METABOLIC RESPONSES TO EXPERIMENTAL DIVING IN ADULT AND FETAL WEDDELL SEALS

by

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ABSTRACT

Metabolic potentials and activities of the heart, lung and brain were examined in the Antarctic Weddell seal, a species displaying outstanding diving abilities. The activities of representative enzymes in oxidative and fermentative metabolism in both the adult and near-term fetus are similar to those in homologous organs of the adult ox, but the brain and heart in both groups of seals contained elevated levels of lactate dehydrogenase. The isozyme data indicate that all three organs in the adult and fetus have the potential for either lactate production or utilization depending upon metabolic conditions.

During experimental diving (up to 30 min) glucose appears to be a critical carbon and energy source for the adult. Glucose is utilized in a mixed aerobic and anaerobic metabolism. The consequent fall in blood glucose levels and rise in lactate levels are due predominantly to peripheral, hypoperfused tissues, but the central organs influence these metabolite pools as well. The brain of the adult seal utilizes glucose at a rate of approximately 0.3 μmol/gm/hr., releasing 20-25% as lactate; this proportion does not change throughout diving-recovery cycles. The lung consumes lactate and thus diminishes its accumulation in the blood during the dive. One, and possibly the main, fate of lactate absorbed by the lung is oxidation since $^{14}CO_2$ is the only measurable derivative found in aortic blood following ^{14}C-lactate infusion into the right ventricle. During recovery, when blood lactate levels rise above 6 μmol/ml, the brain switches from lactate release to lactate uptake at a rate high enough to readily support the normal metabolic rate of this

organ (about 8 μmol ATP/gm/hr). Enzyme and metabolite measurements suggest that the lung and heart also contribute to lactate clearance and re-establishment of metabolic homeostasis following diving in the adult.

Blood metabolite analyses suggest little involvement of the majority of free amino acids during dive-recovery cycles. Only alanine and glutamine increase throughout both phases and both probably function as waste nitrogen carriers. Alanine accumulation could also be caused by anerobic catabolism of protein and/or anaerobic-aerobic transitions in tissues such as skeletal muscles.

Both the physiological data and metabolite recovery profiles indicate fetal peripheral vasoconstriction and a fully developed diving response as a consequence of maternal diving. The fetus has apparently adapted to long-duration diving by significantly increasing its blood storage capacity of glucose to beyond that of the mother. Furthermore, these elevated stores appear to be under tight regulation during the latter stages of experimental diving.

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CHAPTER I

Introduction

Although life is thought to have evolved from non-living organic matter, in a reducing atmosphere, most organisms now depend upon oxygen (0_2) as the ultimate proton acceptor. However, the degree of dependency differs not only from animal to animal but also between different tissues of the same animal. It has now been firmly established that many invertebrates and lower vertebrates (e.g. turtles) reduce their metabolic rates and/or substitute 0_2 with other favorable proton disposal sinks in an effort to survive periods of low oxygen availability. Facultative anaerobes, such as intertidal bivalves, parasitic helminths and annelids, sustain extremely lengthy periods of anoxia by modifying and integrating glycolysis with other catabolic pathways (e.g. amino acid catabolism), resulting in the accumulation of succinate, alanine, propionate etc. These adaptations allow for substrate-linked ATP production, redox balance and the avoidance of an 0_2 debt upon recovery; all of which provide the animal with the opportunity to invade habitats that would otherwise prove hostile. Hochachka (1980) reviews current thinking in this area.

Mammals, on the other hand, display much higher dependencies on 0_2 and hence must compensate for hypoxia or anoxia by evoking a wide variety of 0_2 conserving mechanisms, if they are to be successful in exploiting such habitats. Marine mammals offer the perfect example of such an adaptation. These animals probably made the transition from a terrestrial to an aquatic existence in an effort to secure an ecological niche safe from the interferences and predation of dry-land competitors. Because of their diving habit they have evolved large blood volumes with very high hematocrits and have increased muscle myoglobin content, all of which

provide a large O_2 reservoir. However vital these may be to the overall survival of the organism they must be of secondary importance when compared to the three components of what is commonly termed the 'diving response'. This consists of apnea (cessation of breathing), bradycardia (slowing of the heart rate) and peripheral vasoconstriction, all of which aid in profoundly lowering the extraction of O_2 from the blood. Bradycardia leads to a drop in cardiac output while peripheral vasoconstriction serves to maintain arterial blood pressure and redistribute blood flow to the most needy tissues which were originally thought to include the heart, lung and brain (Scholander, 1940). It is thought that a large fraction of the peripheral circulation is probably restricted upon initiation of these cardiovascular adjustments (Irving et al., 1942).

Studies utilizing microsphere, angiogram and doppler blood flow transducer techniques indicated a more complex situation. Blood flow determinations in restrained harbor (Phoca vitulina), elephant (Mirounga angustuostris) and Weddell seals (Leptonychotes weddelli) suggested that cerebral blood flow (CBF) remains unchanged or decreases slightly during a dive (Bron et al., 1966; Elsner et al., 1966; Kerem et al., 1971; Van Citters et al., 1965; Zapol et al., 1979). On the other hand, a recent study by Elsner et al. (1978) demonstrated CBF increases during the latter stages of diving in the common harbor seal; substantial CBF increases during simulated diving in the sea lion (Zalophus californianus) and the duck (Anas platyrhynchos) have also been reported (Dormer et al., 1977; Jones et al., 1979). In the pinnipeds sampled the myocardial blood flow was found to be reduced to only 7-15% of the pre-dive level (Blix et al., 1976; Zapol et al., 1979). At present nothing is known about the alterations (if any) in bronchial

flow during diving; however, pulmonary flow must decrease since the cardiac output decreases significantly in the diving phase. In the Weddell seal, at least, the unaltered CBF implies that the brain enjoys a normal delivery of O_2 and carbon substrates. Furthermore, all other tissues of this Antarctic seal experience apparent decreases in absolute flow rates (Zapol et al., 1979) which may be consistent with the suggestion that the metabolic rate of the animal actually drops somewhat during diving (Anderson, 1966). In terms of fractional cardiac output, four organs are identified as receiving an increased relative perfusion: the heart, lung, brain and adrenal glands. Peripheral organs such as the kidney, sustain sharp reductions in absolute perfusion, fractional cardiac output and hence in oxygen and nutrient supply. Consequently, these peripheral tissues must sustain lengthy periods of hypoxia and anoxia. For a thorough review on the metabolic or biochemical adjustments involved, refer to Hochachka and Murphy (1979).

Central organs

Although the physiological purpose and consequences of the diving response have been treated by many others (see above), remarkably little has been done on the metabolic capacities and activities of the central organs. However, there are scattered reports on blood metabolite and physiological parameters in these mammals, which offer some hints on the metabolic status of the brain, heart and lung as a consequence of diving.

Before a dive the O_2 content in the venous circulation of the sea mammal increases, presumably for use during the dive episode. The arterial partial pressure of O_2 (PaO_2) falls to 30 mm Hg or lower throughout prolonged diving (Anderson, 1966; Scholander, 1940; Hochachka et al., 1977a; Kerem and Elsner, 1973). In the Weddell seal PaO_2 asymptotes have been observed to occur in the range of 25-30 mm Hg (Hochachka et al., 1977a). Hochachka's group further reported blood glucose levels did not fall monotonically but rather approached an asymptote in prolonged simulated diving. The early decreases in both glucose and O_2 levels suggest mixed aerobic and anaerobic metabolism while the asymptotic behavior of both in later diving implies the initiation of some type of steady state system, protecting the central organs (in particular the brain) against hypoglycemia and anoxia. The precise mechanisms have yet to be ascertained. Past studies have not only detected the classic washout of lactate from peripheral tissues into the central circulation during the recovery phase (Scholander, 1940) but also demonstrated a 2-4 fold increase in lactic acid in this central circulating blood during the dive (Hochachka et al.,1977a; Davis and Kooyman, 1980). This initial increase in lactate could be due to anaerobic fermentation in one or more of the central organs and/or leakage of the

anaerobic end-product out of tissues (such as muscles) that have been isolated from the main circulation.

Beyond these previously published insights very little is known of the 'in vivo' metabolic activity of the functioning central organs. Many outstanding questions remain to be answered. For example: 1) what fuels were burned by which tissues and at what times during and after diving? 2) Does cerebral metabolic rate decrease during diving episodes? 3) How prevalent is intertissue metabolic cycling? Are there any metabolites cycled, and, if so, which one(s) and between which tissues? At the onset of this research it was realized that complete answers to these questions would be impossible but, nevertheless, a beginning could be made to unravel some of the complex interactions involved.

To put such questions in their proper perspective, it is useful to briefly review what is currently known of the metabolic biochemistry of the three central tissues.

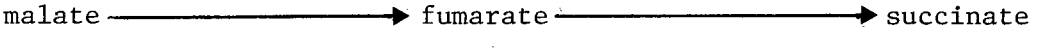
Substrates for the Mammalian Heart

The mammalian heart is considered to be a completely aerobic organ when in resting metabolism, or when only moderately active (Neely and Morgan,1974). This fact is further emphasized by the observation that 40% of the total cellular space is occupied by mitochondria (Fawcett and McNutt, 1969). When there is an ample supply of oxygen, the heart will preferentially oxidize fatty acids, mostly in the form of plasma free fatty acids, but if these are unavailable, it can burn glucose or lactate (Most et al., 1969; Neely et al., 1972). In hypoxic or anoxic hearts the preferred fuel is glucose. Such a situation results in a ten- to twenty-fold increase

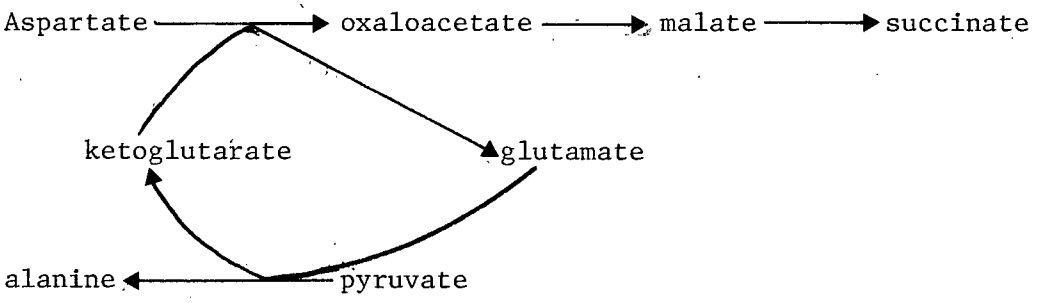
in glycolytic flux (Neely and Morgan, 1974).

In the well oxygenated fat-burning heart glycolysis is inhibited at four loci. Firstly, there appears to be an inhibition of glucose transport by free fatty acids. Secondly, the combined effects of high levels of ATP, G6P and decreased levels of 5'-AMP and inorganic phosphate inhibit glycogen phosphorylase and hexokinase. Thirdly, increased concentrations of citrate and ATP and decreased concentrations of fructose biphosphate, NH_4^+ and AMP during fatty acid oxidation essentially turn off glycolysis by inhibiting phosphofructokinase. This effectively directs exogenous glucose into glycogen. And finally, high levels of acetylCoA during fatty acid oxidation inhibit the pyruvate dehydrogenase enzyme complex. Fat oxidation, also, appears to convert this enzyme into its inactive enzymatic form (Neely and Morgan, 1974).

During periods of hypoxia and anoxia cardiac glycogenolysis and glycolysis are activated by a reversal of the above effects while fatty acid oxidation is retarded. Apparently, hypoxic and anoxic stresses in the mammalian heart are accompanied by a partial reversal of the Krebs cycle with a resulting accumulation of succinate and alanine (Penney and Cascarano, 1970; Sanborn et al., 1979):

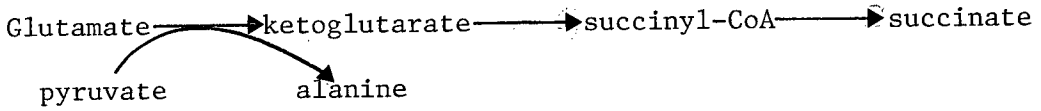


The malate may arise from aspartate through a transamination:



a process that could partially explain the accumulation of alanine that sometimes occurs during hypoxic or anoxic stress (Hochachka et al., 1975; Felig and Wahren, 1971). Collicutt and Hochachka (1975) have shown that this effective "fermentation" of aspartate to succinate is a very active process in bivalve hearts during anoxia, and there is also some evidence for its contribution to metabolism during the hypoxia associated with diving in marine mammals (Hochachka et al., 1975). Sanborneand associates (1979) using ^{14}C -aspartate verified this pathway to be operable in anoxic rabbit heart.

Succinate can, of course, also be formed from intermediates in the first span of the Krebs cycle. Indeed from recent studies it is known that acetyl-CoA is quickly depleted in hypoxia, as are citrate and ketoglutarate (Neely et al., 1976). In the anoxic bivalve heart (Collicutt, 1975) and rabbit papillary muscle (Sanborn et al., 1979), the ^{14}C glutamate that is taken up is converted to succinate. This probably occurs via the glutamate-alanine transamination:

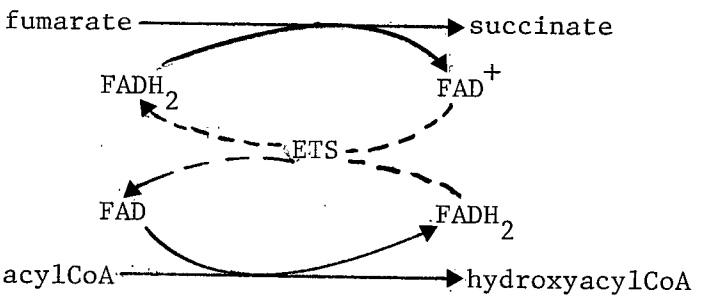


(Hochachka et al., 1975; Collicutt and Hochachka, 1977, Taegtmeyer, 1978; Sanborn et al., 1979).

From these different studies, it is tentatively concluded that when O_2 supply is limited succinate acts as a sink for carbon flowing from both arms of the Krebs cycle. Each span operates until intermediates (and O_2) are largely depleted. It is now assumed that the second span is reversed to allow for redox balance and substrate phosphorylation. In theory, both malate dehydrogenase, functioning in the oxaloacetate ⟶ malate direction, and fumarate reductase, functioning in the fumarate ⟶ succinate

direction, contribute to redox balance. Furthermore, fumarate reduction is thought to be coupled to a phosphorylation, one mole of ATP being formed/mole of succinate accumulated and under hypoxic conditions when energy production may be at a premium, this small rate of mitochondrial ATP production while probably insignificant in overall energy budget may be locally most useful. Cascarano and his coworkers (1976) stated that it is fundamental to sustaining Ca^{++} metabolism of the heart during anoxic stress.

It has been suggested (Hochachka and Murphy, 1979) that $FADH_2$-linked fumarate reductase could possibly form a redox couple with acylCoA dehydrogenase (fatty acid oxidation enzyme), linked by the electron transport system (ETS):



If this postulated couple is operational during hypoxia and anoxia it would imply that the FAD^+-linked dehydrogenase in the β-oxidation of fatty acids is not a significant control point during the aerobic-anaerobic transition. The precise regulatory locus has yet to be ascertained but it is believed that the first step to be "turned off" in β-oxidation when O_2 is limiting would be the NAD^+-linked dehydrogenase (see Hochachka et al., 1977b).

Heart Metabolic Biochemistry in Diving Animals

Unlike the well developed field briefly summarized above, there are only scattered reports in the literature on the metabolic biochemistry of

the heart in diving animals. Electrophoretic work and pyruvate saturation kinetics of lactate dehydrogenase (LDH) from the hearts of common seals (Phoca vitulina vitulina L.), beaver (Castor fiber), pond turtle (Pseudemys scripta elegans), eider (Somateria mollissima), the hooded seal (Cystophora cristata) and several different species of whales have been reported (Blix et al., 1970; Messelt and Blix, 1976; Altman and Robin, 1969; Blix and From, 1971; Shoubridge et al., 1976).

All studies have demonstrated that the isozyme distribution and/or the pyruvate saturation kinetics in the hearts of divers approach the muscle type enzyme (LDH-5), implying a greater potential for anaerobic glycolysis (Holbrook et al., 1975). The hearts of non-diving mammals are known to contain predominantly heart type LDH subunits (Appella and Markert, 1961). Simon et al. (1974) showed that there was increasing pyruvate kinase activity in both the hearts and brains of three different seals with increasing diving time. This, they suggested, implies enhanced glycolytic potential of "aerobic tissues" and may be important in extending diving time. Kerem et al. (1973) found that glycogen concentrations in the cardiac muscle of the Weddell seal are 2-3 times higher than in the hearts of terrestrial mammals, again suggesting an enhanced anaerobic capacity. However, these studies merely put the heart of diving animals into perspective and supply little information on its metabolic status through diving-recovery cycles.

Cerebral Metabolic Rate

Although the mammalian brain is a relatively small organ, its basal oxidative metabolic rate ($CMRO_2$) is high and can account for as much as 20% of the basal metabolic rate of the organism. The human brain utilizes O_2 at an average rate of about 1.5 μmol/gm/min at 37°C, with the more active cerebral cortex metabolizing at a substantially higher rate. Cerebral

metabolic rate, also seems to be influenced by the size of the organism, being high in small animals. The occurrence of so-called "vulnerable areas" in the brain plus this high oxidative capacity has encouraged the view that the brain is one of the most O_2-dependent organs in the mammalian body (see Siesjo et al., 1976, 1977 for reviews in this area).

Substrates for the Mammalian Brain

The unusual oxidative metabolism of the brain is fired predominantly by glucose upon which the brain by and large is assumed to have an absolute dependence. The rate of cerebral glucose consumption normally is regulated by the rate of conversion of fructose-6-phosphate to fructose-1,6-biphosphate by the phosphofructokinase reaction and not by the delivery rate at cerebral capillaries. The rate of consumption can be accelerated several-fold when demand increases (e.g. in hypoxia). In these cases deinhibition of phosphofructokinase reduces the concentration of both fructose-6-phosphate and its metabolic precursor glucose-6-phosphate. Thus, the net effect is to increase the rate of glucose phosphorylation by the hexokinase reaction (see Rappaport, 1976). Recent studies show that the rate of glucose uptake by different regions of the brain closely parallels neural activity (Sokoloff, 1979; Pulsinelli and Duffy, 1979).

No substrate can replace glucose in sustaining brain function indefinitely. However, a number of other substrates can be utilized by the brain, and indeed some of these are also absolutely essential for certain metabolic processes. Of these, ketone bodies and amino acids are the most important, while lactate and pyruvate may take on a significance when blood concentrations are high (Rowe et al., 1959; Nemoto et al., 1974).

The ketone bodies, β-hydroxybutyrate and acetoacetate, can partially support brain function when carbohydrate is in short supply or cannot be utilized. These substrates are generated by the liver and kidney in starvation and diabetic ketoacidosis, and occur in high plasma concentrations in newborn suckling rats. They enter the brain by simple passive diffusion.

At very high blood levels, brain uptake of ketone bodies is limited by metabolic incorporation, which, in turn, depends on intracerebral concentrations of the enzymes involved in acetoacetate-hydroxybutyrate metabolism (Sokoloff, 1973). Although the concentrations of these enzymes, as well as rates of ketone body consumption, are higher in brains of neonates than of adults, the significance of this observation has not been clarified.

Of the amino acids, glutamate, glutamine, aspartate, N-acetylaspartate, and GABA (α aminobutyric acid) are the predominant amino acids of the mature brain and constitute approximately two-thirds of free α-amino nitrogen. The high concentrations and extensive metabolism of glutamate and its derivatives are key hallmarks of brain metabolism. The product of the α-decarboxylation of glutamic acid is GABA, which has been reported to be a major inhibitory transmitter in the vertebrate central nervous system (Rappaport, 1976).

