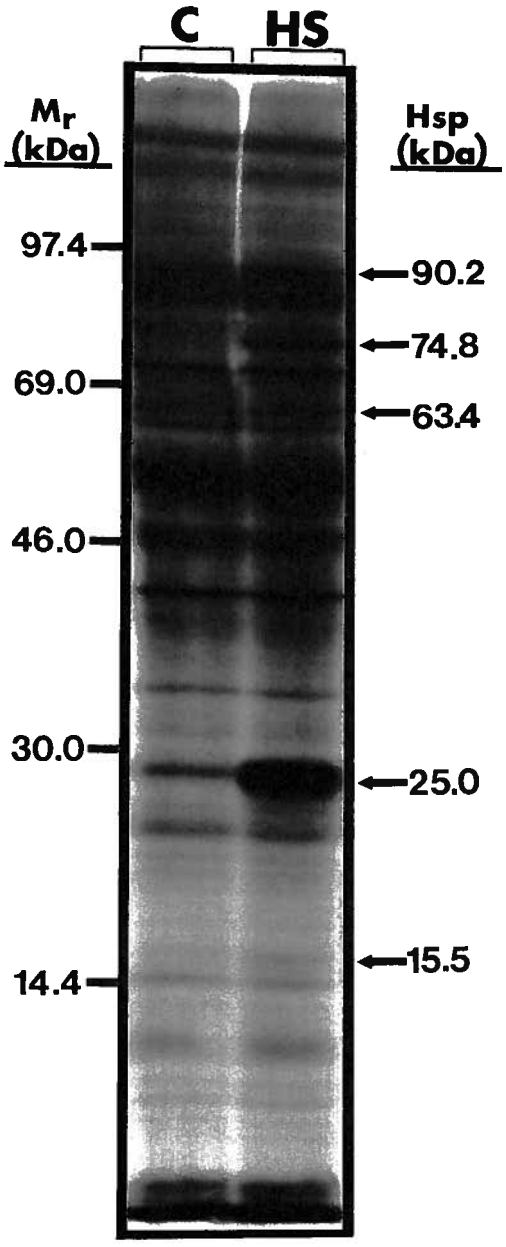

C
HS
Mr (kDa)
Hsp (kDa)
97.4
69.0
46.0
30.0
14.4
90.2
74.8
63.4
25.0
15.5

Figure 18 demonstrates the change in protein expression during the hour following the heat-shock episode. Protein bands of 90.2, 74.8, 63.4, 25 and 15.5 kDa were evident in the heat shocked group and were of a distinct molecular weight range compared to the increased expression of oxygen-sensitive proteins that appeared in anoxia. One possible exception is the 16.1 kDa oxygen- and Co^{2+}/Ni^{2+}-sensitive protein band which is within the margin of standard error to the 15.5 kDa heat shock protein.

Discussion.

The major finding in this study was the presence of nine protein bands that exhibited a differential change in expression directly in response to the presence or absence of oxygen. In the case of the 42.5, 35.3, 17.7 and 16.1 kDa protein bands, expression could be predictably manipulated with cobalt, nickel, carbon monoxide and an inhibitor of heme synthesis. When considered together, this strongly suggests that a conformational change in a heme-group is involved in the transduction of this response. Not all of the described oxygen-sensitive protein bands conformed to this pattern. Increased anoxic expression of the 83 kDa protein band was oxygen-sensitive (fig 12), but was completely unresponsive to any kind of heme protein manipulation. Furthermore, with the exception of the 17.7 kDa protein band, all suppressed oxygen and Co^{2+}/Ni^{2+}-sensitive proteins (panel B figs 13,15,17) were unresponsive to carbon monoxide or an inhibitor of heme synthesis. Since heme-groups differ widely in their affinities for carbon monoxide (Coburn, 1979), this observation may be suggestive of control by a different heme protein and raises the possibility of multiple oxygen-sensing mechanisms governing the expression or suppression of proteins in anoxia.

This overall assessment is influenced by whether aerobic incubation with cyanide was a

realistic mimic of physiological anoxia. In ideal circumstances, the largest difference between aerobic cyanide treatment and anoxia would be the presence or absence of oxygen itself, all other physiological effects of both states being equal. This has been disputed in the mammalian hepatocyte literature where aerobic incubation with KCN results in a rapid loss of cell viability, diminished adenylate energy charge, mitochondrial swelling, increased intramitochondrial phosphate accumulation, and a 50% loss in the mitochondrial proton-motive force. In anoxia without cyanide treatment, these effects occurred on a longer time-scale suggesting that cyanide has a more immediate and profound effect on cellular metabolism (Aw & Jones, 1989). However, anoxia-tolerant organisms demonstrate a markedly different metabolic response to reduced oxygen availability. Although in situ mitochondrial metabolism has not been investigated in cyanide-treated turtle hepatocytes, aerobic incubation with 0.5mM KCN over 10h has no effect on cellular viability or energy charge (Buck *et al.*, 1993b), which agrees with the minimal change in LDH leakage under NaCN incubation noted in this study. Furthermore, turtle hepatocytes demonstrate a similar reduction in metabolic rate and microcalorimetric heat flux when under aerobic incubation with cyanide or under anoxia (Buck *et al.*, 1993a) and in both cases, the plasma membrane potential remains unperturbed from control aerobic conditions (Buck & Hochachka, 1993). Overall, the metabolic response of turtle hepatocytes to aerobic incubation with cyanide clearly represents a much closer mimic of true anoxia than in the case of mammalian hepatocytes. The metabolic differences that might exist are probably minor and are unlikely to be sufficient to cause a differential protein expression in comparison with true anoxia.