The large pool of free glutamate in the brain is in equilibrium with the α-ketoglutarate of the Krebs cycle, and aspartate is in equilibrium with oxaloacetate. After injection of labeled glucose, 70% of the isotope present in the soluble fraction of brain is present in amino acids, primarily glutamate, glutamine, aspartate, and GABA (Guroff, 1972).

Under normal circumstances, lactate and pyruvate are released from the brain into the circulation at rates that account for about 10% of the glucose being utilized. However, under exceptional conditions, when blood

levels are high (for example, following trauma or ischemia), lactate and pyruvate can be utilized by the brain (Siesjo et al., 1976). Lactate and pyruvate as well as other short-chain monocarboxylates (such as acetate, proprionate, and butyrate) cross cerebral capillaries by a common facilitated mechanism (Oldendorf, 1973). The lactate transport capacity, however, is low and apparently saturates at 3-4 times the normal plasma concentration of lactate; that presumably is why lactate uptake becomes significant only at high blood lactate levels.

Circulatory and Metabolic Consequences of Brain Hypoxia

According to Siesjo (1977) brain hypoxia can be defined as a decrease in O_2 availability of such magnitude that there are measurable changes in metabolism or function of the brain. Since hypoxia may occur in prolonged diving (Anderson, 1966; Scholander, 1962 and Ridgeway et al., 1969) it is important to emphasize that hypoxia differs from anoxia (or complete ischemia) in two ways. Firstly, since tissue PO_2 is not reduced to zero, oxidative metabolism continues even if at a reduced rate. And secondly, since cerebral blood flow (CBF) is maintained or increased, there is a continuous supply of glucose for continued anaerobic metabolism.

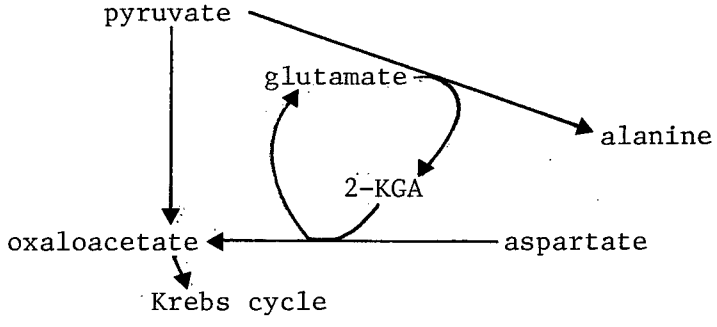
It is now well established that hypoxia in mammals leads to a compensatory increase in CBF while hyperoxia leads to a compensatory decrease (Siesjo et al., 1976; Siesjo and Nordstrom, 1977). Interestingly, variations in PaO_2 from as high as 2100 mm to as low as 20 mm Hg with PvO_2 varying between 57 mm and about 15 mm Hg, do not lead to any changes in $CMRO_2$ or the energy states as assessed by adenylate levels. As Siesjo and Nordstrom (1977) argue, this suggests the cytochrome oxidase reaction runs

at a constant rate despite rather pronounced variations in tissue O_2 levels. Mechanisms underlying this obvious close control of O_2 utilization in the face of wide changes in tissue O_2 availability are not fully clarified.

Another matter that is unclear is why brain perfusion varies over PO_2 ranges that do not cause changes in $CMRO_2$. The earliest detectable metabolic changes in hypoxia are increases in lactate and pyruvate levels and a change in the $NADH/NAD^+$ ratio which is expressed by changes in the lactate/pyruvate ratio. These changes occur at a PaO_2 of about 50 mm Hg, when $CMRO_2$ is still unchanged (see Siesjo and Nordstrom, 1977). At face value, the data imply that both glycolytic and aerobic production of ATP are increased with progressive hypoxia, and that the total energy produced in terms of μmol/ATP/gm/min must presumably rise. Why this should be so is not at all understood.

If hypoxia is prolonged a number of metabolic consequences are now well outlined. Thus, lactate accumulation and glucose utilization both remain elevated. Pyruvate elevation is also sustained and is thought to activate pyruvate carboxylase catalyzed conversion to oxaloacetate (Mahan et al., 1975) and alanine aminotransferase catalyzed conversion to alanine (Siesjo and Nordstrom, 1977).

During maintained hypoxia, there also occurs a redistribution of carbon in the Krebs cycle pool as well as a general increase in size of the pool (Siesjo and Nordstrom, 1977) that is in part initiated by pyruvate and in part by aspartate:



These processes explain rising alanine, but dropping aspartate levels coincident with the increase in Krebs cycle pool size observed at this time. Overall, these adjustments are similar to those to be anticipated when the Krebs cycle is activated (Safer and Williamson, 1973), but they occur in the hypoxic brain presumably due to a gradual decrease in respiration rates and the increasingly reduced state of mitochondrial metabolism.

Although it has been clearly established that neurological changes occur in hypoxia, mechanisms underlying the derangement are still speculative. The classical "vulnerable regions" include small neurons in the neocortex and hippocampus, and Purkinje cells in the cerebellum (Siesjo and Nordstrom, 1977). Recent rather provocative studies imply that incomplete ischemia is more damaging to the brain than complete ischemia (Nordstrom et al., 1976), perhaps because the latter condition prevents 0_2 dependent autolytic processes that may damage cell membranes.

If the mammalian brain experiences total anoxia (e.g. complete ischemia), aerobic energy production drops to zero almost immediately, in about 4 sec. in man and in about 1-2 sec. in the rat cerebral cortex (Siesjo and Nordstrom, 1977). Concomitant with these events, phosphofructokinase is activated, probably due to a drop in ATP and creatine phosphate levels and a rise in AMP, ADP and Pi levels. This leads to a maximum stimulation of glycolysis which ultimately results in complete depletion of glycogen and glucose and the accumulation of lactate, alanine and succinate (Siesjo and Nordstrom, 1977).

During complete ischemia as in hypoxia (see above), the pool of Krebs cycle intermediates increases substantially and there again occurs a significant redistribution of carbon between the various Krebs cycle

components. Key among these are a depletion of 2-ketoglutarate (2-KGA) and oxaloacetate (OXA) and a resulting increase in succinate. The two decreases observed are probably due to increases in the concentrations of NADH, H^+ and NH_3:

$$2\ KGA + NADH + H^+ + NH_3 \longrightarrow glutamate + NAD^+$$

$$OXA + NADH + H^+ \longrightarrow malate + NAD^+$$

Succinate may be viewed as an end product of anaerobic metabolism. The initial increases in most of the Krebs cycle pool are probably the result of anaplerotic reactions such as CO_2 fixation at the stage of phosphoenol-pyruvate or pyruvate and a shift in the alanine aminotransferase reaction. Significant portions of these carbon chains are thought to be tapped off via the glutamate dehydrogenase reaction.

Brain Metabolism in Diving Animals

With respect to diving animals, most of the biochemical work reported on cardiac tissue was also performed on the brain with basically the same overall results, all pointing to an increased capacity to sustain periods of hypoxia or anoxia. Physiological studies (Kerem et al., 1973; Elsner et al., 1970b; Kerem et al., 1971) also have prompted most researchers to infer improved hypoxia tolerance. However, convincing data showing a reliance of brain on anaerobic metabolism during diving are hard to come by; in fact, much of the available information implies a relatively 'normal' aerobic brain metabolism. According to Bryan and Jones (1980), for example, the $NADH/NAD^+$ ratio in the cerebral hemispheres increases by nearly 40% after 1 minute of apneic asphyxia in the fowl while it only increases by 15% in the duck after 2 minutes of asphyxia. However, at a given level of brain

tissue PO_2, both species show the same relative increase in the $NADH/NAD^+$ ratio, implying that both species are equally dependent on an adequate PO_2 for the maintenance of oxidative metabolism in the brain. Moreover, both species show an isoelectric EEG when the $NADH/NAD^+$ ratio increases to within 30-40% of maximum (that occurring at death). Finally, prevention of bradycardia in ducks during apneic asphyxia by atropine causes the $NADH/NAD^+$ ratio to increase at the same rate as in non-atropinized fowl. The authors, therefore, conclude that O_2 conserving cardiovascular adjustments are responsible for the increased cerebral tolerance to apneic asphyxia in ducks, with no specific biochemical mechanisms being involved. However, the non-invasive fluorometric technique used bears information only upon the redox state of the mitochondria and yields no insight into cytoplasmic (i.e. glycolytic) events. Along with a large increase in cerebral blood flow, there automatically occurs a greatly increased delivery of glucose to cerebral energy metabolism in the duck during diving (or during apneic asphyxia). If aerobic glucose metabolism is unchanged, what happens to the excess supply of this carbon and energy source remains to be clarified. Is it fermented? Is it 'dumped' into the free amino acid pool? Or does it have some other fate? Only further work will ascertain the answers.

Other indicators of the metabolic status of the brain of divers are helpful but, thus far at least, also equivocal. Blix (1971), for example found concentrations of creatine phosphate to be similar in seals and sheep. LDH isozyme patterns in at least 2 cetaceans (Vogl, pers. comm.) imply that both heart and muscle type subunits are involved in generating LDH holo-enzymes; however, heart type subunits definitely predominate and overall

LDH isozyme patterns are therefore not strikingly different from those observed in terrestrial mammals. In this connection, Shoubridge et al. (1976) found a rough correlation between the fraction of muscle type subunits in brain LDHs and depth (thus duration?) of diving in large whales, a correlation that is almost implicitly assumed to occur by most workers in the field.

All the above kinds of data, of course, are circumstantial. Rigorous demonstration of the metabolic status of the brain in diving mammals must come either from direct tissue sampling (thus far unavailable) or from AV gradients across the brain.

Substrates for the Mammalian Lung

Eight decades ago it was suggested that the lung may play useful metabolic roles (Bøhr and Henriques, 1897), yet the mapping of metabolic organization in the mammalian lung has only recently been initiated, and surprisingly little is known compared to other tissues such as the brain or heart. This situation stems not only from a general lack of interest by biochemists but also because of the many difficulties of working with the lung both in vitro and in vivo. Despite this, some general characteristics of the lung's substrate preferences have been documented and deserve a brief description.

Although the rates of 0_2 consumption and energy utilization are relatively low compared to the rest of the body (Weber and Visscher, 1969), the many different cell types of the lung parenchyma (including the alveolar-capillary interfaces) are considered to be very metabolically active (Terney, 1974a). This apparent paradox arises from the high percentage of inert structural lung tissues. The intense metabolism of the

parenchyma was initially considered to be largely fueled by glucose and lipids (Felts, 1964; O'Neil and Tierney, 1974; Rhoades, 1974); however, within the last few years some compelling evidence in support of lactate as a preferential pulmonary substrate has been presented (Wolfe et al., 1979; Rhoades et al., 1979; Hochachka et al., 1977a; Wallace et al., 1974).

Free fatty acids (e.g. palmitate) are considered to be oxidized quite readily by lung tissue (Salisbury-Murphy et al., 1966; Wang and Meng, 1972; Wolfe et al., 1970; Rhoades, 1974). In vivo the fatty acids utilized by the lung may be derived from nonesterified free fatty acids as well as plasma lipoproteins (Felts, 1964). However important these substrates may be to pulmonary energy production their main function probably lies in the production of surfactants, which are primarily composed of phospholipid. Surfactants which line the alveolar surface are responsible for the unusually great surface activity present in mammalian lungs. Because these important compounds have very short half-lives the pulmonary parenchyma must be continuously involved in their biosynthesis. The type II alveolar cells which have been identified as among the titans of lipid metabolism (Tierney, 1974a) are now believed to be the main sites of surfactant production and secretion (Klaus et al., 1962, Tierney, 1974a).

Although lung function is apparently not totally dependent upon glucose, the substrate probably plays several vital roles.

1. NADPH Generation. NADPH is required not only for pulmonary synthesis of lipid (surfactant) and protein (Tierney, 1974a) but also for the maintenance of reduced glutathione, which may serve to protect against damage to the lungs by oxidants (Tierney et al., 1973). Most of the pulmonary NADPH is derived from a very active pentose phosphate cycle, which relies upon

glucose as its ultimate substrate (Bassett and Fisher, 1976b; Tierney et al., 1973); the 'malic' enzyme is an unlikely source since its activity levels are so low in lung tissue (Scholz and Rhoades, 1971).

2. Biosynthesis of Lipid and Glycoprotein. Two- and three-carbon products (acetylCoA and α-glycerophosphate) derived from glucose provide intermediates for lipid metabolism (Bassett et al., 1974; Godinez and Longmore, 1973). The α-glycerophosphate (αGP) which is produced from dihydroxyacetone phosphate by the catalysis of a very active αGP dehydrogenase (Lee and Lardy, 1965), may be a determinant of the rate of lung lipid synthesis and in particular dipalmitoyl lecithin, a major component of the alveolar surface-active lining layer (Felts, 1964). It has also been reported (Yeager and Massaro, 1972) that glucose carbon atoms appear in pulmonary glycoproteins.

3. Energy Production. Glucose probably fuels most of the pulmonary cell types to varying degrees but is perhaps the major substrate for some of the more anaerobic cells such as type I alveolar epithelial cells (Tierney, 1974a). However, Tierney (1974b) suggested that the isolated rat lung can survive for up to 2 hours without any exogenous glucose, implying utilization of other substrates such as lipids or amino acids. Parenthetically, a recent study by Scholz and Evans (1977) demonstrated the ability of the rat lung to incorporate various amino acids into CO_2 and lipids. Yeager and Massaro (1972) determined glucose utilization by rabbit lung slices and found it to be remarkably high (1.83 mg/g lung/hr). Interestingly, only 19% of ^{14}C in the glucose that was consumed appeared in $^{14}CO_2$, whereas 30% appeared in lactate, a value similar to that observed in perfused rat lung (Rhoades, 1974). The elevated lactate production has also been observed in lung slices which were incubated under high O_2 tensions and lactate levels (Tierney,

1971; Rhoades et al., 1978). Presumably, a major fraction of the labelled glucose carbons were channeled into the pentose phosphate shunt (Bassett and Fisher, 1976a) and into anabolic pathways (Tierney, 1974a).

It now appears that the lung not only produces lactate but also employs the metabolite as a potentially important substrate (Searle et al., 1974; Wallace et al., 1974; Wolfe et al., 1979; Rhoades et al., 1978; Hochachka et al., 1977a). Wolfe and associates (1979), utilizing perfused rat lung, have proven lactate to be a good energy substrate which can actually inhibit glucose oxidation by 50% at concentrations as low as 1 mM. A similar phenomenon of preferential pulmonary oxidation of lactate was confirmed by Rhoades et al. (1979). Furthermore, this group demonstrated the incorporation of a sizable portion of the lactate carbons into lung lipids. Quite surprisingly, their data showed that lactate concentrations of 5 mM in the perfusion medium had no significant effect on the lactate production even though glucose utilization was inhibited. They suggested non-carbohydrate sources (e.g. alanine) fueling the lactate production under their conditions.

Although lactate production by the lung was apparently unaltered by the availability and uptake of lactate, it has been shown that this organ exhibits a Pasteur effect (Bassett and Fisher, 1976b). However, glycolytic compensation in the absence of oxidative metabolism was not considered to be sufficient to maintain ATP content or its supply for synthetic activity (Bassett and Fisher, 1976b). On the other hand, Fisher and associates (1972) have demonstrated that the intact organ remained capable of maintaining functional and structural homeostasis during rather prolonged periods of hypoxic stress (Fisher et al., 1972). Longmore and Mourning (1976) report during hypoxia the rate of lactate production doubles

but only about 50-60% is apparently derived from glucose. They postulated amino acid catabolism to be the most likely source of the excess lactate production. Unfortunately, this postulate has yet to be verified and must await future experimentation.

For an organism to withstand hypoxia it is advantageous not only to develop efficient anaerobic machinery and redox balancing mechanisms but also to develop inter-tissue cooperation. For example, certain end products of anaerobic metabolism from one tissue could be shipped to some less hypoxic tissue for conversion (i.e. reduction) to intermediates which would be utilized by the more hypoxic tissue. The lung, of course, is a prime candidate for a role as an acceptor organ for during hypoxic or even anoxic stress it probably remains in a somewhat more aerobic state. Such a realization probably led Cascarano and associates (1976) to postulate that the lungs of rats, subject to low tensions of oxygen, will actually take up the anaerobic end product, succinate, produced by other tissues, and release fumarate and malate for further use in energy metabolism by the hypoxic heart. This process, they suggested, sets up an electron shuttle between the heart and the lungs. Such a shuttling process would be of great advantage to organisms which are routinely exposed to hypoxic or anoxic stress (e.g. the marine mammals).

Lung Metabolism in Diving Mammals

Before the 1977 study of Hochachka and associates, pulmonary metabolism in the marine mammal had been largely ignored, but this report has provided a very provocative interpretation of the suspected role of the seal lung during restrained diving episodes. In working with adult Weddell seals, these investigators focused attention on the impressive ability of this

animal's lung to absorb significant quantities of lactate during the dive phase when the metabolite's concentration in central whole blood is 3.0 mM or less. Depending on the animal and the submersion time, between 0.1 and 0.25 μmoles of lactate/ml of blood can be taken up by the lung. The authors also reported low but detectable releases of glucose during this period. They thus, extrapolated that the seal's lung conditions central circulating blood by reducing lactate accumulation through the preferential conversion of lactate to glucose and by the actual release of glucose into the main bloodstream. However, the ultimate fate of absorbed lactate was never demonstrated in this study.

Heart-Lung-Brain Metabolism in the Weddell Seal

In Scholander's original formulation, the diving reflexes were seen to play a primary role in the conservation of O_2 for the most O_2-dependent tissues. Of these, the heart, lung and brain were considered the most important. However, in most physiological studies, these have been considered separately, if at all (e.g. Kerem and Elsner, 1973) while in metabolic studies they have usually been lumped together as a "black box" (Scholander, 1962; Hochachka and Murphy, 1979). The only concerted attempt to integrate the metabolism of the different central organs has been generated by Hochachka and associates (1977a), a study discussed in the preceding section of this chapter. This general lack of insight into 'in vivo' metabolic activities of the functioning central organs is the direct result of the many difficulties associated with studying diving mammals. Therefore, advantage was taken of the opportunity to join an interdisplinary group studying the physiological and metabolic consequences of diving in

the Antarctic Weddell seal. This aquatic mammal offered many useful advantages (e.g. easy capture and maintenance along with many previous field and laboratory studies by Kooyman and Elsner). However, the seal's phenomenal diving capacities provided the most attractive quality. Recent field studies by Kooyman et al. (1980) now indicate that the adult Weddell seal is capable of breath-hold dives for up to 70 minutes. If any metabolic or biochemical modifications were associated with the heart-lung-brain machine during diving they would certainly be accentuated in this pinniped.

For this study of the Weddell seal, a variety of techniques were used to approach the problem at various levels of organization. These included: (1) enzyme profiles of all three organs, (2) tissue slice studies using C^{14} metabolites, (3) arteriovenous concentration differences, (4) in vivo metabolite infusions using C^{14} labelled and unlabelled metabolites, and finally (5) transient changes of blood metabolite levels prior to, during, and following diving. Furthermore, in closely collaborative studies, organ perfusion, heart rate, blood pressure, cardiac output, blood gases, and blood pH were simultaneously monitored (Zapol et al., 1979; Liggins et al., 1980). Thus physiological parameters could be used to assist in interpreting biochemical data and vice versa. These physiological parameters will be presented throughout this thesis; however, for thorough discussions of these phases of the study refer to Zapol et al. (1979); Liggins et al. (1980); Qvist et al., (1980).

Fetal Metabolism

The mammalian fetus utilizes the placenta as an avenue through which it receives all substrate and O_2 requirements from the mother. Maternal-

derived O_2 is also considered to be of prime importance for it fires a very active fetal catabolism. In all species thus far studied, fetal oxygen consumption ranges from 6 to 9 ml STP. min^{-1} kg^{-1}, indicating a constancy across mammalian species (Battaglia and Meschia, 1976). Therefore, in an animal like the Weddell seal fetal tissues would be a site of comparatively high O_2 utilization within the maternal organism, due to the normal scaling of O_2 consumption to body size (Coulson et al., 1977). Under normal conditions, it is now believed that anaerobic metabolism plays a very minor role in the generation of energy. Nevertheless it is traditionally considered that the mammalian fetus displays a higher tolerance to hypoxemia than that of the mother (Dawes, 1968).

In the ovine fetus the principal exogenous oxidative fuels are glucose, lactate and amino acids. In most mammals thus far studied, the glucose uptake from the placenta is inadequate to meet the caloric requirements of the fetus (Battaglia and Meschia, 1976). Under normal conditions glucose uptake is capable of sustaining 50-70% of fetal oxidative metabolism. Unfortunately, the rate of fetal gluconeogenesis (if any) and its regulation has yet to be ascertained. Lactate uptake by the ovine fetus can account for approximately 25% of the total fetal O_2 consumption if the substrate is completely catabolized. The amino acids delivered to the umbilical circulation are utilized by the fetus to build new tissues and, in part, as fuels of oxidative metabolism. At present, no quantative determinations of the individual amino acid's contribution to catabolism are available; however, it is known that their relative contribution to energy production

increases during maternal hypoglycemia (Battaglia and Meschia, 1976). Although most fetuses are capable of free fatty acid (FFA) and ketoacid oxidation, it is not clear if these substrates are important sources of energy in those species whose placenta is permeable to them.

Heart Metabolism

Unlike the adult heart, the fetal myocardium relies very little on free fatty acids but instead consumes predominantly glucose under aerobic conditions (Breuer et al., 1967; Battaglia and Meschia, 1976). The degree to which this holds true depends upon the species and the stage of gestation (Beatty et al., 1972; Clark, 1971). This peculiarity of the fetal heart is considered to be due to low carnitine concentrations and low enzyme activities in the β-oxidation spiral of the fetal myocardium (Wittels and Bressler, 1965). Furthermore, fetal and neonatal hearts are considered to be relatively resistant to hypoxia, probably a result of increased concentrations of myocardial glycogen (Dawes, 1968; Hoerter, 1976). No relevant enzyme data are yet available.

Brain Metabolism

Glucose is considered the major fuel of, at least, the ovine fetal brain; a situation akin to that of the adult. Recent information suggests that the high rates of cerebral oxygen and glucose utilization are a general phenomenon, irrespective of size and stage of development (Battaglia and Meschia, 1976). However, there are varying reports regarding the contribution of anaerobic metabolism in the normal fetal brain (Benjamins and McKhann, 1972; Jones et al., 1975). Regarding alternative energy

sources, it has been suggested, but not demonstrated, that ketone bodies play an important role in fetal cerebral metabolism of some species, replacing glucose when the carbohydrate supply is at a premium (Battaglia and Meschia, 1976).

Fetal Metabolism in the Diving Mammal

Prior to these current studies there had been only two physiologically orientated reports pertaining to the fetus of the aquatic mammal, cited in the literature. Neither dealt directly with any aspect of the metabolic organization of the fetus. One decade ago, Lenfant's group (1969) studied the respiratory properties of blood in both the fetal and maternal blood of the Weddell seal. The pregnant seal was found to have higher hemoglobin concentration, hence higher oxygen capacity, than its near-term fetus. Qvist et al. (1980), from our group, have verified this phenomenon. This condition is noteworthy for it is the reverse of that in most mammals (Dawes, 1968). Furthermore, the O_2 affinity of the fetal seal blood is higher than the maternal blood, a situation common to most mammals. Therefore the fetal seal is capable of extracting more O_2 from the maternal blood than its terrestrial counterpart at low PaO_2 and thus would reduce the requirement for uterine blood flow during submergence. Elsner and associates (1970) followed fetal heart rate and uterine blood flow in one asphyxiated gravid Weddell seal, paralyzed by doses of succinyl choline (Elsner et al., 1970). The authors reported a slow but gradual decline in fetal heart rate (66 beats/min to 20 beats/min after 29 minutes) followed by a slow recovery upon resumption of maternal ventilation. Utilizing Doppler ultrasonic blood flow transducers this group also observed an uninterrupted uterine blood

flow throughout the entire episode. They concluded that the continuation of blood flow to the fetus during asphyxia served as a defense for the fetus by continuing its oxygen supply.

Strategy of Study

Given the opportunity to study intact Weddell seals, it was reckoned that a good attempt could be made at unravelling some of the numerous questions relating to the metabolic functionings and cooperation between at least the three central organs during dive-recovery cycles. To recap the basic questions addressed in this thesis are as follows: 1) what fuels were burned by each of the three central organs during rest, dive and recovery phases? Are the preferences during rest periods similar to those of homologous tissues in comparatively sized terrestrial mammals or have the organs been adapted for the diving habit? 2) Does the brain experience any increases in anaerobic glycolysis during the dive episode? 3) Are there changes in cerebral metabolic rate in response to diving? 4) Finally, are there any inter-tissue cycling of metabolites that may be operational during dive-recovery cycles? One of the most likely candidate is considered to be lactate which is the main anaerobic end product of the more hypoxic tissues.

Since forced diving appears to elicit a maximum diving response (i.e. profound bradycardia and intense peripheral vasoconstriction) akin to that of long duration diving, it was hoped that the chosen experimental techniques would shed some light on the metabolic functionings of the central organs during a time when the animal is preparing for the distinct possibility of extreme taxes on its oxygen and substrate supplies. The techniques included: 1) blood metabolite (glucose, lactate, pyruvate and

amino acids) sampling prior to, during and following diving; 2) enzymatic profiles of all three organs; 3) A-V samplings across the brain and lung; 4) in vivo metabolite infusion using C^{14} labelled and unlabelled lactate and 5) finally tissue slice studies utilizing C^{14} labelled metabolites (glucose and lactate).

Although the fetal questions were peripheral to the main problems addressed here, it was initially hoped that fetal arterial concentration profiles of lactate in conjunction with the physiological data could indicate whether or not the fetus elicits its own diving response, as a consequence of maternal diving. In the process, other fetal blood metabolite levels could be plotted as functions of rest and dive-recovery cycles and thus compared with the maternal profiles. Furthermore, enzymatic profiles of the fetal heart, lung and brain could be compared to the respective maternal organs. Technical problems precluded any planned measurements of arteriovenous concentration differences across the three organs in question while time limitations prevented the thorough study of any of the peripheral tissues such as skeletal muscle.

CHAPTER II

Materials and Methods

Experimental Animals and In Vivo Manipulations

Gravid and non-gravid adult Weddell seals (Leptonychotes weddelli), weighing 350-500 kg, were captured near Turtle Rock on the Ross Island Antarctic fast ice and transported by sled to the Eklund Biological Laboratory at McMurdo Station. The seals were anesthetized with intramuscular ketamine hydrochloride (2 mg/kg). Surgery was performed in a lateral position on a mobile operating table. During operations, anesthesia was maintained by spontaneous breathing of 1-4% halothane in O_2 through a to-and-fro CO_2 absorption cannister. Diving experiments were not initiated until 8-12 hours after surgery when control heart rate, PaO_2, $PaCO_2$, and pH_a had returned to resting values.

For diving experiments, the adult seal was restrained on a mobile table with the webbing of a cargo net. The mobile table was supported on two tracks, and the seal's head inserted through a sponge rubber gasket into a reinforced wood and plastic tank fixed to the tracks. Ambient temperature in the laboratory was maintained at $4^{\circ}C$, and the seal's flippers and dorsum were frequently bathed with iced seawater. To simulate diving by head submersion, the table was tilted 20° head down and the tank was filled rapidly with iced seawater.

For monitoring metabolite profiles in arterial blood of the adult seals, 2 ml samples were drawn from a PE 190 catheter positioned in the thoracic aorta via a flipper artery. Fetal blood sampling involved a midline laparotomy incision approximately 25 cm in length) of the mother, followed by a uterine incision (7.5 cm). The most accessible flipper was drawn out until the proximal part of the flipper was firmly wedged in the uterine

wound. The fetal membrane could then be opened without spillage of amniotic fluid. The fetal thoracic aorta was catheterized via the left radial artery. Samples were taken at various times prior to diving, during simulated diving, and during recovery for up to several hours. All blood samples were added directly to 2 ml of chilled 1.4 M perchloric acid and stirred vigorously. The PCA extracts were then centrifuged and the supernatant solutions were neutralized with 1.4 M KOH or K_2CO_3. Following another centrifugation to remove the precipitated perchlorate salt, the extracts either were immediately analyzed for unstable metabolites (e.g. pyruvate) or were stored at $-80^{o}C$ in a Revco freezer until required.

To assess the capacity of the adult lung to metabolize lactate, a 150 cm long, 8 French diameter, balloon flotation Swan Ganz thermodilution catheter was surgically introduced into an exposed internal jugular vein at the thoracic inlet and advanced into the pulmonary artery with pressure monitoring. The proximal port of the Swan Ganz catheter in the right ventricle was used as an injection site, while the distal port was used for collecting blood samples from the pulmonary artery. The lactate injectate or bolus contained ^{14}C-U-lactate (4 μC/l blood), 2 gm carrier lactate to bring concentrations up to about 3-4 mM, and 200 mg Evans blue dye; the injectate was made up in a 15 ml total volume of normal saline (9 g/l) at pH 7.4. At 20 second intervals following rapid (5 sec) manual injection of the lactate bolus into the right ventricle, 5 ml blood samples were drawn simultaneously from the sampling port in the pulmonary circulation and from the thoracic aorta. The blood samples taken from the pulmonary artery and aorta were added directly to equal volumes of 1.4 M PCA in stoppered tubes containing hyamine hydroxide CO_2 traps. Thus, $^{14}CO_2$ formed from ^{14}C-lactate

during a single circulation through the lung could be readily detected. On removal of the CO_2 traps, these blood samples were treated as before, and assayed for dye content, lactate concentration, and ^{14}C-lactate radioactivity. The marker dye concentration was estimated in arbitrary optical density units read at 600 nm; lactate was measured enzymatically, following NAD^+ reduction at 340 nm; ^{14}C-lactate radioactivity in aliquots dissolved in Aquasol (New England Nuclear, Boston) was determined using a Nuclear Chicago Unilux 2A Liquid Scintillation Counter; $^{14}CO_2$ trapped in hyamine hydroxide was taken up in 10 ml of Aquasol for counting. Using the methods of Wolfe et al. (1979), no evidence of blood ^{14}C-glucose formation from ^{14}C-lactate was evident in these short-term experiments. Thin layer chromatography indicated that no metabolic derivatives of ^{14}C-lactate other than $^{14}CO_2$ were released into the blood during the 3 minute time course of these experiments.

In this study, right ventricular cardiac output was determined in the resting control state, at 10 minutes of a 17 minute dive (just prior to the ^{14}C-lactate injection) immediately after the end of sampling, and finally during recovery from diving. Right cardiac output was estimated by the thermodilution method (Maruschak et al., 1974).

For monitoring cerebral venous metabolites, a PE 50 catheter was introduced via a 13 ga steel Tuohy needle into an epidural vein in the cervical region and advanced in an anterior direction to within a few centimeters of the occiput. In pinnipeds, a cerebral venous blood is principally drained by these veins without admixture by venous blood from other tissues (King, 1977).

In all in vivo experiments, catheters were routinely flushed with saline solution (9 g/l) containing heparin (5,000 units/l) and connected to Statham

Model 1280C transducers and a Hewlett Packard 7758B recorder. Heart rate, vascular and intracardiac pressures and EKG were intermittently recorded. Arterial blood pH and gas tensions were determined intermittently with a Radiometer Model PHM72 blood gas analzyer.

After full recovery from anesthesia (8-12 hours), blood samples were drawn to establish metabolite levels in the control or resting state. This was usually followed by a relatively short simulated dive (10-20 minutes) and a recovery period. Another simulated dive was performed when the seal had fully recovered (usually 2-3 hours) as judged by the return to resting levels of pHa, PaO_2, $PaCO_2$, and heart rate. When the desired in vivo experiments were completed, the animal was sacrificed with an overdose of anesthetic and the position of all catheters was verified at autopsy.

The harbor seals (Phoca vitulina) used in these studies were borrowed from Sea World, San Diego, California. Venous blood samples from the unanesthetized seals were obtained from the extradural vein, catheterized near the posterior end of the animal. Human blood samples were collected from the antecubical veins of 4 male volunteers by a qualified medical technician. Samples from these two groups were not only treated with 1.4 M PCA, as previously described, but some were also added directly to 4 volumes of 3.75% sulfosalicyclic acid (SSA), centrifuged and stored at $-20^{\circ}C$ for future analysis. The latter treatment is preferred for amino acid analysis.

Enzyme Extraction and Assay

Tissues for enzyme extraction were excised as quickly as possible after sacrificing the animal; because of the massive size of the Weddell seal, this process took 20-30 minutes. A transmural left ventricular myocardial

sample was obtained from each seal. Each sample contained both endocardium and epicardium. The same regions of the brain (anterior cerebral cortex) containing both grey and white matter, and of lung parenchyma (left lower lobe periphery without major vessels or airways) were sampled in each seal.

The samples of heart, lung, and brain were immediately placed in $0^{o}C$ Ringers solution and washed several times. All further manipulations were at $0^{o}C$. Small samples of brain, heart, and lung were removed, blotted dry on filter paper, weighed and then homogenized using a Polytron tissue processor (Brinkman Instruments). The homogenization medium was 50 mM imidazole, pH 7.4, 50 mM KCl, with 0.1% Triton-X100. The homogenate was well stirred, then centrifuged in an RC-2B Sorvall centrifuge at $4^{o}C$ to remove cellular debris. The supernatant solution was used to assess the activities of several oxidative and glycolytic enzymes. For comparative purposes, samples of ox heart, lung and brain were prepared in identical manner. All enzymes were assayed in a Unicam SP1800 recording spectrophotometer with a thermostated cell holder maintained at $37^{o}C$ with a Lauda constant temperature bath and circulator.

Citrate synthase was assayed by the method of Srere (1969). Assay conditions were 0.15 mM acetylCoA, 0.5 mM oxaloacetate, in 50 mM imidazole buffer, pH 7.4, 7.5 mM Mg^{++}, 50 mM K^{+}, at $37^{o}C$. The release of CoA was monitored at 412 nm with 0.25 mM DTNB.

Glutamate dehydrogenase was assayed by following the oxidation of 0.1 mM NADH at 340 nm. Assay conditions were 2-ketoglutarate, 7 mM; NH_4^{+}, 50 mM; and NADH, 0.1 mM; 1 mM ADP was always included to fully activate the enzyme (Smith et al., 1975). Imidazole buffer (50 mM), pH 7.4, was used with 7.5 mM Mg^{++}, 50 mM K^{+}, at $37^{o}C$.

The activity of β-hydroxybutyrate dehydrogenase was monitored by following the oxidation of NADH at 340 nm. Assay conditions were 0.1 mM NADH, 2 mM acetoacetate, in 50 mM imidazole buffer, pH 7.4, 7.5 mM Mg^{++}, 37°C. The catalytic activity of β-hydroxybutyrylCoA dehydrogenase was assayed under identical conditions except that the substrate was 0.4 mM acetoacetylCoA.

The hexokinase assay depended upon coupling the production of glucose-6-phosphate to glucose-6-phosphate dehydrogenase and monitoring NADPH production at 340 nm. Assay conditions were 2.5 mM glucose, 5.0 mM ATP, 7.5 mM Mg^{++}, 50 mM K^{+}, 0.4 mM $NADP^{+}$, 2 units of glucose-6-phosphate dehydrogenase, 50 mM imidazole, pH 7.4, at 37°C.

Measuring phosphofructokinase depended upon coupling the reaction to aldolase, triose phosphate isomerase, and α-glycerophosphate dehydrogenase. Assay conditions were 1 mM ATP, 1 mM fructose-6-phosphate, 0.1 mM NADH, and excess (over 2 units) aldolase, triose phosphate isomerase, and α-glycerophosphate dehydrogenase. AMP (1 mM) was included in the reaction mixture to fully activate the enzyme (Tsai and Kemp, 1975). Imidazole buffer at pH 7.4 and 37°C was used.

Pyruvate kinase was assayed by coupling to lactate dehydrogenase. Assay conditions were 1.0 mM phosphoenolpyruvate, 1.0 mM ADP, 7.5 mM Mg^{++}, 50 mM K^{+}, 0.1 mM NADH, and excess lactate dehydrogenase, in 50 mM imidazole buffer, pH 7.4, at 37°C.

Lactate dehydrogenase activity was assayed by following NADH oxidation. Conditions were 2.5 mM pyruvate, 0.1 mM NADH, in imidazole buffer (50 mM), pH 7.4, at 37°C. Separation of the lactate dehydrogenase isozyme in skeletal muscle, lung, blood cells, heart and brain of the Weddell seal was

accomplished by the use of starch gel electrophoresis. The electrode buffer consisted of 0.05 M dibasic phosphate buffer, pH 7.0 (adjusted with sodium-free citric acid). The stationary phase used was 14% (W/V) starch suspended in a 1:20 dilution of the electrode buffer. Depending upon LDH activity, 5-10 µl were placed onto approximately 3 mm square wicks of the filter paper. These wicks were then placed into slits cut into the starch gel. Electrophoresis conditions were: 25 m A, 200 volts run for 12 hours at 4°C. The stain medium contained NAD (1 mM), phenazine methosulfate (0.1 mM), nitro blue tetrazolium (1 mM), lactic acid (100 mM), Tris-HCl (50 mM) at pH 7.5.

Glucose-6-phosphatase was assayed by following the release of inorganic phosphate which was measured according to Nordlie (1971). Assay conditions were 5 mM glucose-6-phosphate, 7.5 mM Mg^{++}, 50 mM K^{+}, imidazole buffer, pH 7.4, 37°C.

A unit of enzyme activity converts 1 µmole substrate to product per minute. All enzyme activities are expressed in terms of units/gm wet weight of tissue at 37°C.

In addition to the above enzymes, attempts were made to measure glycogen phosphorylase. This seal tissue enzyme was found to be extremely unstable, and we were unable to stabilize it. Drummond (pers. comm.) also found glycogen phosphorylase from diving animals difficult to work with.

Lung Slice Studies

Samples of lung tissue were excised and washed in chilled Ringers solution as previously described. Small samples were blotted on filter paper and weighed to the nearest mg. Slices weighing 25-50 mg were placed

in Ringers solution of the following composition: NaCl (122 mM), KCl (3 mM), $MgSO_4$ (1.2 mM), $CaCl_2$ (1.3 mM), KH_2PO_4 (0.4 mM) and $NaHCO_3$ (25 mM), pH 7.8. The O_2 uptake rate by lung slices was determined at 37°C using a Gilson Oxygraph. The oxidation of ^{14}C-U-lactate and ^{14}C-6-glucose by lung slices was determined using Warburg-type flasks containing a small hyamine hydroxide CO_2 trap. Slices were incubated for 20-30 minutes in the presence of labelled and carrier substrates (protocol given below), holding specific activity of glucose and lactate constant but varying total concentration. The lung slice experiments were terminated with PCA to stop the reaction and release the CO_2 formed. $^{14}CO_2$ collected in the hyamine hydroxide traps was counted as described above.

Metabolite Assays

Lactate and pyruvate concentrations in PCA extracts were determined by following the reduction of NAD^+ or the oxidation of NADH in the presence of purified lactate dehydrogenase. Glucose concentrations in PCA extracts were determined routinely with the hexokinase assay measuring the change in optical density at 340 nm due to $NADP^+$ reduction by glucose-6-phosphate dehydrogenase (Bergmeyer et al., 1974). In several extracts, glucose was also determined by the glucose oxidase method, the Benedict assay system and by an automated glucose analyzer with a linear response over the range of 5-50 µmol/ml. All three assay procedures yielded the same absolute glucose concentrations. In addition, whole blood extracts were analyzed by gas liquid chromatography (Albersheim et al., 1967) to show that glucose was the only major sugar present in seal blood; fructose, galactose, mannose, and ribose did not occur in measurable concentrations.