The identity of the expressed and suppressed protein bands was not established in the present study. Previously it was noted that anoxic gene expression in turtle liver was associated

with de novo synthesis and increased expression of three mRNA transcripts that were subsequently translated in vitro into proteins corresponding to 19.5 (de novo), 28.6 and 79.9 kDa (Douglas *et al.*, submitted). It was suggested that these protein translates could be part of a stress response that is active in stabilising cellular constituents both during anoxia and on re-oxygenation. To determine whether the anoxic protein bands observed in this study were part of an overall stress response, we compared them to the turtle hepatocyte heat-shock response. Of the proteins expressed in anoxia, only the 16.1 kDa protein band demonstrated a molecular weight overlap with a heat-shock protein at 15.5 kDa, and all others bands were completely distinct. It therefore appears that the increase in the expression of specific proteins in anoxia is not part of a stress response *per se*. Whether the mRNA translates corresponded to any of the protein bands found in the present study is difficult to assess since in vitro translation does not produce a mature protein product. Nevertheless, the discrepancy between the number of protein bands found in this study, and the number of mRNA transcripts found by Douglas *et al.* (submitted) could be suggestive of control at both transcription and translation. With regard to the latter, Lefebvre *et al.* (1993) have demonstrated that a possibility exists for oxygen-sensitive control of translation in rat hepatocytes: progressive hypoxia induced rapid and coordinate decreases in protein synthesis rates which occurred well before any change in cellular energy charge, metabolite or mRNA concentration.

A number of oxygen-sensitive genes that are controlled through conformational changes in a heme-protein have been characterised in liver tissue. Particularly significant is the observation that hormonally regulated gluconeogenic and glycolytic enzyme expression along the periportal-perivenous oxygen gradient, may be under modular control by a cobalt and carbon monoxide-sensitive, oxygen-sensing mechanism. Under high periportal oxygen tensions, glucagon induces

the expression of PEPCK mRNA and protein, an effect which is diminished with progressive hypoxia. Incubation with cobalt relieves the oxygen effect and carbon monoxide reinstates it (Keitzmann *et al.*, 1992 & 1993). Although hormones, metabolites and autonomic input are all involved in defining basal expression of periportal-perivenous enzymes, it appears that oxygen-sensing plays an adaptive role in altering enzyme profiles during periods of hypoxia (Jungermann & Katz, 1989). Under normoxia, incubation of isolated hepatocytes with either glucagon or insulin preferentially mimics periportal gluconeogenic, and perivenous glycolytic enzyme profiles respectively. However, reducing oxygen concentrations significantly lowers PEPCK activity in glucagon-treated cells and increases pyruvate kinase (PK) and glucokinase activity in both insulin and glucagon-treated cells (Nauck *et al.*, ,1981; Wölfle & Jungermann, 1985). The control of this response appears to be different in the case of PEPCK and PK. In whole liver in vivo, PEPCK mRNA and specific activity is found predominantly in the periportal region (Bartels *et al.*, 1989), whereas pyruvate kinase mRNA is found homogenously, but the active protein translate is only located in perivenous areas (Lames *et al.*, 1987). Thus, oxygen appears capable of modulating PEPCK enzyme concentrations through a heme-protein oxygen-sensor at the transcriptional level and in the case of PK, through an unknown mechanism at the translational level.

This agrees with the initial assessment that turtle liver also appears to demonstrate oxygen-sensitive control at transcription and translation, but whether the anoxic profile of protein expression observed in this study reflects an oxygen-sensing phenomenon similar to that found in liver zonation remains unknown. Whereas the expression and suppression of protein bands occurred in the absence of added exogenous factors such as hormones, no single protein in this study demonstrated absolute induction or suppression (from or to zero) in anoxia. Likewise,

incubation with metal ions, carbon monoxide or DHA did not result in an absolute loss or gain of the protein bands of interest. It seems clear that the background expression of these proteins is governed by an oxygen-independent factor, which can be modulated by an oxygen-sensitive heme protein based mechanism during anoxia.

The transduction pathway of an oxygen signal, through a heme-protein has been examined in studies on the regulation of the Epo gene in liver and kidney (Goldberg *et al.*, 1988; Maxwell *et al.*, 1993; Eckhardt *et al.*, 1993). In liver, the basal expression of this gene occurs almost entirely in parenchymal cells in the hypoxic perivenous region of the acinus and deepening hypoxia increases its expression several fold (Koury *et al.*, 1991). The epo gene is flanked at both ends by a series of positive and negative regulatory elements. Oxygen-sensitive induction of this gene is believed to involve the expression of one or more oxygen regulated inducer element(s) (Tsuchiya *et al.*, 1993) which relieve the binding of a trans-acting repressor at a site upstream from the epo gene. The inducer element(s) also remove inhibitory binding of a ribonucleoprotein to an upstream promoter site, allowing the binding of a constitutive 47kDa protein, and consequent expression of the epo gene (Imagawa *et al.*, 1994). The intracellular signalling pathway that transduces the change in heme conformation into a genetic response has not yet been characterised but there is some evidence to suggest that phosphorylation via protein kinase C is not involved (Eckhardt *et al.*, 1993). The nature of this kind of oxygen-sensitive control of gene expression remains unknown although there are numerous parallels in the prokaryotic literature (e.g Lois *et al.*, 1993). Nevertheless, studies on epo, and other oxygen-sensitive genes may provide good working models for future studies concerning oxygen sensing mechanisms in facultative anaerobes.

In conclusion, evidence is provided for an oxygen-sensing mechanism that is based on

conformational change in a heme-protein and that is involved in the expression and suppression of numerous proteins during anoxia in turtle hepatocytes. In addition, there is some evidence to suggest that other oxygen-sensitive, non-heme based mechanisms may have been involved in regulating an 83 kDa protein band. Because the oxygen-sensitive response is not absolute, the primary role for this kind of mechanism appears to be in the adaptive modulation of existing protein concentrations in response to progressive hypoxia and anoxia. Given that oxygen-sensitive control of gene expression is phylogenetically conserved and widespread between organ systems (Hochachka, 1994; Fanburg *et al.*, 1992), there is a good probability that this kind of oxygen-sensing mechanism may play a fundamental role in adjusting cellular phenotype to the demands of anoxic survival.