Amino Acid Analysis

Aliquots of the PCA and SSA treated blood samples from the Weddell seals, harbor seals and male volunteers were filtered using Millipore filters (mesh size of 0.4 μM); the PCA samples were adjusted to a pH of approximately 2.2 with concentrated hydrochloric acid. One hundred microlitre portions were then injected into a Beckman ^{C119} amino acid analyzer, packed with a type AA20 cationic exchange column. Individual amino acid concentrations were determined by comparison of sample peak areas with those of a standard. No significant differences in amino acid concentrations between the two extraction procedures were observed.

CHAPTER III

Blood Metabolite Profiles as a Consequence of Diving

INTRODUCTION

In studying metabolic events in animals under stress, it is usually advantageous to monitor blood metabolite concentrations prior to, during and following the stressful episode. Itwas just such a tactic that enabled P. F. Scholander (1940) to postulate the probable existence of a selective blood redistribution during diving in aquatic mammals; his main, and in fact only, evidence stemmed from observations of a rapid increase of lactate level in the animal's blood upon surfacing. Scholander reasoned that anaerobic glycolysis and the concomitant lactate production would be associated with peripheral vasoconstriction. Parenthetically, Irving first suggested a peripheral vasoconstriction in 1939 but it was Scholander who first offered any tangible proof for such a phenomenon during diving.

Since very few studies have focused upon blood metabolite profiles in diving animals (see Hochachka et al., 1975, 1977a) advantage was taken of the opportunity to measure levels of some of the more important metabolites in the central circulation prior to, during and after a simulated dive of the Weddell seal. The primary aim of these investigations was to monitor glucose, pyruvate and lactate profiles but in the process a wide spectrum of ninhydrin reactive compounds (amino acids and the tripeptide glutathione) were also surveyed.

Hochachka et al. (1977a), under identical experimental conditions to this model, reported significant decreases in central blood levels of glucose, along with well defined elevations in the lactate profiles during simulated diving. Whereas lactate levels rose sharply upon termination of the dive, glucose concentrations usually remained relatively

constant or actually dropped for the first couple of minutes, followed by abrupt increases in the whole blood levels. These results appear to be in direct contradiction to those of Blix and Kjeskshus (unpublished work; see Blix, 1976) as they not only observed a glucose increase during the forced dive of a harbor seal, but also recorded a consistent decline in the plasma free fatty acids. Unpublished work by our group and that of Kooyman and Davis (pers. commun.) on trained harbor seals demonstrated distinctive drops in whole blood glucose during routine diving. It can only be assumed that Blix and Kjeskshus were observing a harbor seal who was mainly sustained by aerobic metabolism.

Prior to this study, no thorough investigation of amino acid profiles during diving and recovery cycles had been undertaken. Hochachka and associates (1975) did measure blood levels of alanine prior to and after diving in the harbor seal (no sampling was performed during the dive phase). By about 5 minutes into recovery the amino acid had increased to approximately 200% over the predive values, indicating a sizable flush-out of the peripheral tissues. This observation led the authors to postulate the existence of an anaerobic pathway with the amino acid as one of its endproducts.

RESULTS AND DISCUSSION

Blood Glucose, Pyruvate and Lactate Profiles

The present set of data, generated from simulated diving of approximately 10-30 min. predictably demonstrate steady declines in glucose levels of the central (or main) circulation, followed by continued decreases or sharp declines during the first 5-10 min. of recovery, when the total 60 l. of blood volume is well-mixed (Fig. III, 1). The continued drop in glucose levels in early recovery may indicate that glucose is depleted in the total blood volume and not only in the central blood. If only the latter occurred, glucose levels would return to near-normal levels within the first minute of recovery as cardiac output rose and fully mixed the blood. (In the above argument, it is assumed that there is a central circulation, which is slowly exchanging with the greater volume of blood trapped in the peripheral regions of the animals. See Chapter V for a thorough discussion.) The eventual increase in blood glucose is presumably due to rising glucagon concentrations. Although no such analyses are available for the Weddell seal, Robin (pers. commun.) observed the presence of this phenomenon in the common harbor seal.

In contrast to glucose profiles during diving, blood lactate concentrations consistently rise in arterial blood usually from less than 1 μmol/ml in predive states to over 3 μmol/ml at the end of the diving period (Fig. III, 2). On the other hand, pyruvate levels can increase, decrease or remain relatively constant during diving, but following simulated diving, a large washout of both pyruvate and lactate is always observed (Fig. III, 3). What was not expected was the large difference

Fig. III, 1. Change in glucose concentration of whole arterial blood during diving and recovery in 6 representative seals whose initial blood glucose supplies varied by nearly 2-fold. Duration of dives, standardized to an arbitrary scale, varied between 10 and 20 minutes. Dive times: (○), 20 minutes; (△), 20 minutes; (■), 10 minutes; (□), 15 minutes; (●), 20 minutes; (▲), 20 minutes.

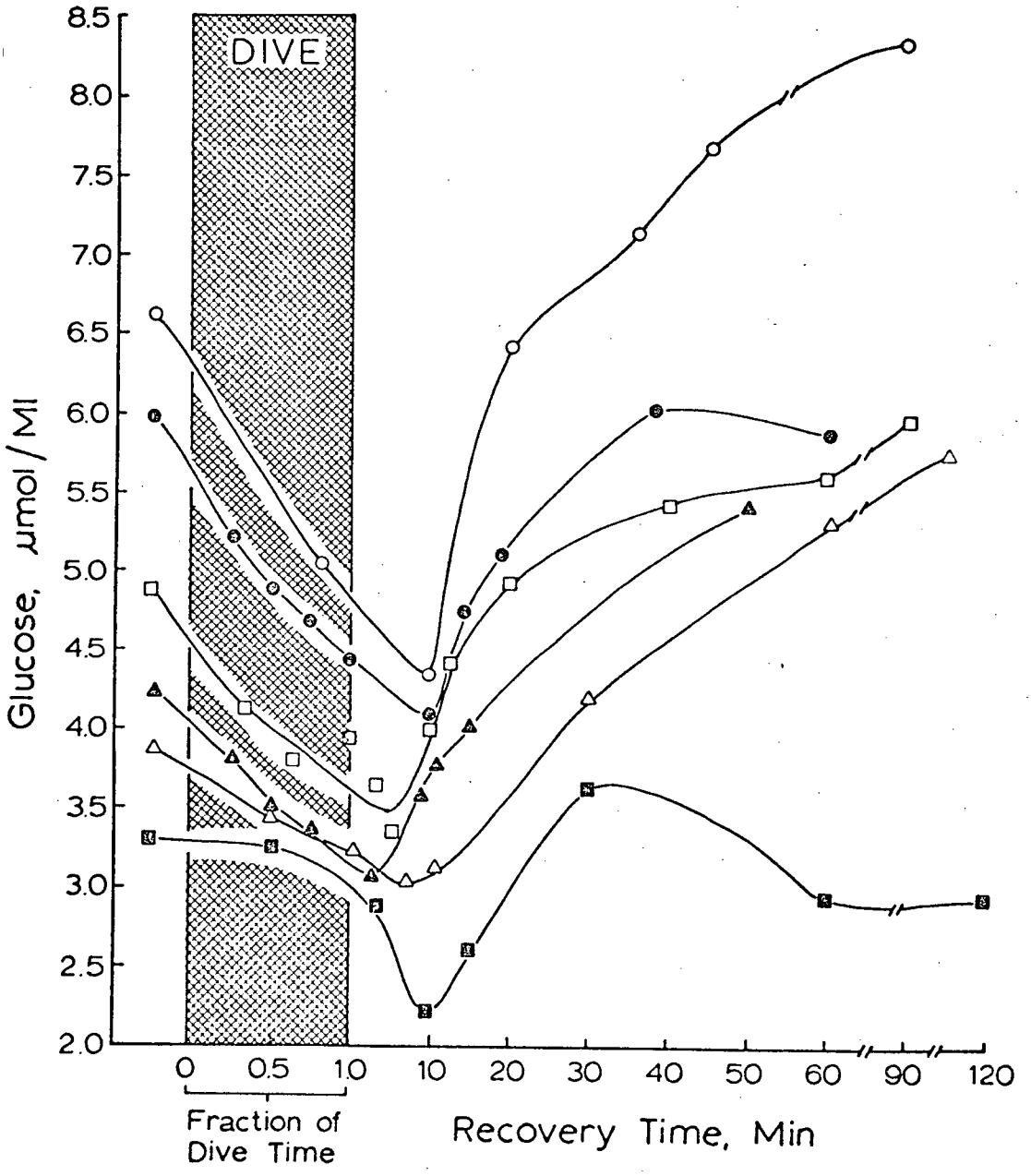

DIVE
Glucose, µmol/Ml
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
0
0.5
1.0
10
20
30
40
50
60
90
120
Fraction of Dive Time
Recovery Time, Min

Fig. III, 1

Fig. III, 2. Change in lactate concentration of whole arterial blood during diving and recovery in 5 representative seals. Dive duration varied between 10 and 20 minutes but was standardized to facilitate comparison on an arbitrary scale. Dive times: (●), 20 minutes; (△), 20 minutes; (○), 10 minutes; (▲), 20 minutes; (□), 15 minutes.

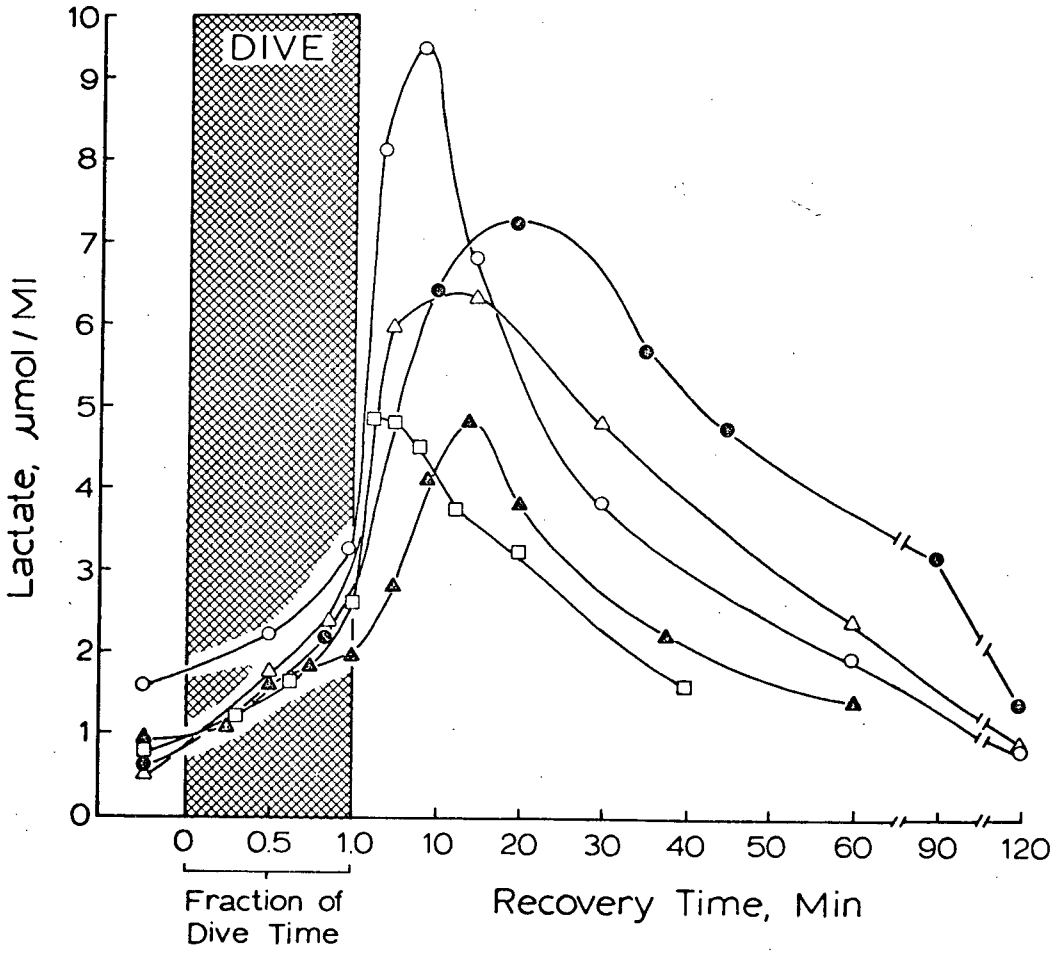

DIVE
Lactate, µmol / Ml
0
1
2
3
4
5
6
7
8
9
10
0
0.5
1.0
10
20
30
40
50
60
90
120
Fraction of
Dive Time
Recovery Time, Min

Fig. III, 2

Fig. III, 3. Change in pyruvate concentration of whole arterial blood during diving and recovery in 5 represenative seals. Dive duration varied between 10 and 47 minutes, but was standardized to facilitate comparison on an arbitrary scale. Dive times: (●), 10 minutes; (□), 20 minutes; (○), 46 minutes, (▲), 15 minutes, (△), 20 minutes.

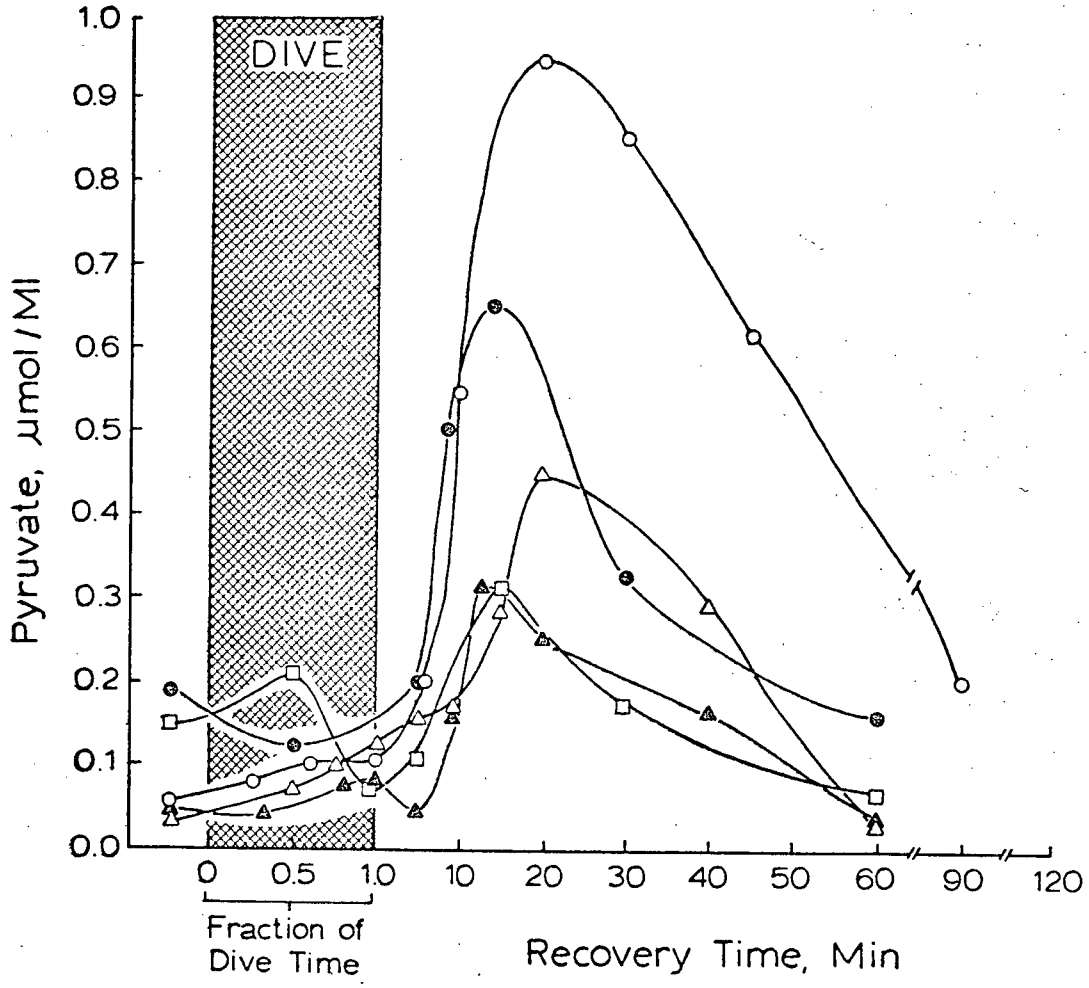

DIVE
Pyruvate, µmol/Ml
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
0.5
1.0
10
20
30
40
50
60
90
120
Fraction of
Dive Time
Recovery Time, Min

Fig. III, 3

in the kinetics of pyruvate and lactate appearance in the blood, with the pyruvate washout peak always lagging behind the lactate washout peak (Fig. III, 4). As a result, during early stages of recovery, lactate concentrations may rise well before there is any measurable change in pyruvate levels. This means transient, and very large changes in lactate/pyruvate ratios may occur in early recovery periods (Fig. III, 4).

Since pyruvate and lactate are thought to be in equilibrium in all tissues, the question arises as to what processes account for their differing behaviour during diving and recovery. One possibility is that pyruvate and lactate form the basis for a kind of inter-tissue hydrogen cycling mechanism, organs in different redox states exchanging one for the other. This kind of hydrogen shuttling mechanism was predicted on theoretical grounds (Hochachka and Storey, 1975) and has been previously observed in the perfused mammlian heart during extreme hypoxia (Lee et al, 1973). Furthermore, it is quite plausible that the high activity levels of blood lactate dehydrogenase (Vallyathan et al., 1969) may contribute to redox balancing throughout the diving episode. A second possibility is that lactate and pyruvate exchange rates between tissue and blood differ by a large enough factor to account for the lag in pyruvate release into the blood. At present, the lack of relevant information prohibits ascertainment of the precise situation.

The above data raise further questions pertaining to the source(s) responsible for metabolite changes in the central blood volume during the dive phase. Peripheral leakage and/or anaerobic fermentation in one or more of the central organs are likely candidates. This problem will be addressed and treated in later chapters (Chapters V, VI, VIII).

Fig. III, 4. Change in lactate/pyruvate concentration ratio of whole arterial blood during diving and recovery in 3 representative seals. Duration of dives, standardized to an arbitrary scale, varied between 10 and 20 min. Dive times: (●), 10 min; (0) 20 min; (△), 15 min.