Chapter 5.
General Discussion.

Partitioning of ATP Demand in Metabolically Suppressed Tissues.

A schematic representation of the normoxic-anoxic transition in turtle hepaotcytes is presented in figure 19. Although metabolic rate falls precipitously on entry into anoxia, remaining anoxic metabolism accounts for about 10% of the normoxic metabolic rate. This can be extinguished by addition of the glycolytic inhibitor, iodoacetate which also results in the rapid death of the cell. This demonstrates two important points: firstly, metabolism during metabolic suppression is maintained by a reduced rate of glycolysis and secondly, a slow, but sustained rate of metabolism is vital for the continued survival of tissues during metabolic suppression.

From the research presented in this thesis, together with other studies on turtle hepatocytes we are now in a position to ascribe and rank energetic costs to the metabolic processes active during both normoxia and anoxic metabolic suppression (Figure 19). Under normoxia, rates of ATP turnover for Na^+/K^+ ATPase, protein synthesis, protein degradation and urea synthesis occupy a similar proportion of total metabolic rate as found in other cell types (eg. Ascites tumour cells, Müller *et al.*, 1986) with protein turnover (i.e. protein synthesis and degradation) accounting for a principal proportion of normoxic ATP turnover. Since we can account for 89% of normoxic ATP turnover, processes not measured, such as RNA synthesis, Ca^{2+} ATPase and so forth, presumably account for the remainder. Normoxic gluconeogenesis rates suggest a gross ATP requirement of 11.4μmol ATP.g $cells^{-1}.hr^{-1}$ but without knowledge of rates of glycolysis under normoxia it is impossible to infer a net energy cost for this process.

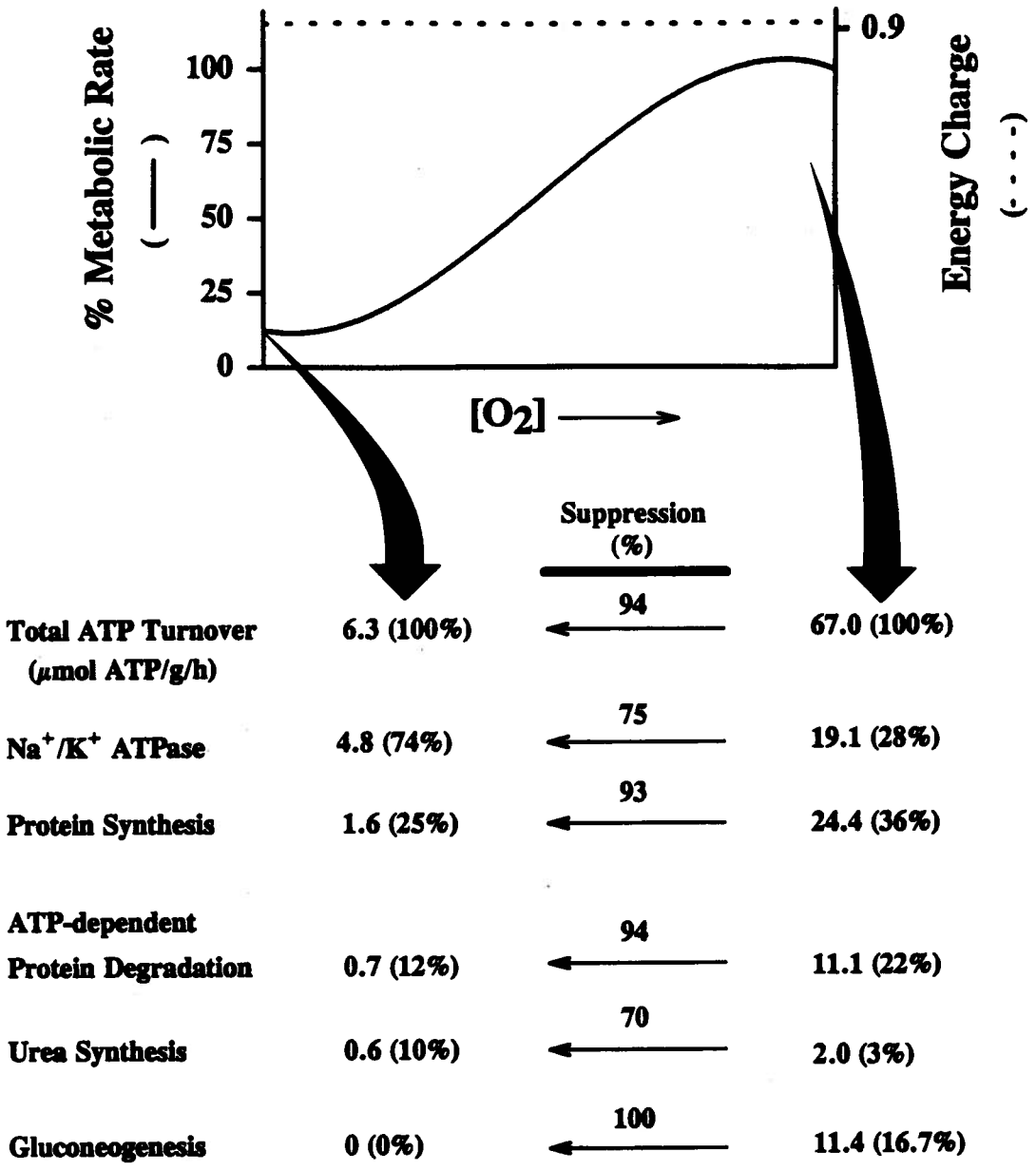


FIGURE 19. Partitioning of energy demand from normoxia to anoxia in turtle hepatocytes. Compiled from chapter 2, chapter 3, Buck *et al.*, 1993b and Buck & Hochachka, 1993. Percentages are data means from each source.

Under anoxia, a particularly significant finding is that the suppression of whole cell metabolism is matched by all individual ATP-dependent cellular processes investigated in these cells so far. This supports the concept that entry into metabolic suppression is highly regulated and coordinated. Of the remaining cellular metabolism, however, 76% is required by Na^+/K^+ ATPase followed by a further 35-45% required by protein turnover. Although this actually accounts for more than 100% of anoxic ATP metabolism (differences in culture conditions and seasonal variation in the donor animals most likely account for this) it is clear that there is a significant, primary energy expenditure for the maintenance of membrane potential and a secondary expenditure for the maintenance of protein turnover during anoxic metabolic arrest. Urea synthesis accounts for a diminishingly small proportion of anoxic metabolism and since ATP concentrations remain steady, and glycogen content falls in proportion to the rate of lactate production and glucose mobilisation it is assumed that gluconeogenesis is not active under anoxia.

Does the remaining ATP demand in anoxia represent a set-point beyond which metabolism cannot be suppressed without excessive tissue damage and possibly cell death? The answer would appear to be dependent upon the effectiveness of membrane stabilisation by channel arrest and probably also the ability of cell proteins to remain stable. In endothermic tissues, this set-point can be considered to be very much higher due to the porosity of cellular membranes and naturally higher rates of protein turnover. Singer *et al.* (1992) have proposed that the minimal metabolic rate (MMR) of all mammals, including those that enter dormant states, can be suppressed down to a minimal level that is defined by the lowest metabolic limit for sustained structural integrity of tissues. Interestingly, this MMR set-point coincides with the basal mass-specific metabolic rate of the largest mammal, the blue whale. Suppression of metabolism

beyond this point (as would occur in hypothermia or ischemia) results in an inability to recover tissue function, also supporting the notion of vital cell processes in defining the set-point for MMR. In turtle hepatocytes, complete inhibition of ATP supply is clearly lethal. However, the absolute limit for sustaining viability with increasingly severe metabolic suppression has not been experimentally probed.

Recoverable, near ametabolic states do exist in nature however, as in the case of dormancy in *Artemia* embryos (Hand, 1990; Clegg, 1992). The key to survival here appears to lie in the relatively low complexity of the system (cells are barely differentiated) and significantly, in the ability to encyst. The cyst wall is impermeable to ions and metabolites (Clegg & Jackson, 1989) and in ensuring a constant internal environment, presumably stabilises cells against large changes in external ion concentrations. However, the question of protein stability remains a major area of interest during dormancy in this species (Anchordoguy & Hand, In Press).

Importance of Protein Turnover in Metabolic Suppression.

Protein synthesis presents an excellent example of how energetically expensive cellular processes are balanced against the requirement to conserve energy during metabolic suppression. In chapter 2, the specific cost for peptide bond synthesis in turtle hepatocytes was calculated at 5.3 ATP equivalents per peptide bond. Theoretically, this would be distributed in translation as 1 ATP and 1 GTP in initiation and 2 GTP in elongation. In addition, the binding of an amino acid to tRNA involves the hydrolysis of two phosphate bonds in the potentiation of the aminoacyl-tRNA complex for peptide bond formation in elongation. Since most amino acids are co-transported with Na^+, 1 ATP is included as the incidental cost of amino acid transport by Na^+ extrusion by Na^+/K^+ ATPase. Theoretically then, 5 ATP equivalents are involved directly

and indirectly in the synthesis of a single peptide bond (amino acid transport, binding to tRNA and elongation), and a further 2 are involved in forming the initiation complex. Assuming that the hydrolysis of high-energy phosphate bonds yields a standard ΔG° of -7.3kcal/mol, then the cost of peptide bond synthesis possesses a ΔG° of 36.6kcal/mol (assumes a negligible cost for initiation which occurs once per peptide chain). The standard ΔG° of the peptide bond is 5kcal/mol and therefore the energetic efficiency of protein synthesis is only about 13%. Clearly, the high degree of fidelity required by protein synthesis commands the vast proportion of energy expenditure in protein synthesis.

Once formed, peptide bonds are extremely stable. This results from resonance within the π-electron orbital between the carboxyl and amide groups along the length of the bond (Pauling *et al.*, 1951). This resonance hybrid resists twisting in the plane of the bond and creates an extremely stable, low energy linkage between amino acids. Given the high cost of synthesis and high stability of peptide bonds, it would seem counter-productive to maintain protein turnover during metabolic suppression. Nevertheless, in turtle hepatocytes, about 40% of anoxic energy metabolism is invested in sustaining protein turnover; why so?

Maintenance of reduced protein synthesis rates ensures that control of protein expression does not shift too far towards protein degradation. Consider equation 1 (chapter 1) where the steady state concentration of a protein was a function of its rate of transcription and translation, divided by its rate of degradation. Complete inhibition of protein synthesis would result in a change in the control of protein expression towards degradative processes alone. Therefore, maintaining protein synthesis rates at some level helps to defray the influence of protein degradation on protein expression during metabolic suppression. The results presented in chapters 2 and 3 support this view since the control of protein expression did indeed shift

towards protein degradation in anoxia but remaining rates of protein synthesis were significant. However, protein degradation rates were also reduced in anoxia, resulting in a two-fold increase in protein half-lives for both stable and labile protein pools. Clearly proteins are generally present for longer during metabolic suppression before they are degraded and replaced. Taken together, the shift in control of protein expression towards protein degradation and the overall increase in protein half-life points to a cellular environment that favours the accumulation of damaged and denatured proteins.