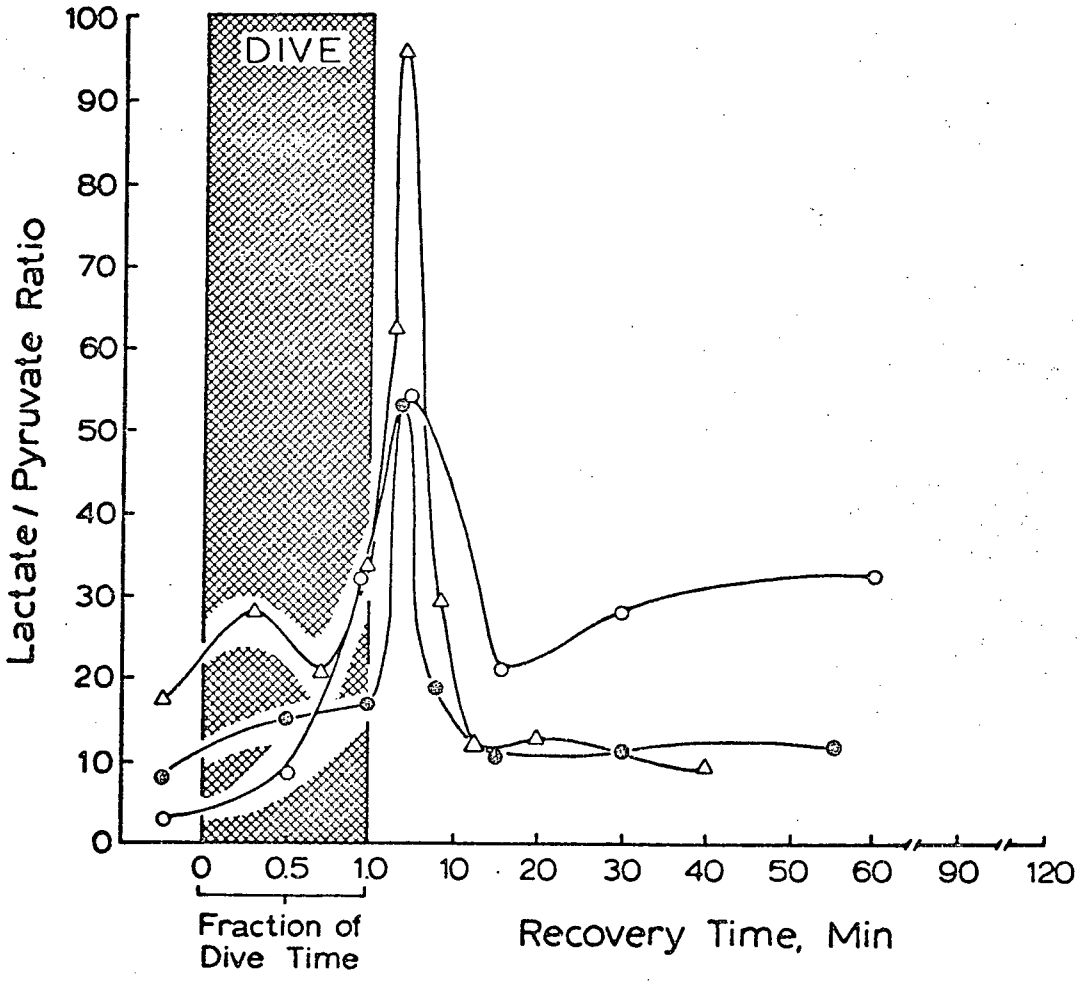

DIVE
Lactate / Pyruvate Ratio
100
90
80
70
60
50
40
30
20
10
0
0
0.5
1.0
10
20
30
40
50
60
90
120
Fraction of
Dive Time
Recovery Time, Min

Fig.III, 4

Free Amino Acids and Glutathione in Resting Animals

In an effort to decipher whether the diving habit in marine mammals has dictated any alterations in the free amino acid blood pool, blood samples from Weddell and harbor seals (2 phocids with dramatically different diving abilities) and male volunteers were assayed for the more important amino acids. Table III, 1 gives absolute concentrations (nmoles/ml whole blood) of the sampled amino acids in the three different groups analyzed. Unfortunately, sampling sites were not consistent in the three groups; venous blood was collected from both the harbor seals and the human volunteers while the Weddell seal profiles were derived from arterial samples (see Materials and Methods). It should be realized that comparisons between arterial and venous amino acid pools must be handled with caution for some amino acids display significant arterio-venous concentration differences, depending on the position of the catheters (Felig et al., 1973). Be that as it may, statistical evaluations of the mean differences in concentrations between all three groups (Table III, 2) were performed.

Where differences were detected, the human levels were usually the more concentrated, the major exception being taurine, whose levels appear to be two fold higher in the harbor seal than that of both the human and Weddell seal. The metabolic implications of the high taurine concentrations in harbor seal blood are unclear since the precise biological functions of this sulphur-containing amino acid have yet to be ascertained (Awapara, 1976). The terrestrial mammal, as represented by the male humans, has significantly higher concentrations of serine, glutamate, glycine and ornithine than those of both species of seals. Alanine profiles

Table III, 1. Whole blood concentrations (μM) of free amino acids in male Weddell seals (arterial samples), harbor seals (venous) and human volunteers (venous). Concentrations are presented as means ± standard deviations of 3 to 5 subjects.

Amino Acid	Whole Blood Concentration (nmol/ml) Weddell seal	Harbor seal	Human
Taurine	172.6 ± 20.5	326.5 ± 12.5	170.5 ± 13.4
Aspartate	26.6 ± 5.9	35.3 ± 4.0	35.7 ± 3.4
Threonine	137.2 ± 18.5	99.6 ± 25.5	133.2 ± 23.3
Serine	72.8 ± 10.0	91.3 ± 4.8	131.9 ± 5.8
Glutamate	97.9 ± 18.5	63.5 ± 8.6	142.3 ± 17.2
Glutamine	493.3 ± 230.0	481.7 ± 59.3	556.7 ± 109.9
Glycine	107.5 ± 10.8	178.3 ± 34.1	317.3 ± 29.0
Alanine	83.6 ± 2.6	398.4 ± 59.8	336.7 ± 37.9
Valine	167.4 ± 16.0	170.7 ± 5.9	170.6 ± 15.2
Isoleucine	38.4 ± 6.5	51.0 ± 2.8	54.4 ± 7.2
Leucine	155.1 ± 24.3	94.1 ± 5.8	103.8 ± 14.0
Tyrosine	18.5 ± 2.9	57.2 ± 8.0	55.8 ± 6.9
Phenylalanine	64.6 ± 7.2	53.1 ± 3.6	55.9 ± 7.4
Lysine	103.3 ± 4.8	174.4 ± 48.2	158.7 ± 30.5
Ornithine	28.2 ± 1.2	54.4 ± 9.7	91.8 ± 23.4
Histidine	78.4 ± 12.6	74.2 ± 16.1	66.9 ± 4.5
Arginine	61.8 ± 9.4	173.5 ± 43.9	99.3 ± 34.0
Total Branched Chained Amino Acids	391.4 ± 42.6	315.8 ± 14.2	328.7 ± 29.5
Total Amino Acid Pool	2145.0 ± 132.0	2692.0 ± 176.0	2769.8 ± 156.9
Glutathione	1535.4 ± 127.2	998.2 ± 187.4	678.0 ± 46.6

Table III, 1

Table III, 2. Statistical evaluation of mean differences of amino acids concentrations presented in Table III, 1. Subscripts indicate the animal with the higher concentration. Abbreviations used: W = Weddell seal;, H = Harbor seal; hu = human; N.S. = not significant.

Amino Acid	Male Weddell Seal vs Male Human	Male Weddell Seal vs Male Harbor Seal	Male Harbor Seal vs Male Human
Taurine	N.S.	$p < .001$ (H)	$p < .001$(H)
Aspartate	N.S.	N.S.	N.S.
Threonine	N.S.	N.S.	N.S.
Serine	$p < .001$ (hu)	N.S.	$p < .001$(hu)
Glutamate	$p < .01$ (hu)	$p < .01$ (W)	$p < .001$ (hu)
Glutamine	N.S.	N.S.	N.S.
Glycine	$p < .001$(hu)	$p < .02$ (H)	$p < .01$ (hu)
Alanine	$p < .001$(hu)	$p < .001$ (H)	N.S.
Valine	N.S.	N.S.	N.S.
Isoleucine	$p < .02$ (hu)	$p < .05$ (H)	N.S.
Leucine	$p < .01$ (W)	$p < .001$ (W)	N.S.
Tyrosine	$p < .001$(hu)	$p < .001$ (H)	N.S.
Phenylalanine	N.S.	N.S.	N.S.
Lysine	$p < .02$ (hu)	$p < .05$ (H)	N.S.
Ornithine	$p < .01$ (hu)	$p < .01$ (H)	$p < .05$ (hu)
Histidine	N.S.	N.S.	N.S.
Arginine	N.S.	$p < .01$ (H)	N.S.
Total branched chained amino acids	N.S.	N.S.	N.S.
Total amino acid pool	$p < .001$(hu)	$p < .05$ (H)	N.S.
Glutathione	$p < .001$(W)	$p < .01$ (W)	N.S.

Table III, 2

provide the most dramatic differences; the levels of this metabolically active amino acid (see Goldberg and Chang, 1978) are three fold higher in both the harbor seal and human than that of the Weddell seal. To assign any definitive significance to this finding would be quite risky considering the availability of so little information. However, it has been suggested that free blood alanine is involved in an alanine-glucose cycle between the skeletal muscle and liver in mammals (Felig, 1973). According to this model alanine, derived from amnio acid or protein catabolism in the muscles, is used in hepatic gluconeogenesis and carries amino groups to the liver for disposition as urea. If this postulated role is valid for the Weddell seal, perhaps the extremely low levels of alanine could indicate an active gluconeogenic process (Young and Hill, 1973). Since most phocid seals have very low carbohydrate diets (Blix, 1976) it is intuitively obvious that most of their glucose must be derived gluconeogenically. Of course, this does not explain the relatively high levels of the amino acid occurring in harbor seal blood since this seal is also a member of the genus Phocidae. The situation may be more complex and hence must be thoroughly investigated before a precise explanation becomes apparent.

Glutathione (GSH) profiles, as determined by the Beckman C119 amino acid analyzer, indicate that this tripeptide is significantly more concentrated in the Weddell seal than in human and harbor seal whole blood (see Tables III, 1 and III, 2). However, the human values are low in comparison to other literature reports; Bernt and

Bergmeyer (1974) report whole blood levels in the 1000 µmol/ml range. Until more data is generated it would be inappropriate to attach much importance to the above finding.

Therefore, the current data demonstrate that most free amino acids in mammalian blood remain relatively constant in all members of the class thus far studied. The differences, as recorded in this thesis, could be attributed to sampling site selections, fasting durations and different dietary regimes.

Diving Profiles of Amino Acids and Glutathione

During restrained diving in the Weddell seal only alanine and glutamine levels in whole blood increase , (glutamate and glycine also rise, but are considered to be involved in the glutathione changes; this will be discussed later in the chapter). Alanine (Fig. III, 5) displays a slow but steady increase throughout the dive, followed by a more rapid rise (up to 200% increase over pre-dive levels) early in the recovery period. Glutamine profiles (Fig. III, 6) are qualitatively similar to those of alanine; both amino acids have been implicated to function as waste nitrogen carriers, transporting amino nitrogen out of various tissues to the liver, kidney and other sites (Chang and Goldberg, 1978). Since some amino acids are thought to be fermented in hypoxic muscles it is quite possible that both are acting as nitrogen transporters which are flushed-out in the recovery phase of diving. The alanine increase may also be due to an activation of muscle alanine aminotransferase. It has been postulated that during diving the hypoxic or anoxic muscles will channel pyruvate to lactate and alanine (Owen and Hochachka, 1974). The alanine and

Fig. III, 5. Percentage change of alanine concentration in whole arterial blood during diving and recovery in 4 representative seals. Dive duration varied between 10 and 20 min, but was standardized to facilitate comparison on an arbitrary scale. Dive times: (0), 10 min;(X),18 min; (△), 20 min;(□), 17 min.

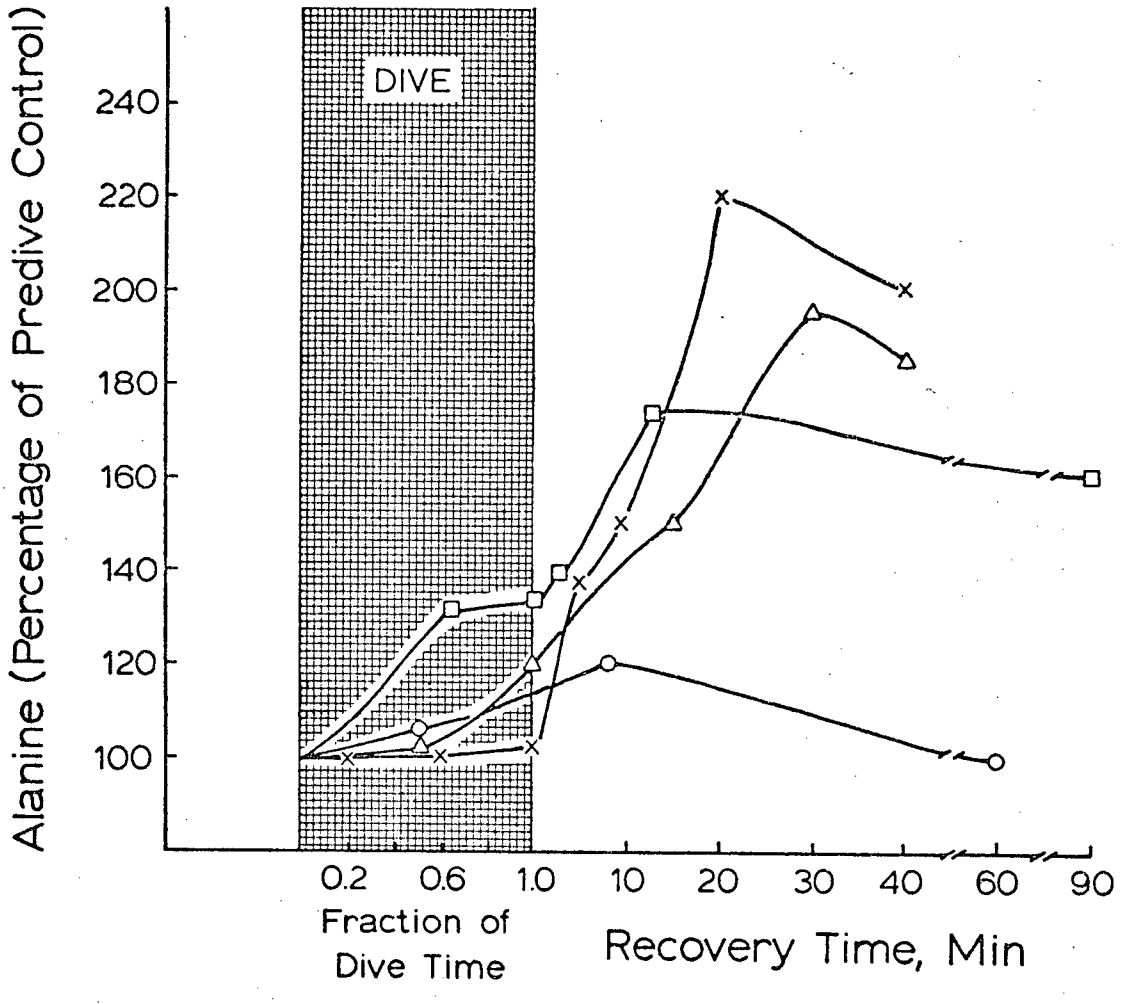

Alanine (Percentage of Predive Control)
240
220
200
180
160
140
120
100
DIVE
0.2
0.6
1.0
10
20
30
40
60
90
Fraction of Dive Time
Recovery Time, Min

Fig. III,5

Fig. III, 6. Percentage change of glutamine concentration in whole arterial blood during diving and recovery in three representative seals. Duration of dives, standardized to an arbitrary scale, varied between 10 and 20 min. Dive times: (0), 10 min; (X), 10 min; (□), 20 min.

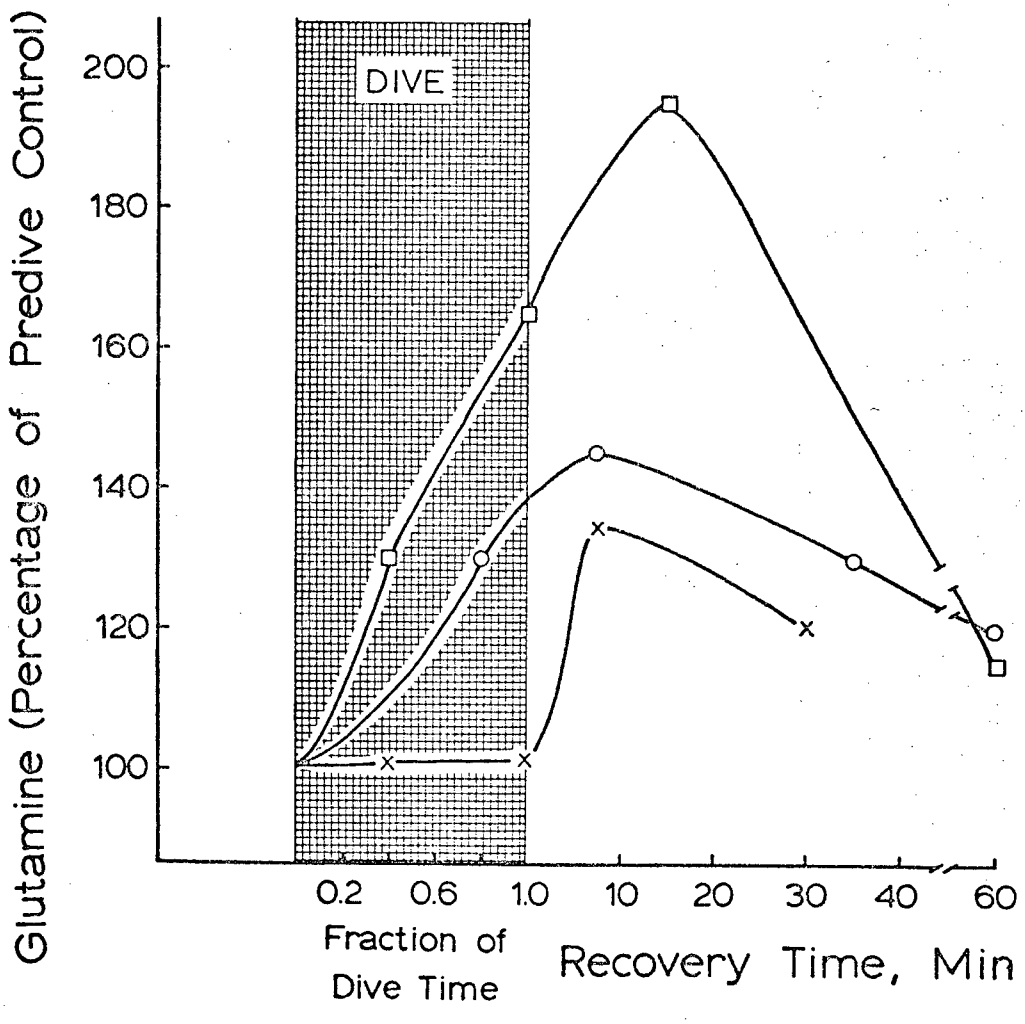

Glutamine (Percentage of Predive Control)
200
180
160
140
120
100
DIVE
0.2
0.6
1.0
10
20
30
40
60
Fraction of Dive Time
Recovery Time, Min

Fig. III,6

co-substrate oxo-glutarate are produced by the action of alanine aminotransferase on pyruvate and glutamate. The alanine presumably accumulates in the cytoplasm whereas the oxo-glutarate is transported to the mitochondria where it is thought to be eventually converted to succinate. This pathway not only aids in balancing redox but also increases energy supply in the form of GTP (Owen and Hochachka, 1974). Part of the alanine increase associated with recovery could also stem from the sparking of the tricarboxylic acid cycle in the muscles during anaerobic-aerobic transition (Hochachka and Murphy, 1979). Whatever the source, most of the alanine probably is absorbed by the liver where its carbon skeleton is used for the synthesis of glucose and the nitrogen atoms excreted in the form of urea (Felig, 1973). Glutamine, on the other hand, may ultimately be utilized by the liver, the gastro-intestinal tract (Chang and Goldberg, 1978) and/or catabolized by the kidney (Baruch et al., 1976).

Of the ten different dive sequences analyzed for glutathione, five displayed rather dramatic drops in blood concentrations of GSH. These decreases in the dive phase were usually followed by rapid increases to above pre-dive levels upon recovery (Fig. III, 7). Glutathione, a ubiquitous tripeptide (α-glutamyl-cysteinglycine), is postulated to have at least four major biological roles: 1) it can conjugate with many different substances, priming them for eventual excretion (Flohe et al., 1974); 2) it may act as a co-enzyme (Jocelyn, 1972); 3) it probably functions as an anti-oxidant, protecting membranes and maintaining sulfhydryl-containing intracellular enzymes in their active states (Beuther, 1971) and 4) GSH has been postulated to participate in the

Fig. III, 7. Change in glutathione concentration of whole arterial blood during diving and recovery in 5 different seals. Dive duration varied between 10 and 30 min, but was standardized to facilitiate comparison on an arbitrary scale. Dive times: (0), 20 min; (□), 10 min; (△), 15 min; (●), 22 min; (X), 17 min.