What are the principal sources of protein damage in these conditions? Post-translational protein modification and damage can occur by a variety of mechanisms. A particularly interesting discovery which may have some significance to protein conformation during metabolic arrest is the ability of asparagine and glutamine residues to undergo spontaneous chemical deamination to aspartic and glutamic acids (Robinson, 1979; Yuan *et al.*, 1981). The appearance of deaminated protein forms is common among proteins and these usually constitute a precursor to their catabolism. In the case of triosephosphate isomerase (TPI) for example, under conditions of normal protein turnover, spontaneous deamination of two specific asparagines results in the separation and unfolding of the TPI dimers leading to the appearance of a more acidic enzyme prior to its subsequent degradation (Yuan *et al.*, 1981). However, if protein turnover rates are reduced, as in the degenerative disease progeria, denatured TPI accumulates, with potentially lethal consequences (Gracy *et al.*, 1984).

Another source of protein modification which may be particularly relevant to tissues that store and release concentrations of fermentable substrate results from non-enzymatic attachments of glucose to free amino groups (Valssara *et al.*, 1985). This association occurs in tissues with glucose concentrations in excess of 50mM and is characteristic amongst proteins with slow

turnover times. The resulting glycosylated proteins (known as advanced glycosylation end-products - AGE) selectively bind to an AGE receptor and are degraded. In turtle liver, glucose concentrations reach 200mM during the first 10h of anoxia at 25°C (Buck *et al.*, 1993b), a time when protein turnover is extended suggesting that AGE accumulation may be a very real problem during metabolic suppression. Other potential sources of protein damage arise from limited proteolysis, and racemization. Proteolytic modification of functionally important proteins may be particularly problematical given the relative significance of ATP-independent proteolysis in anoxia. Racemization (the substitution of L to D amino acid stereoisomers within a protein via racemase) occurs in proteins with extremely long turnover times and may be a significant source of protein damage to slow-turnover structural proteins during dormancies that are extremely chronic.

Recovery from chronic anoxia represents a potentially critical period in terms of reinstating aerobic cellular function. After 6h of forced anoxic submergence at 24°C, arterial pO_2 rapidly recovers to pre-dive within the first 30 min of recovery. The decline in plasma lactate is immediate in onset, but much less rapid, returning lactate concentrations to pre-dive levels over 15h (Robin *et al.*, 1981). This suggests that after prolonged anoxia, aerobic conditions, suitable for supporting oxidative metabolism are quickly reinstated. With the potential for oxygen free radical formation during this time (chapter 1), compounded by anoxia-induced protein damage, the reinstatement of aerobic metabolism could be severely hampered. It seems likely therefore that efficient animal anaerobes should possess mechanisms for stabilising proteins during anoxia and dealing with oxygen free radical formation on recovery.

A major mechanism of stabilising proteins against denaturation during many kinds of metabolic insult (heat, oxygen and glucose deprivation, metabolic toxins etc) involves the

synthesis of stress proteins. A key feature of stress protein synthesis is that repeated exposure to the given stress results in increased tolerance and a lower incidence of protein damage (Morimoto *et al.*, 1990). Although there are approximately 30 different kinds of stress protein synthesised under differing stresses (belonging to classes of heat shock proteins (HSPs) and glucose regulated proteins (GRPs), for example), the best characterised are those of the heat shock protein 70 (HSP 70) class. These proteins possess both constitutive and inducible forms and appear to play a role in stabilising nascent protein structures during formation of the tertiary and quaternary structure. Other members of the HSP70 family (Munro & Pelham, 1986) and GRP78 (Hendershot *et al.*, 1988) have been identified as similar to immunoglobulin binding protein (BiP), a protein involved in stabilising nascent protein chains prior to glycosylation or during periods of low glucose exposure (Kim *et al.*, 1987). If the nascent protein chain remains bound to BiP due to denaturation, BiP selectively identifies that protein chain for removal by proteases. Since protein folding is an ATP dependent process requiring the interplay of numerous stress protein classes and several bind-release stages, some workers have noted that expression of inducible forms of stress protein does not occur if ATP concentrations are high and constant (Benjamin *et al.*, 1992; Williams *et al.*, 1993). Once again, this typifies the situation in tissues during metabolic arrest. In turtle hepatocytes, during recovery from anoxia, ATP concentrations have been found to remain high with no synthesis of stress proteins (Land *et al.*, 1993).

There are numerous other mechanisms of protein stabilisation, all of which require further research under conditions of metabolic suppression. Receiving some current attention is the effect of reduced metabolism on ubiquitinisation of proteins, a mechanism that ear-marks proteins for removal by ubiquitin-dependent cytoplasmic proteases (Hershko, 1988). During the

early stages of estivative dormancy in *Artemia* gastrulae, there is a 93% reduction in the number of proteins conjugated to ubiquitin (Anchordoguy & Hand, 1994). The first step in ubiquitin-protein conjugation requires ATP and involves a protein whose action may be pH dependent. With the large and rapid fall in pH that occurs during the first stages of dormancy in this species, it is possible that pH, combined with a change in adenylate ratios may be important factors in stabilising proteins against this kind of proteolytic degradation (Anchordoguy & Hand, 1994). Another exciting possibility is whether proteins from facultative anaerobes possess innate features of protein primary sequence that preferentially stabilise proteins against proteolytic attack. For instance, certain amino acid sequences such as those rich in proline, glutamic acid, serine and threonine appear to destabilise proteins (the PEST hypothesis, Rogers *et al.*, 1986) or features of amino terminal residues that appear to determine ease of degradation by the ubiquitin-proteasome pathway (The N-end Rule, Bachmair *et al.*, 1986). In studies of metabolic suppression, this is a wide-open and important area for future investigation.

If rates of protein synthesis and protein degradation are indeed imbalanced to favour liberation of amino acids, what is their fate or possible role? Clearly, in anoxia, they are not utilised as metabolic or gluconeogenic substrates although they likely fulfil this role during recovery where reinstatement of oxidative metabolism enables flux through gluconeogenic and oxidative pathways and also the re-instatement of aerobic protein synthesis rates. In the context of cellular maintenance, a role for free amino acids in the stabilisation of cell structure has been suggested. In hypoxia-intolerant cell-lines, a depolarisation resulting from diminished ion gradients precedes cell death with the result that intracellular accumulations of amino acids are lost. However, it appears that if cells are provided with glycine or alanine in low mM concentrations, viability is markedly extended. In mammalian kidney proximal tubules, the

protective effects of glycine and alanine have been characterised to include the preservation of intracellular ATP concentrations, stabilisation of cell membrane structure, inhibition of Na^+/K^+ ATPase (although K^+ leakage still occurs) and a delayed increase in intracellular free Ca^{2+}. The precise mechanism behind this effect is not yet known but it appears that protection is independent of amino acid catabolism and there is a strict dependency for amino acids with a glycine-related structure (Venkatachalam & Weinberg, 1993).

Implications of Oxygen Sensing in Metabolic Suppression.

Changes in metabolism during progressive hypoxia are clearly linked, in some way, to changes in oxygen. The question is, how coarse is this control and are there signalling mechanisms that sense changes in extracellular O_2 over a wide range of ambient concentrations? Closer examination of individual biochemical pathways reveal apparent "set-points" for hypoxia-associated rate changes. Figure 20 demonstrates the oxygen dependence for various cellular processes over the lower half of the curve presented in figure 1 in Chapter 1. The points indicated on the curve for each cellular process are given as the half-maximal ($V_{0.5}$) response to a decrease in oxygen concentration, measured at the cell surface. The most striking observation from this figure is the apparent change in rates of glycolysis and gluconeogenesis which occur at the same point, and in opposite directions, well before decreases in protein synthesis rates, and in turn, well before any fall in ATP concentrations. As with the fall in whole tissue VO_2 (figure 1, Chapter 1) $V_{0.5}$ for each of these processes occurs at points that are several-fold higher than the standard km of O_2 at cytochrome c oxidase.

This response can be interpreted in two ways: i) in intact hepatocytes, there is a substantial gradient of O_2 across the plasma membrane that results in diffusional limitation of in situ

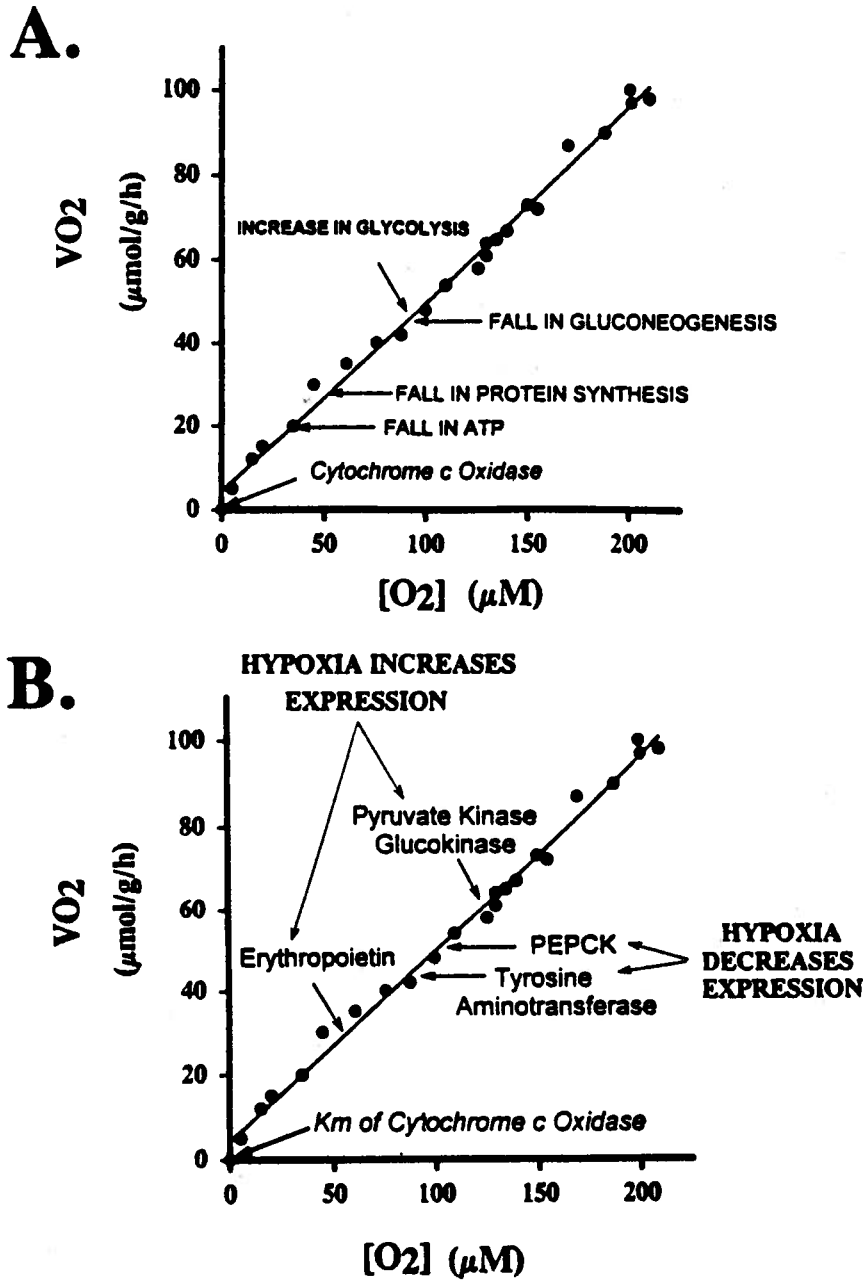


FIGURE 20. Control of anaerobic processes appears independent of mitochondrial oxygen consumption and ATP concentrations in rat hepatocytes. A) $V_{0.5}$ points for up- and down-regulated cell processes in anoxia. B) Induction and suppression points for hypoxia sensitive gene and protein expression in anoxia. Compiled from Nauck *et al.*, 1981, Wölfle & Jungermann, 1985, Eckhardt *et al.*, 1993, Lefebvre *et al.*, 1993, Thurman *et al.*, 1993).