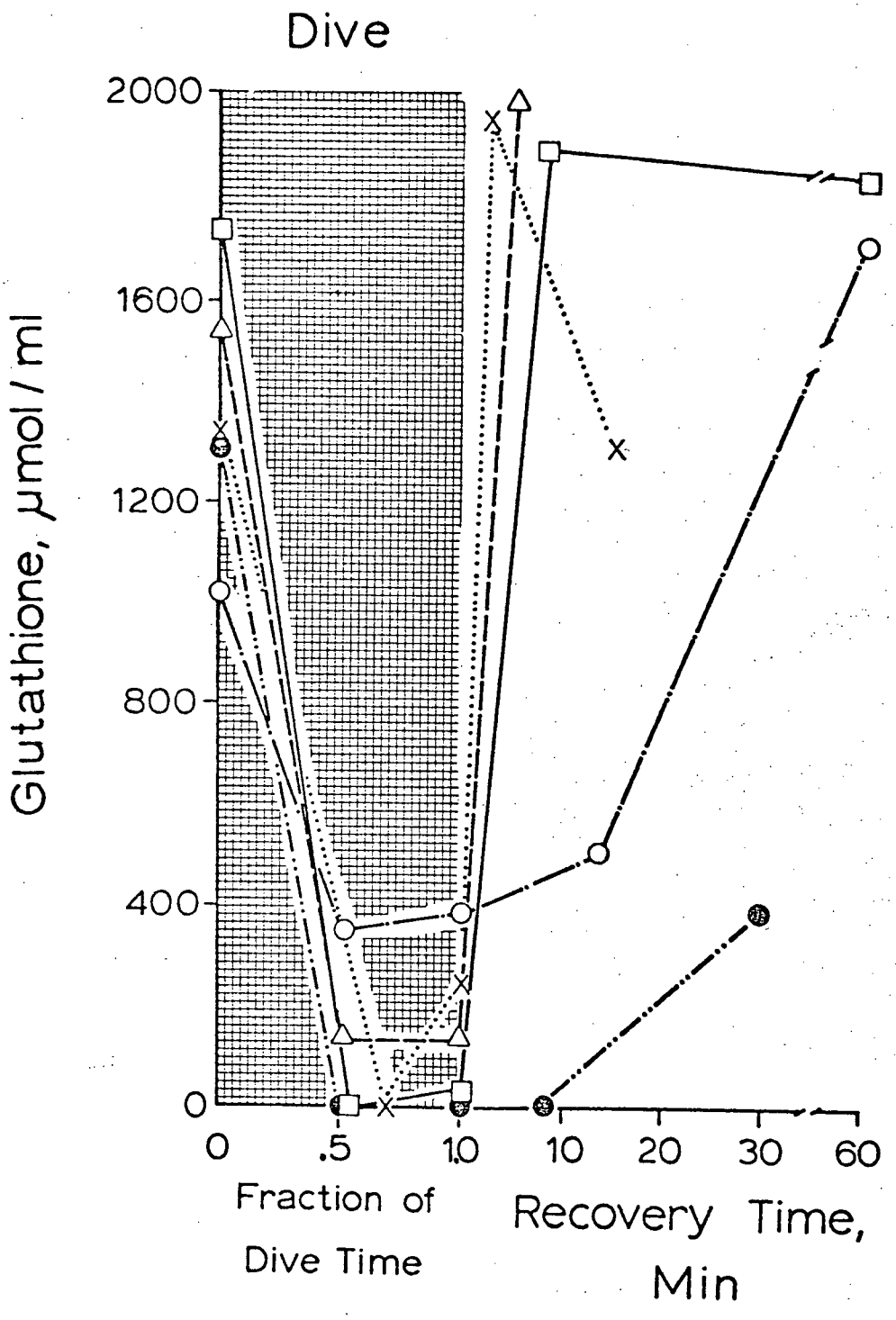

Dive
2000
1600
1200
800
400
0
Glutathione, μmol/ml
0
.5
1.0
10
20
30
60
Fraction of
Dive Time
Recovery Time,
Min

Fig. III, 7

transport of amino acids across cellular membranes (Meister, 1973).

Although early dive data are sketchy, it appears that by mid-dive, at the latest, 80-100% of the GSH had disappeared from the central circulation. Erythrocytes, as most other tissues, possess the necessary enzymes needed to convert the tripeptide into its three amino acid components: glutamate, cysteine and glycine (Palekav et al., 1974). Table III, 3 gives a summary of the mean percentage retentions and disappearances of blood glutamate and glycine as a function of GSH changes in the central blood volume. Cysteine values are not presented as this amino acid was not detectable by the techniques employed, probably due to the lack of sulfhydryl protection and/or to the relatively lengthy storage times. During diving only 50-55% of the degraded GSH can be traced to increases in free blood glutamate and glycine, whereas approximately 35% of the resynthesized GSH appears to be derived from the blood pools of the two amino acids. At the moment, it is impossible to account for the total amino acids released from the breakdown of GSH (45-50% unaccountable) and their subsequent uptake for the resynthesis of the tripeptide (65% unaccountable). Possibly, a portion of the released amino acids or glutathione, itself, are stored (or even metabolized) in some organ or tissue in the peripheral blood during the dive. Unfortunately no solid physiological significance to this set of data can be offered, and furthermore the presence of an artefact cannot be ruled out. However, if this were a manipulative error, one would expect to observe the same phenomenon throughout the entire study.

From the amino acid study it is concluded that routine diving episodes (of up to 30 minutes) have very minimal effects on the majority of free

Table III, 3. Mean percentage changes (± standard errors) in whole blood glutamate and glycine as functions of GSH changes during diving-recovery cycles.

$$\frac{\Delta \text{ free amino acid}}{\Delta \text{ GSH}} \times 100$$

Sample sizes = 5

	Mean Percentage Change	
Condition	Glutamate	Glycine
Dive	50.6 ± 4.6	54.2 ± 10.4
Recovery	37.0 ± 3.1	35.5 ± 6.2

amino acids in whole blood of the adult Weddell seal; a result consistent with that of recent studies on anoxia in the freshwater turtle, Pseudemys scripta elegans and the common goldfish, Carassius auratus (B. Emmett and E. Shoubridge, pers. commun.). The impressive homeostasis of all these animals could be due to a very finely tuned regulation of the amino acid blood pools or, more likely, to a lack of dependence on this potential energy source during transient hypoxic and anoxic episodes.

CHAPTER IV

Enzymes of Aerobic and Anaerobic Metabolism in the Brain, Heart and Lung of the Weddell Seal

INTRODUCTION

In any comprehensive metabolic study it is sometimes useful to explore the 'enzymatic potentials' of the tissues or organs under investigation. However, the mapping of enzyme titers will, at the best, provide a gross index of the complex metabolic organization in the tissue. Invariably, such enzymatic assays are performed under optimal experimental conditions of substrate, cofactors, pH, activator concentration, etc. A more valid and revealing procedure would be to mimic the precise 'in vivo' environment under the different conditions to which the enzyme is exposed; unfortunately this still remains a formidable task. Without such data one is compelled to extrapolate from optimal activities to actual 'in vivo' rates. Such conjecture may render the analysis meaningless, especially if the enzyme is near equilibrium or non-regulatory, for such enzymes do not accurately mirror the pathway's potential. Nevertheless, a careful selection of enzymes to be tested can allow for valid insights into metabolic directions and potentials. For example Crabtree and Newsholme (1972) have demonstrated that glycolytic rates can be directly correlated with the activities of phosphorylase, hexokinase (HK) and phosphofructokinase (PFK). Simon and Robin (1971, 1972) observed a close relationship between pyruvate kinase (PK) activity and lactate production during anaerobiosis and cytochrome oxidase activity with basal oxygen consumption. More recently, Guppy and co-workers (1979) have demonstrated that the tuna white muscle, a very intense anaerobic tissue, contains not only unusually high activities of phosphorylase and lactate dehydrogenase (LDH) but also α-glycerophosphate dehydrogenase,

malate dehydrogenase and glutamate oxaloacetate transaminase, implying high anaerobic production of energy via glycolysis with a very efficient mechanism to balance redox. Simon and associates (1979) report actual increase in muscle PK activities during prolonged submergence of the freshwater turtle (Pseudemys scripta).

To recapitulate, it is realized that there are many inherent limitations associated with the extraction of relevant metabolic information from maximal velocity data of enzymes; but if used (with care and restraint) in conjunction with other metabolic parameters a clearer picture of the overall metabolic state of the tissue will probably emerge.

The following study was performed in an attempt to place some ground work for the eventual elucidation of the metabolic profiles or potentials of the heart, lung and brain in the experimental animal. Some key enzymes of both glycolysis and oxidative metabolism were measured; all are compared to homologous enzymes of a comparably-sized terrestrial mammal (ox).

RESULTS AND DISCUSSION

Oxidative Enzymes

Four mitochondrial marker enzymes were assayed to measure the oxidative capacities of the seal heart, lung and brain: β-hydroxybutrylCoA dehydrogenase and β-hydroxybutyrate dehydrogenase, functioning in β-oxidation of fatty acids and ketone body metabolism, respectively; citrate synthase, catalyzing the entry of acetylCoA carbon into the Krebs cycle and thought to represent an important control site (Tischlev et al., 1977); and glutamate dehydrogenase, a key regulatory enzyme (Srere, 1969), catalyzing the entry of glutamate carbon into the Krebs cycle. As the heart, lung and brain differ greatly in metabolic organization (Siesjo and Nordstrom, 1977; Neely and Morgan, 1974; Tierney, 1974a), their enzyme activity profiles also differ greatly (Table IV, 1). However, the activities of these mitochondrial marker enzymes in seal heart, lung, and brain are similar or somewhat lower than those measured under identical conditions in homologous ox tissues. Parenthetically, Ballantyne (personal communication) found cardiac carnitine palmitoyl-transferase, an enzyme considered to be rate limiting in the oxidation of fatty acids (Pande and Blanchaer, 1971), to be similar in activity levels to that occurring in bovine heart. These results imply that when oxygen is available, all three organs can sustain oxidative metabolism at rates per gm. tissue that will be similar to other mammalian species. This is not surprising since a vigorous 0_2-based metabolism is well documented in seals (Ashwell-Erickson and Elsner, 1977; Gallivan and Ronald, 1979).

Enzymes of Anaerobic Glycolysis

Hexokinase, phosphofructokinase, and pyruvate kinase, which are all

Table IV, 1. Enzyme activities in brain, heart and lung of the Weddell seal and ox. Values are means, with ranges in parentheses, expressed as μmole substrate converted/min/g wet tissue at 37°C, pH 7.4, and saturating levels of substrates, cofactors or coenzymes. Detailed assayed conditions are presented in Chapter II. Sample sizes for the seal and bovine assays were 6 and 2, respectively.

*n = 1; almost identical values obtained from two cetaceans (W. Vogl, unpublished data).

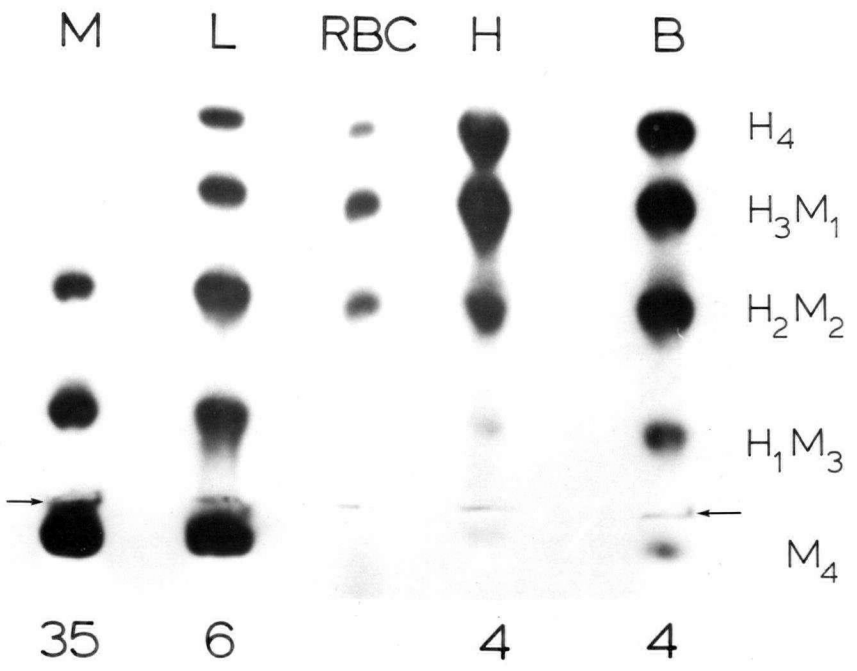
+n = 3.

Enzymes	Brain		Heart		Lung	
	Seal	Ox	Seal	Ox	Seal	Ox
Citrate Synthase	17.8 ± 2.5 (15.6 - 20.5)	16.8 (15.4 - 18.1)	28.8 ± 6.9 (18.8 - 35.3)	61.7 (45.8 - 79.9)	1.52 ± 0.9 (0.8 - 1.7)	6.6 (6.4 - 6.9)
Glutamate Dehydrogenase	7.5 ± 1.6 (5.7 - 8.6)	4.5	4.4 ± 1.3 (3.5 - 6.5)	2.8 (2.4 - 3.2)	0.90 ± 0.4 (0.6 - 1.7)	0.9
β - hydroxybutrate dehydrogenase	0.40 ± 0.10 (.28 - 0.5)	0.3 (1.9 - 3.6)	2.42 ± 0.7 (1.9 - 3.6)	2.8	1.7 ± 1.0 (0.5 - 2.5)	0.2
β-hydroxybutyryl CoA dehydrogenase	3.4 ± (0.3) (3.2 - 3.7)	-	16.0 ± 3.5 (12.5 - 20.5)	-	1.2 ± 0.4 (0.5 - 2.5)	-
Hexokinase	5.2 ± 1.8 (3.0 ± 5.7)	1.3 (1.3 - 1.4)	2.0 ± 0.9 (1.2 - 3.4)	2.0 (2.8 - 3.0)	1.7 ± 0.8 (1.1 - 2.9)	2.2 (2.2 - 2.1)
Glucose 6-phosphatase*	0.61	-	0.48	-	0.72	-
Phospho-fructokinase	8.6 ± 2.8 (5.3 - 12.3)	8.6 (9.2 - 9.0)	16.7 ± 5.9 (9.9 - 24.1)	14.0 (13.9 - 14.0)	3.7 + 1.3 (2.3 - 5.4)	4.7 (4.2 - 5.2)
Pyruvate kinase	167.3 ± 11.1 (157 - 350)	196 (194 - 198)	217.5 ± 51.5 (183.3 - 294.0)	133.1 (128 - 137.9)	45.6 ± 17.7 (21.6 - 66.3)	98.0 (94.4 - 101.6)
Lactate dehydrogenase	228.8 (200 - 350)	128.2 (125.8 - 130.6)	1032.0 ± 45.7 (1013 - 1050)	556.0 (508 - 604)	69.6 ± 30.7 (50.6 - 107.8)	91.9 (79.8 - 104.0)

potential regulating sites in anerobic glycolysis (Scrutton and Utter, 1968), and lactate dehydrogenase, catalyzing the terminal step in glycolysis, were measured to assess the potential for anaerobic glycolysis in these organs. In the seal brain, hexokinase and lactate dehydrogenase occur at 4- and 2-fold higher levels than in the ox brain, respectively, while pyruvate kinase and phosphofructokinase occur at similar concentrations in the ox and seal brain (Table IV, 1). In the seal lung, these four glycolytic enzymes occur at levels similar to those in the ox lung. In the heart of both species hexokinase and phosphofructokinase occur at similar levels. In contrast, the activities of seal heart PK and LDH are 1.5- to 2-fold higher than in the ox heart, the latter occurring at levels of about 1000 μmoles product/min/gm at 37°C. This is the highest concentration of LDH of any comparatively sized vertebrate heart thus far studied.

Interestingly, electrophoretic studies show that in all three organs both heart- and muscle-type subunits of LDH are synthesized; thus multiple isozymes occur in all three organs (Fig. IV, 1). In the heart and brain, the heart-type subunits have higher activity than the muscle-type subunits. Nevertheless, all three organs clearly have the potential either for lactate production, catalyzed most effectively by muscle-type lactate dehydrogenase, or for lactate utilization, catalyzed most effectively by heart-type lactate dehydrogenase (Holbrook et al., 1975). Empirically this is indicated by the ratio of pyruvate reductase activity to lactate oxidase activity, which is strikingly higher for the skeletal muscle lactate dehydrogenases (Fig. IV, 1). Similar results have been generated from other diving animals including seals (see Chap. I).

Fig. IV, 1. Starch gel electrophoretic separation of lactate dehydrogenase isozymes in skeletal muscle (M), lung (L), red blood cells (RBC), heart (H), and brain (B) of the Weddell seal. Electrophoresis conditions: 25 mA, 200 volts, 12 hours at $4^{o}C$, anode at the top, origin marked with an arrow. The subunit composition of each isozyme is shown on the right. The numbers below refer to the ratio of pyruvate reductase activity to lactate oxidase activity at pH 7.4 at saturating coenzyme and substrate concentrations, assayed at $37^{o}C$.


M
L
RBC
H
B
H_4
H_3M_1
H_2M_2
H_1M_3
M_4
35
6
4
4

Glucose-6-Phosphatase

Glucose-6-phosphatase catalyzes the terminal step in the formation of glucose either from triose precursors or glycogen (Scrutton and Utter, 1968). Interestingly, the enzyme occurs in all three organs of the Weddell seal (Table IV, 1). The ratio of glucose-6-phosphatase to hexokinase activities, which may supply an indication of the potential for glucose release vs. glucose phosphorylation, is highest for the lung (0.4) and lowest (about 0.1) for the brain. These results may explain two previous findings. Firstly, it has been reported that the seal lung can release small amounts of glucose into the blood (Hochachka et al., 1977a), a process that would necessitate glucose-6-phosphatase function. And secondly, the Weddell seal heart metabolically may serve as a glucose-storage organ (Kerem et al., 1973) since it accumulates huge amounts of glycogen which could be mobilized and released as glucose under hypoxic situations. Although glucose release by the heart has not been demonstrated, if it occurs, it too would require glucose-6-phosphatase function.

The occurrence of significant activities of glucose-6-phosphatase in brain tissue is in agreement with recent studies by Anchors and coworkers (1977). The brain is usually regarded as having an absolute dependence upon glucose as a substrate and, as discussed below, the seal brain also utilizes it (see Chapter VI). However, infrequently and only when blood lactate levels are high, the seal brain appears to release measurable amounts of glucose into the blood (P. W. Hochachka and B. Murphy, unpubl. data). The phenomenon may be common to marine and terrestrial mammals alike since the same enzyme profiles emerges from studies of the heart, lung, and brain in two species of whales (Vogl, 1979) as well as from

nervous tissue and nerve cell lines of terrestrial species (Anchors et al., 1977).

From the above enzyme profiles, it is tentatively concluded that the potential for anaerobic glycolysis in the seal brain and heart may be somewhat greater than that of the terrestrial species, while oxidative potential is unchanged or slightly reduced; however, differences, where found, are rather modest. Such a situation may imply quite a different level of biochemical adaptation, which could include kinetic modifications of certain pivotal regulatory enzymes.

The enzyme data presented unequivocally indicate that all three organs are capable of using or releasing both glucose and lactate. With this in mind, it was decided to monitor organ arterio-venous (A-V) concentration differences of both metabolites in the intact animal; this could provide insights into glucose and lactate metabolism from resting, diving and recovery phases. Such information would also be helpful in ascertaining central organ contributions to the observed metabolite changes in the main circulation during diving episodes (see Chapter III).