mitochondrial metabolism even at high oxygen concentrations, and/or ii) cellular metabolism is regulated by a mechanism that is capable of sensing and transducing changes in extracellular oxygen concentrations (possesses a high K_m for O_2) into a coordinated cellular response. Jones *et al.* (1990), has calculated that under normoxic steady-state conditions, the gradient of O_2 from the unstirred extracellular boundary layer to the mitochondrion is about 6μM O_2. During acute hypoxia, there is a dramatic increase in the relative resistance of oxygen diffusion over this distance, due to functional anoxia around mitochondria (Jones *et al.*, 1990). Therefore, metabolism *is* functionally anaerobic before ambient O_2 reaches zero. Nevertheless, the magnitude of the oxygen gradient is too small to account for the $V_{0.5}$ of oxygen sensitive cellular processes demonstrated in figure 1.

A further issue that is not settled by the suggestion that O_2-gradients may bring about the coordinated change towards anaerobic metabolism, is the observation that cellular changes occur whilst metabolic signals remain relatively unperturbed. Changes that do occur, cannot account for the global suppression of ATP synthesis and demand that is associated with numerous metabolic processes during metabolic suppression. Clearly, something else is controlling flux through metabolic pathways, that is sensitive to oxygen concentrations and is capable of coordinately modulating numerous cellular pathways.

In an attempt to examine the relationship between signal and response, the studies in chapter 4 investigated oxygen-induced changes in protein expression and suppression. The results suggest that oxygen plays a significant role in modulating the expression and suppression of proteins over their basal concentrations through conformational changes in a heme-protein oxygen receptor. This kind of oxygen sensing mechanism is significant because it presents a mechanism where, depending on the O_2-binding kinetics of the heme group, changes in oxygen

concentration can be detected early in hypoxia, and ensuing oxygen-sensitive changes titrated appropriately with increasingly severe hypoxia. Furthermore, the results suggested that oxygen control of protein expression could over-ride the primary factors controlling basal protein expression (eg hormones). This is therefore a system that operates independently of oxygen supply until a critical point is reached in hypoxia, at which time the system is capable of invoking oxygen sensitive-control and readjusting to functioning in a low O_2 environment. Figure 20B demonstrates the points of induction for various oxygen sensitive gene products in mammalian liver, over the lower half of the curve presented in figure 1 (Chapter 1). As with the pattern of protein induction in turtle hepatocytes, expression and suppression of proteins occurs in an oxygen-sensitive fashion, well before ATP concentrations fall, and long before O_2 is limiting at the mitochondrion. The advantage of modulating protein profiles in this way is clear: as progressive hypoxia becomes increasingly severe, so the profile of functional proteins can be readjusted towards favoured function in anoxia well before oxygen and energy supply become limiting.

Switching over control from the primary signals that govern cell functions, to an oxygen-dependent modulation of cell function requires an oxygen-sensing mechanism that can interact directly with normal modes of cellular signalling. Guanylate cyclases offer an excellent example of a possible bridge between hormone and oxygen based signalling mechanisms (fig 21). Receptor guanylate cyclases (GCases) are found in both insoluble (membrane-bound), and soluble (cytosolic) forms and in each case, respond to the binding of agonists to produce cyclic GMP (cGMP). By activating protein kinases, cGMP modulates Na^+ channel activity in numerous tissues and can abrogate cAMP-induced responses by activating cAMP-hydrolysing phosphodiesterases (Hille, 1991). In metabolic suppression, cGMP and protein kinase G's have

been implicated in the inhibition of PK activity by phosphorylation in the anoxic whelk tissues (chapter 1). Although the membrane-bound GCase appears largely responsive to hormones, the soluble form appears to be activated by other agonists. This protein possesses a ferro-heme subunit which can change conformation to directly affect the rate of hydrolysis of GTP → cGMP+2Pi at the catalytic site and as such, allows alternate signals to be fed into the cGMP signalling pathway. The principal agonist at this site appears to be nitric oxide which interacts directly with the central iron position to effect large changes in cGMP concentrations (Ignarro, 1989). To my knowledge, no studies have been carried out to examine if changes in cytosolic oxygen can similarly activate GCase, or whether this is represents a physiologically important signalling route in hypoxia in vivo. However, the association of two signalling mechanisms within one signal-transducing pathway is highly suggestive of the theoretical requirements for an oxygen-sensing process that operates to modulate existing cell functions (fig 21).

How important are oxygen sensing mechanisms in the control of metabolic suppression? This is difficult to assess at this stage although the potential is enormous. The fact that protein concentrations change via an oxygen-sensing mechanism is indicative of fundamental phenotypic change within cells during metabolic suppression. Further investigation may reveal subsequent modulation of metabolic pathways or cellular processes as a result. Perhaps the most important feature of this mechanism is its demonstrated ability to bring about a coordinated change in protein concentrations directly in response to a change in oxygen, as would be required for cellular responses during metabolic suppression.

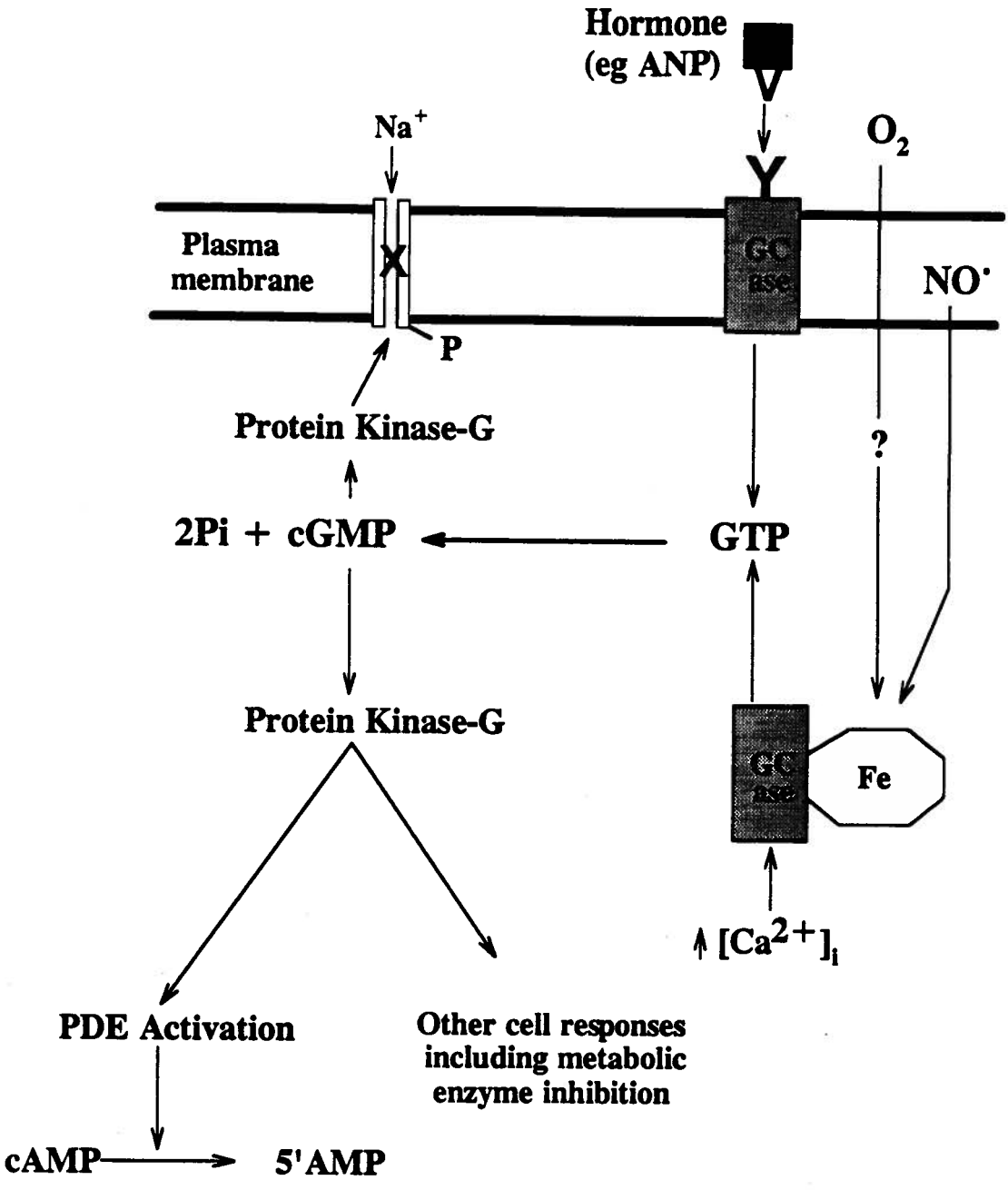


FIGURE 21. The oxygen-sensing potential of the cGMP signalling pathway: feeding two messages into one second-messenger system. Abbreviations: PDE-phosphodiesterase, ANP-Atrial naturietic peptide, GCase-guanylate cyclase.

Perspective.

The research presented in this thesis supports the concept of metabolic suppression as a highly coordinated and regulated response to anoxia. The principal contribution made here is that we can now assign energetic costs to individual cellular processes during normoxia and anoxia, and view changes in the partitioning of these costs across each environmental condition. In turtle hepatocytes, it was found that the maintenance of the membrane potential and protein turnover account for the vast proportion of energy usage in anoxia. Is this observation transferrable to other tissues during metabolic suppression? Almost certainly yes, in the case of tissues that normally possess high metabolically activity such as heart and brain. As established in chapter 1, membrane ion pumping accounts for the vast proportion of energy utilisation in the brain and despite changes in synaptic activity, the large number of ion channels present points to a good likelihood that ion pumping would be the most active component of metabolism in metabolic suppression. Metabolically active tissues, are usually also associated with higher rates of protein turnover where the consequences of accumulations of damaged protein would otherwise be high in normoxia and anoxia.

The control of this energy partitioning is particularly intriguing since in some cases relative increases in the overall proportion of energy utilised occur, during a time when ATP synthesis, together with ATP demand are in a balanced suppression. The coordination of these events remains a mystery, however this thesis also demonstrated that in the case of altering hepatocyte protein profiles, oxygen itself was the key player. That oxygen can directly control numerous physiological and biochemical processes through oxygen sensing mechanisms represents a potentially crucial and unexplored area of future research in facultative anaerobes. Depending on the mechanism, there could exist the faculty to detect changes in environmental oxygen

concentrations, and accordingly respond, well before oxygen becomes truly limiting.

Therefore, the concept of metabolic suppression requires to be refined somewhat to place emphasis on a number of inter-related points: i) Metabolic suppression probably occurs down to a tissue-specific metabolic set-point that is governed by membrane and protein stability, ii) There is a repartitioning of remaining metabolism in metabolic suppression to favour important ATP sinks and iii) In the case of certain active processes in anoxia (demonstrated here as protein expression), oxygen itself is the primary signal for invoking physical changes in cell function.

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