CHAPTER V

Impact of Diving on Lung Metabolism

INTRODUCTION

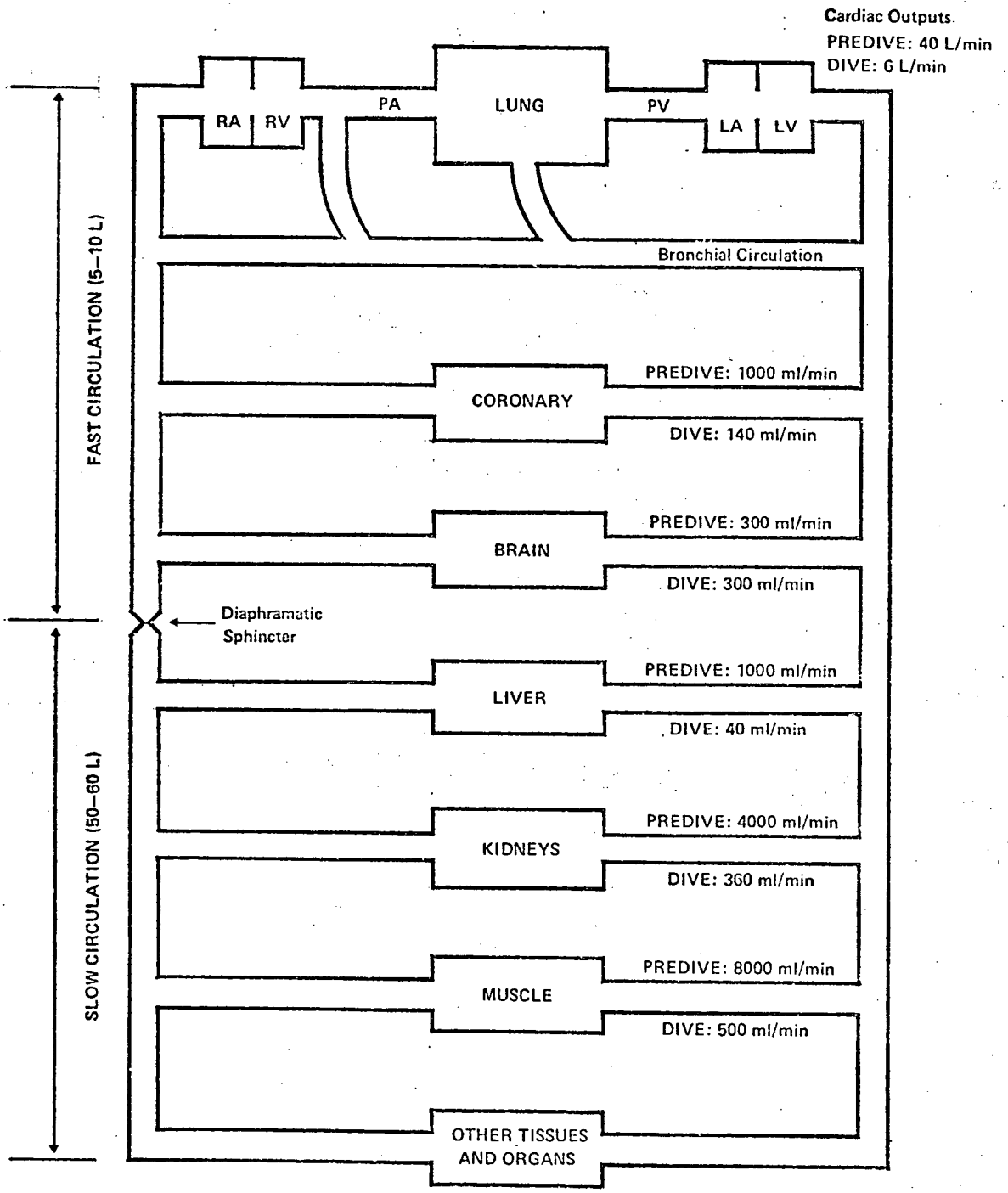
Since the metabolic and biochemical organization of the mammalian lung has, until recently, been largely ignored, it is understandable that the present knowledge of pulmonary metabolism in diving mammals is in an expanding phase. Recent and innovative 'in vivo' lung research (Wolfe et al., 1979; Rhoades et al., 1978, Hochachka et al., 1977a) have uncovered some revealing insights into the metabolic status of this organ in both the terrestrial and aquatic mammals (see Chapter I for a general review of mammalian lung metabolism). According to Hochachka et al. (1977a) the Weddell seal lung is capable of using or releasing both lactate and glucose. The initial studies showed that A-V concentration gradients are small and therefore a more powerful approach was needed to ascertain the metabolic fate of circulating lactate or glucose arriving at the lung. That is why in these studies emphasis was placed on isotope experiments; it was reckoned that more definitive data could be generated by utilizing radioactive tracers in both 'in vivo' preparations and tissue slice incubations. ^{14}C -lactate was used todeduce the metabolic fate of absorbed lactate while ^{14}C -lactate and ^{14}C - glucose competition experiments were utilized to compare relative lung preferences for the two substrates.

RESULTS AND DISCUSSION

"In Vivo' Preparation

These experiments essentially consisted of tracing metabolic derivatives (e.g. CO_2) of ^{14}C-lactate infused into the blood on the afferent side of the lung. A bolus of ^{14}C-lactate (about 4 μC/l blood) plus dye and carrier lactate (whose final concentration was about 3 mM, equivalent to what the lung might experience towards the end of 15-20 minute dive periods) was rapidly injected into the right ventricle after 10 minutes of simulated diving. Blood samples were taken every 20 seconds at two ports representing right (pulmonary artery) and left (aorta) sides of the circulation (Scheme V-1). Most of the ^{14}C-lactate (as well as the unlabelled bolus) traversed the pulmonary circulation simultaneously with the tracking dye (Fig. V, 1). A small fraction of the lactate bolus rapidly traversed the pulmonary circulation so that at the time of the first two samples (at 20 and 40 seconds), significant amounts of ^{14}C-lactate were already present in arterial blood. At the same time (20-40 seconds after injection) although the ^{14}C-lactate and absolute lactate concentrations were decreasing in the pulmonary circulation, $^{14}CO_2$ had already appeared in aortic blood and could only have been generated by lung metabolism since at this time the lung was the only tissue (other than blood) to have received ^{14}C-lactate. The majority of the $^{14}CO_2$ pulse appeared on the left side of the heart, peaking at about 35 seconds after injection; the smaller initial peak

Scheme V-1. Diagrammatic representation of the Weddell seal circulation, indicating blood sampling ports. RV, right ventricle; RA, right atrium; PA, pulmonary artery; PV, pulmonary vein; LA, left atrium; LV, left ventricle. Blood flow rates from Zapol et al. (1979). Bronchial circulation assumed to be similar to terrestrial mammals (Nagarshi, 1974). The diagram emphasizes two (coronary and cerebral) and possibly three (bronchial) 'fast-circulation pathways' for tracer to return to injection site, as well as numerous 'slow circulation pathways'.


Cardiac Outputs
PREDIVE: 40 L/min
DIVE: 6 L/min
RA
RV
PA
LUNG
PV
LA
LV
Bronchial Circulation
FAST CIRCULATION (5–10 L)
PREDIVE: 1000 ml/min
CORONARY
DIVE: 140 ml/min
PREDIVE: 300 ml/min
BRAIN
DIVE: 300 ml/min
Diaphramatic
Sphincter
PREDIVE: 1000 ml/min
LIVER
DIVE: 40 ml/min
PREDIVE: 4000 ml/min
KIDNEYS
DIVE: 360 ml/min
PREDIVE: 8000 ml/min
MUSCLE
DIVE: 500 ml/min
SLOW CIRCULATION (50–60 L)
OTHER TISSUES
AND ORGANS

Scheme V-1

Fig. V, 1. U-^{14}C lactate oxidation by the Weddell seal lung in vivo. Multiple openings in right ventricular injection port of Swan-Ganz catheter assured well-mixed introduction of ^{14}C lactate bolus. Rapid manual injection was performed after a stable bradycardia (heart rate of 15 beats/min) was established at 10 min into a 17 min simulated dive. At 20-s intervals simultaneous 5-ml blood samples were withdrawn from pulmonary artery (PA) and aorta and treated as described in Chapter II. Cardiac output in this experimental seal (350-kg male) was 24 l/min before diving and decreased to 4 l/min at 10 min into dive. Qualitatively similar results were obtained in a preliminary experiment with another seal during entry into a simulated dive.

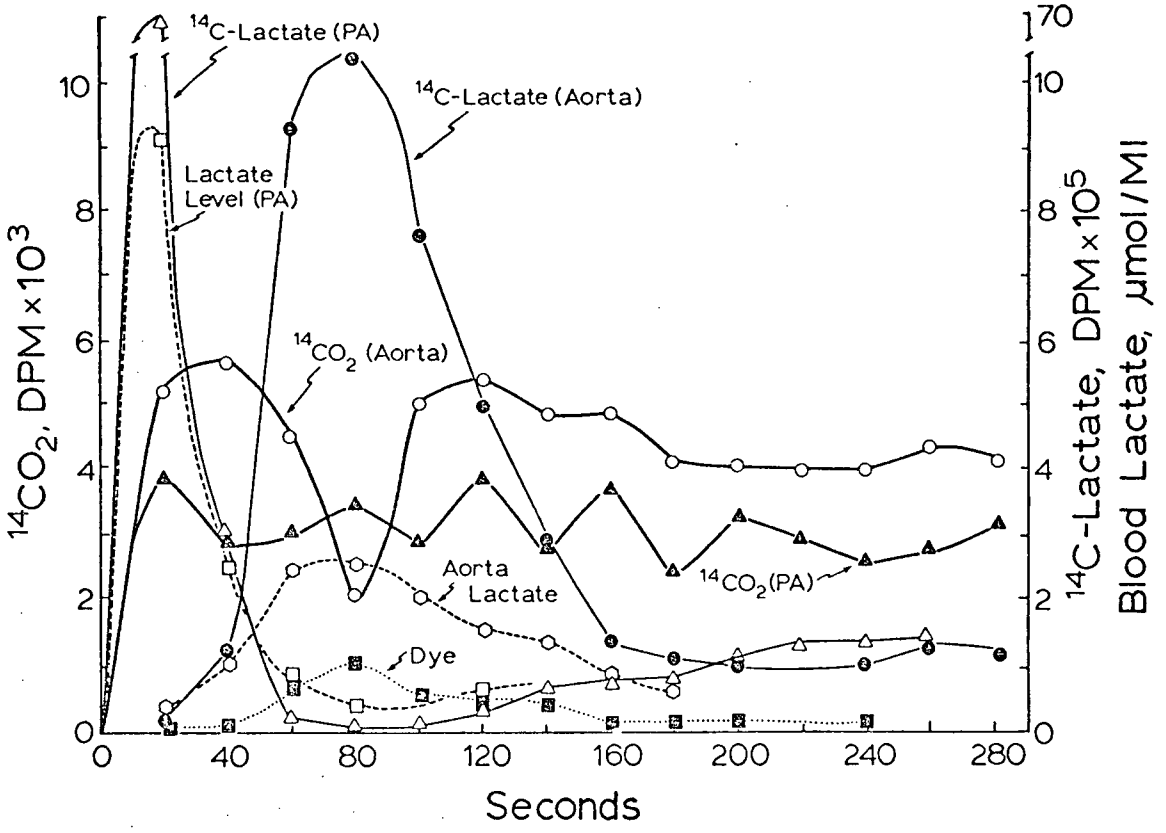

^{14}C-Lactate (PA)
^{14}C-Lactate (Aorta)
Lactate Level (PA)
$^{14}CO_2$ (Aorta)
Aorta Lactate
$^{14}CO_2$(PA)
Dye
$^{14}CO_2$, DPM×10^3
^{14}C-Lactate, DPM×10^5
Blood Lactate, μmol/Ml
Seconds
0
40
80
120
160
200
240
280
2
4
6
8
10
70

Fig. V,1

of $^{14}CO_2$ in the pulmonary arterial blood probably arrived via the coronary and/or bronchial circulations (see Scheme V-1). Consistent with this interpretation and predicted by it is the observation that the oscillations in blood $^{14}CO_2$ levels on the left and right sides of the heart initially are about 90^{o} out of phase with each other. This situation holds for about the first 100 seconds. Nevertheless, for the duration of the experiment (7 minutes, of which only the first 280 seconds are shown in Fig. V, 1), it appears that lung oxidation of ^{14}C-lactate exceeded lactate oxidation by any other organs, a process which would maintain the consistently higher levels of $^{14}CO_2$ in the aorta than in the PA blood. The mean pulmonary-to-thoracic aorta circulation time (time for traversing from the right to the left side) is about 30-40 seconds under simulated diving conditions, with heart rate reduced from 55 to 15 beats/min and cardiac output reduced from about 24 to 4 l/min. Thus a complete circulation time is estimated to be about 60-80 seconds (Fig. V, 1). Since the average cardiac output is 6 l/min (Zapol et al., 1979), it can be estimated that a "central" blood volume of 8 l exchanges slowly with the rest of the 60 l blood volume, which is presumably pooled in the venous system. Unfortunately, the present data do not allow for a concrete estimation of the exchange rate between the two blood pools. Nevertheless, this rate is assumed to be relatively low since the lactate profiles change so dramatically immediately upon recovery from the dive episodes (see Fig. III, 2).

Tissue Slice Studies

The above experiments supply direct 'in vivo' evidence that a major metabolic fate of lactate taken up by the seal lung is complete oxidation.

Further evidence indicating that lactate is a good substrate for this organ comes from tissue slice experiments. Using air-equilibrated Krebs-Henseleit Ringer solution, the $\dot{Q}O_2$ for lung tissue was found to be similar to that reported for other mammals (Wallace et al., 1974), at 30.19 µl O_2/hr/100 mg wet weight (n=14; standard error = ± 1.63), and remained stable for over an hour. Lung slices were found to oxidize ^{14}C-lactate at relevatively high rates but ^{14}C-6-glucose at substantially reduced rates. Increasing glucose concentration from 1 to 10 mM caused a three-fold increase in the rate of oxidation, while increasing lactate concentration from 1 to 10 mM increased oxidation rate by nearly five-fold (Table V, 1A). Lactate oxidation exceeded glucose oxidation rates at all substrate concentrations, but this was accentuated at high levels. The maximum oxidation rates compared quite closely with those observed in perfused rat lung (Wolfe et al., 1979; Rhoades et al., 1979); interacting effects of glucose and lactate were modest (Table V, 1B), in contrast to the perfused rat lung, in which physiological levels of lactate (1-10 mM) inhibit glycolysis up to 60%. In part, the differences between ^{14}C-U-lactate and ^{14}C-6-glucose oxidation may be artefactual because the pyruvate dehydrogenase reaction sequence allows $^{14}CO_2$ production from uniformly-labelled lactate but not from ^{14}C-6-glucose while complete oxidation in the Krebs cycle releases $^{14}CO_2$ from them both.

From the available data it is tempting to speculate that there would have been very little teleological need for the sea mammal to improve upon the pulmonary metabolic equipment in the transition from a terrestrial to an aquatic environment. It, like other mammalian lungs, will burn lactate in preference to glucose; an arrangement that may be of

Table V, 1. $^{14}CO_2$ production from ^{14}C-U-lactate and from ^{14}C-6-glucose by lung slices of the Weddell seal. Conditions are given in Materials and Methods. In part A, glucose and lactate concentrations are varied independently, while in part B, both are varied simultaneously. The rate of $^{14}CO_2$ release is expressed in nmoles/hour/gm wet weight of tissue at 37°C. Number of experiments given in brackets. Specific activity of glucose and lactate was constant under all conditions. All data are means $\pm$ S.E.

		$^{14}CO_2$ from:	
A:	Conditions	^{14}C-6-glucose	^{14}C-U-lactate
	10 mM glucose	247.8 ± 62.0 (6)	
	5 mM glucose	122.2 ± 16.6 (5)	
	1 mM glucose	63.3 ± 8.1 (6)	
	10 mM lactate		531.5 ± 71.8 (6)
	5 mM lactate		396.5 ± 35.3 (6)
	1 mM lactate		201.3 ± 25.7 (6)
B:	10 mM glucose		
	1 mM lactate	204.0 ± 23.2 (11)	197.8 ± 15.2 (12)
	1 mM glucose		
	10 mM lacate	50.1 ± 7.0 (12)	848.4 ± 74.7 (11)

Table V, 1

paramount importance during a prolonged dive, for not only will this preserve vital blood glucose stores for the central nervous system but will also aid in 'mopping up' a potentially hazardous end product. However, it should be realized that these experiments were performed without any thoracic compression, which the animal will inevitably encounter upon diving to deep depths (e.g. 500 m for the Weddell seal). Thoracic compression could not only alter bronchial and perhaps pulmonary blood flow but it may drastically diminish the alevolar oxygen stores. However, it can be safely assumed that the lung does have an important function during the normobaric recovery period. When large quantities of lactic acid are washed out of peripheral tissues, glycolysis will likely be dampened and the lung will primarily burn the anaerobic end product. This coupled with an increased heart rate (M. Snider, pers. commun.) and hyperventilation may allow for a greater oxidizing capacity of the lung during this critical phase when blood lactate levels can leap to over 20 μmol/ml (Kooyman, pers. commun.). If these levels were unchecked they could pose serious buffering and oxygen loading problems for the organism.

Since no pulmonary A-V concentration gradients were measured in this study it was impossible to determine the lung's contribution to metabolite changes in the central blood volume during simulated diving. Nevertheless, if the findings of Hochachka et al. (1977a) are real, it can be tentatively concluded that this organ cannot contribute to the observed increases in lactate levels during diving. In fact, one can extrapolate that an A-V concentration gradient of +0.1 μmol of lactate/ml whole blood (Hochachka et al., 1977a) by a 4 kg. lung will

effectively decrease lactate blood levels at a rate of 0.5 mmol/min., assuming a cardiac output of 5 ℓ/min. Such an arrangement also has the added advantage of sparing vital glucose stores for other organs (e.g. the brain).

CHAPTER VI

Impact of Diving and Recovery on Cerebral Metabolism

INTRODUCTION

It is commonly thought that the mature mammalian brain uses glucose as its major substrate (Sokoloff, 1973) and oxygen as the terminal proton acceptor (Siesjo et al., 1976). Any substantial interruption in the flow of these two substrates could spell grave consequences to the brain and inevitably to the organism. In terrestrial species, hypoglycemia results in the metabolism of endogenous substrates (Siesjo et al., 1976) while reductions in the mean PaO_2 (down to about 15 mm Hg) cause concomitant increases in the cerebral blood flow (CBF), effectively maintaining a constant O_2 delivery rate. Such an O_2 compensatory mechanism has been observed in the duck (Anas platyrhynchos), the sea lion (Zapophus Californianus) and most recently in the harbor seal (Phoca vitulina) during routine diving (Jones et al., 1979; Dormer et al., 1977; Elsner et al., 1978). However, other studies on the harbor seal and the northern elephant seal (Mirounga angustirostris) indicated little or no CBF changes throughout routine and long term diving (Bron et al., 1966; Kerem et al., 1971; Van Citters et al., 1965), whilst that of the grey seal (Halichoerus grypus) showed a marked reduction (Dormer et al., 1977). At the time of this study, parallel work by Zapol et al. (1979) demonstrated that CBF remained unaltered during simulated diving (of the Weddell seal) when PaO_2 levels fell to about 25 mm Hg. Since these low O_2 tensions are considered to be hypoxic to the mammalian brain (Seisjo et al., 1976) one would intuitively expect an increased CBF and/or an increased dependence upon anaerobic glycolysis.

Prior to this study there were no available data concerning the anaerobic versus aerobic contributions to cerebral energy metabolism nor

on substrate preferences of the brain under normal states and dive-recovery episodes. Some indirect estimates suggested an activated reliance on anaerobic glycolysis, inferred from substrate (i.e. glycogen) storage data (Kerem et al., 1973), enzyme profiles (Simon et al., 1974) and electrophoretic patterns (Blix et al., 1970; Messelt and Blix, 1976; Altman and Robin, 1969; Blix and From, 1971). However two physiologic investigations did provide more tangible evidence. Ridgeway et al. (1969) in measuring 0_2 tensions in expired air samples from a trained bottlenose porpoise (Tursiops truncatus) extrapolated that towards the end of a routine dive the porpoise brain was by necessity, deriving most of its energy requirements anaerobically; the simulated diving experiments of Kerem and Elsner (1973) showed marked increases in cerebral lactate production in the latter stages of a prolonged harbor seal dive (20 minutes), again strongly suggesting an activation of anaerobic glycolysis.

It was reasoned that assaying A-V differences for glucose and lactate it would be possible to ascertain:

1) if there is any increase in cerebral anaerobic metabolism as a function of low $Pa0_2$ levels associated with diving.

2) If there is any change in substrate preferences during diving and recovery episodes.

3) If cerebral metabolism could account for glucose and lactate concentration changes in the central circulation throughout the dive phases.

RESULTS AND DISCUSSION

A-V Concentration Differences

By simultaneous sampling of arterial blood in the aorta and epidural vein close to occiput (King, 1977) it was found that glucose uptake by the brain during simulated diving (0.4 μmole/ml) is somewhat higher than during control, resting states (Table VI, 1). Parenthetically predive estimates (.28 μmole/ml) are in the same range as those reported for the terrestrial mammalian brain (Maker et al., 1976).

Only a small fraction (about 1/11) of the total brain hexokinase activity (Table IV, 1) would be needed to sustain these rates of glucose metabolism. The change in the A-V concentration gradient during diving could be caused by changes in uptake or by a reduction in blood flow to the brain. However, parallel microsphere studies of organ flow showed that brain perfusion, at least in 8-12 minutes of diving, is largely unchanged or actually rises somewhat (Zapol, et al., 1979). Thus, the 140% increase in the glucose concentration gradient (Table VI, 1) probably somewhat underestimates changes in glucose uptake by the brain during simulated diving. The estimate is nevertheless instructive, for if brain oxygen supplies were limiting during diving, glucose uptake would presumably increase by up to 18-fold due to the energetic inefficiency of anaerobic glycolysis; similarly, the fraction of glucose appearing as lactate should rise. The estimated 140% increase in glucose uptake, therefore, indicates that the brain's dependence upon anaerobic glycolysis does not rise greatly during simulated diving of up to 20 minutes duration, despite PaO_2 levels (as low as 25 mm Hg) which may be hypoxic to non-diving

Table VI, 1. Whole blood glucose and lactate concentration gradients across brain of the Weddell seal before and during simulated diving. Values are means $\pm$ SE, expressed as the difference in μmol/ml between arterial and venous blood samples drawn simultaneously. Maximum metabolic rate sustained by glucose catabolism is calculated in terms of μmol ATP/g/min assuming a flow rate of 0.6 ml/g/min in the control predive state and 0.75 ml/g/min during diving. Numbers in parenthesis in table refer to number of samples. Duration of dives in minutes shown in square brackets.

Cerebral

Arteriovenous Differences

Seal No.	Condition	Lactate Production	Glucose Uptake	% Glucose Fermented	Rate of ATP Formation
9	Predive	0.13 ± 0.039(5)	0.38 ± 0.042(5)	17	6.9
17	Predive	0.18 ± 0.064(6)	0.26 ± 0.031(6)	34	3.9
	Dive [10]	0.24 ± 0.125(2)	0.46 ± 0.030(2)	23	10.8
18	Predive	0.13 ± 0.120(3)	0.26 ± 0.141(3)	25	4.4
	Dive [15]	0.13 ± 0.115(3)	0.30 ± 0.109(3)	22	6.3
18	Predive	0.13 ± 0.052(4)	0.25 ± 0.106(3)	26	4.2
	Dive [20]	0.31 ± 0.071(4)	0.50 ± 0.038(3)	31	9.5
20	Predive	0.14 ± 0.122(4)	0.26 ± 0.092(3)	26.9	3.6
	Dive [20]	0.11 ± 0.087(4)	0.34 ± 0.119(4)	16	7.8
Predive mean		0.14 ± 0.032(20)	0.28 ± 0.033(21)	25.0	4.7
Dive mean		0.16 ± 0.068(12)	0.40 ± 0.051(12)	20.0	8.3

Table VI, 1

mammals (Siesjo and Nordstrom, 1977). This was confirmed by lactate measurements which estimate that the lactate released by the brain accounts for 20-25% of the cerebral glucose uptake under both control and diving conditions (Table VI, 1). In the rat, the brain releases lactate (5-15% of absorbed glucose) because of limited pyruvate dehydrogenase function (Cremer and Teal, 1974) and it is assumed a similar mechanism operates in the seal brain. Because of the higher total lactate dehydrogenase activities (Table IV, 1) and relatively more muscle-type isozyme (Fig. IV, 1) a large fraction of the pyruvate pool may be diverted to lactate in the seal brain. The routine and apparently "wasteful" release of lactate by the brain may therefore represent a minor cost of increasing its anaerobic potential somewhat.

Since the mean PaO_2 levels never fell below 20-30 mm Hg in any of the Weddell seals studied, it would be informative to follow cerebral metabolism in the animal during prolonged diving (50-70 min.) when arterial O_2 tensions may dip below 20 mm Hg. Elsner and associates (1970b) suggest Weddell seals have greater cerebral tolerance for low O_2 than terrestrial mammals. Furthermore, harbor seals, apparently, show increased cerebral V-A lactate content towards the end of prolonged diving (Kerem and Elsner, 1973). It can only be surmised that prolonged 'hypoxic' diving in the Weddell seal would also be accompanied by a greater reliance on anaerobic metabolism with a possible increase in CBF. However, Kerem and Elsner (1973) report no such compensatory increases in harbor seal CBF even at PaO_2 levels approaching 10 mm Hg.

Since the mean blood flow to the seal brain was measured, the glucose and lactate gradients allow estimation of metabolic rates in terms of μmol

ATP/gm/min that can be sustained with glucose as the carbon and energy source (Table VI, 1). The calculated metabolic rates sustainable by glucose catabolism (assuming complete oxidation) are somewhat lower than for brain metabolism in man and other mammals (Siesjo and Nordstrom, 1977). This may be expected from the scaling effects of body size since brain metabolic rates of large mammals are reported to be lower than in small-sized species (Siesjo and Nordstrom, 1977). The estimates of cerebral metabolic rates do not take into consideration the flow of glucose carbon into glycogen, the pentose cycle, the free amino acid pool, or other metabolic pathways, and this may explain the apparent differences between the diving and control states (Table VI, 1). Under some conditions, glucose incorporation into the free amino acid pool accounts for a large fraction of the glucose uptake by the brain in terrestrial mammals (Siesjo and Nordstrom, 1977). Thus, the higher estimated values for cerebral metabolic rates during diving (about 1.8 fold increases) may merely reflect an increased pooling of glucose carbon in non-oxidative metabolic pathways.

Are these metabolic rates high enough to cause significant depletion of blood glucose reserves during diving? The answer is evident in a simple set of calculations. If one assumes an average A-V concentration gradient across the brain of about 0.4 μmol/ml, and an average blood flow of about 700 ml/kg/min, then a 500 gm Weddell seal brain can take up glucose at a rate of 0.14 mmol/min, or about 3 mmoles/20 min dive. Assuming an 8 l volume exchanging slowly with total blood volume during diving (see Chapter V), this rate of glucose uptake would lead to an overall concentration change of 0.37 μmol/ml blood over a 20 minute dive. This

glucose utilization rate would decrease blood glucose levels by less than 0.05 μmol/ml when the total blood volume was well mixed. Complex mixing would be expected very early in the recovery process since cardiac output tends to overshoot prediving control levels, reaching values as high as 60 l/min during the first minute of recovery (M. Snider, unpubl. data).

Although in fermenting some glucose to lactate, the seal brain is not unusual, it is unusual in the high fraction (20-25%) that appears to be fermented, so the question arises as to whether brain lactate production rate is high enough to significantly increase blood lactate levels during diving. The same kind of calculation can be made as above to demonstrate that brain anaerobic glycolysis can lead to an overall increase in blood lactate concentration of only 0.18 μmol/ml over a 20 minute diving period, assuming 8 l of circulating blood; this increase would be less than 0.03 μmol/ml when the blood was fully mixed in recovery. If these calculations are correct, they imply that during routine diving periods (of about 20 minutes duration) brain metabolism on its own does not markedly alter total blood pools of glucose or lactate. For this reason, and because lung metabolism alone contributes to opposite changes in blood glucose and lactate levels, it is evident that these two organs cannot account for the rather marked changes in both these metabolites during diving (Chapter III). It can be, therefore, assumed that such metabolite alterations are due to peripheral leakage and, possibly, cardiac metabolism (see Chapter VIII for a discussion on cardiac metabolism).

Lactate Uptake by the Brain

In addition to being capable of releasing lactate, the Weddell seal brain can also consume it. Routine lactate A-V measurements upon recovery

show a net cerebral uptake of this anaerobic end product whenever the mean arterial concentration reaches critical levels of about 7 µmol/ml (Fig. VI, 1). This finding was verified by means of a lactate infusion experiment (Fig. VI, 2). For experimental details refer to Materials and Methods. In both cases, once this lactate concentration is surpassed, the brain vigorously consumes the substrate generating an A-V gradient of up to 1.25 µmol/ml (Figs. VI, 1 and VI, 2). The capacity for either lactate production or lactate uptake is consistent with the occurence in seal brain of high levels of lactate dehydrogenase, kinetically well suited for bi-directional function (Fig. IV, 1, Table IV, 1). If all the lactate consumed were fully oxidized, it could support a metabolic rate of 9 µmol/ml ATP/gm/min, assuming that brain blood flow were normal. This value is equal to, or greater than, that sustainable by glucose metabolism and indicates that lactate metabolism under these conditions can readily supply all of the energy demands of the brain. However, at present, there are no experimental estimates on the ultimate fate(s) of the absorbed lactate.

The experiments described in this chapter pointedly demonstrate that, during simulated diving of 30 minutes or less, the Weddell seal brain derives ample supplies of glucose and oxygen from arterial blood to meet its demanding energy requirements. Whether adjustments in the CBF could perfectly compensate for the possible decreasing levels of both oxygen and glucose during longer duration diving still remains unclear and, unfortunately, beyond the scope of the thesis. However, it has been shown that this brain will absorb and probably oxidize lactate when arterial concentrations reach a critical level of approximately 7 µmol/ml. Such an ability will likely aid in the speedy removal of the acidic, anaerobic

Fig. VI, 1. Change in lactate concentration in arterial and epidural venous blood samples during diving and recovery in a seal showing an unusually large lactate washout. Dive time: 20 min.

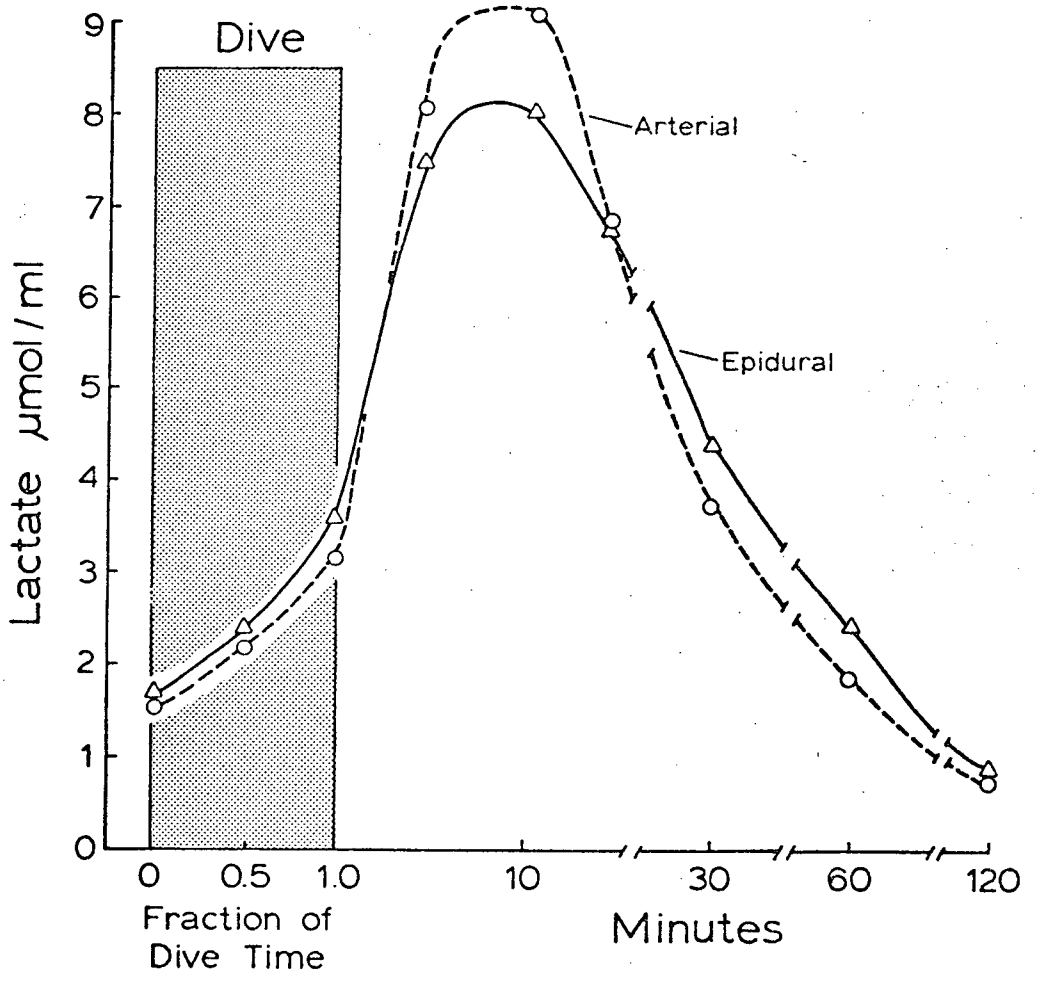

Dive
Arterial
Epidural
Lactate µmol/ml
0
1
2
3
4
5
6
7
8
9
0
0.5
1.0
10
30
60
120
Fraction of
Dive Time
Minutes

Fig. VI, 1

Fig. VI, 2. Lactate concentration changes in arterial and epidural venous blood following infusion of 176g of lactate. Lactate was infused in normal saline at pH 7.3 at a rate of about 100 ml/min.

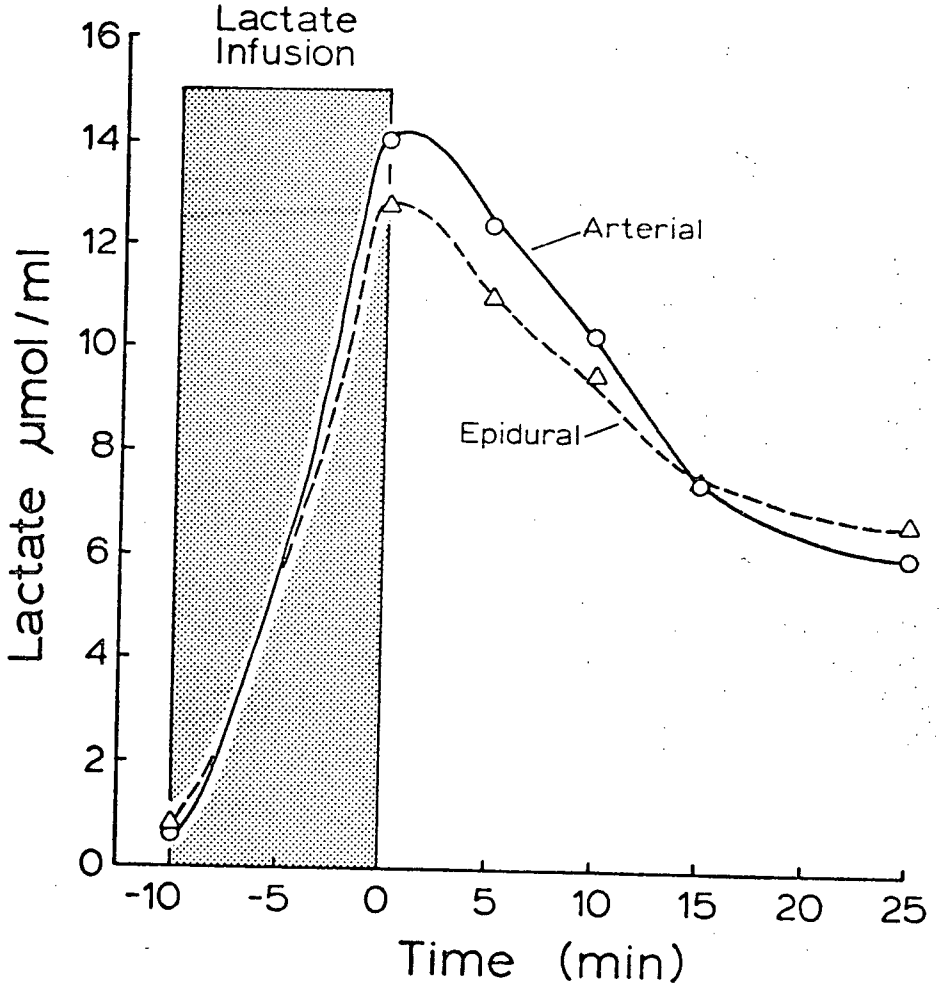

Lactate
Infusion
Arterial
Epidural
Lactate µmol/ml
Time (min)
0
2
4
6
8
10
12
14
16
-10
-5
0
5
10
15
20
25

Fig. VI,2

end-product during the recovery period. It is also expected that, if for any reason (e.g. peripheral leakage) arterial lactate concentrations reach intolerable levels during diving, cerebral metabolism may switch from a glucose to a lactate based oxidative metabolism (assuming O_2 is available).

CHAPTER VII

Fetal Responses to Maternal Diving

"I sometimes wonder why it is that, when a pregnant whale dives, and the fetus in her womb begins to feel that awful weight and the slowing pulse, the little thing is not expelled like a popping cork. Perhaps, when the oxygen level drops, it too begins to "dive" in its own small way, to obey the automatic signals in her blood."

- From the Year of the Whale, Victor B. Scheffer, Charles Scribner's Sons, New York, 1979 -

(i) Blood Metabolite Profiles as a Consequence of Maternal Diving

INTRODUCTION

In overall outline the general metabolic consequences of diving (e.g. peripheral organs sustained by anaerobic metabolism, leading to lactate accumulation and its eventual release into the circulation) have been appreciated, at least, since the classic studies of Scholander (1940) four decades ago. What has not been clarified at all, however, is the metabolic status of the seal fetus during maternal diving. There are two possibilities: either the fetus simply tolerates the consequences of the maternal dive or it too evokes the above diving responses. With respect to bradycardia, direct measurements implicate the latter strategy. Thus, soon after the maternal bradycardia is elicited, a fetal bradycardia develops, heart rates typically falling from about 90 to 30 beats/min by the end of a maternal dive (Liggins et al., 1980). Although absolute flow rates are not known, Liggins et al. (1980) demonstrated that the placenta probably receives an increased fraction of the maternal cardiac output during diving and that the mean arterial pressure of the fetus remains relatively constant (91 to 96 mm Hg) throughout this stressful episode

(Liggins et al., 1980). The implications are, therefore, that the diving response is already developed in the late term fetus. In that event, similar metabolic profiles should be obtained in the fetal circulation as in the maternal system. With this in mind arterial blood profiles of lactate, pyruvate and glucose, a wide spectrum of amino acid and glutathione in feto - maternal pairs of the Weddell seal prior to, during and following normobaric diving were monitored.

RESULTS AND DISCUSSION

Blood Glucose, Pyruvate and Lactate Profiles

In only 3 of 6 fetal-maternal pairs utilized did the fetus show good recovery, with heart rate, PO_2, PCO_2, and blood pH quickly returning to normal levels following diving (Liggins et al., 1980). These 3 pairs were used in 4 different diving experiments summarized in Figs. VII, 1 and VII, 2. The overall pattern is similar in both. Except in one short dive (Fig. VII, 1), maternal blood glucose levels typically decrease slightly during simulated diving. During recovery, glucose levels are gradually restored presumably at the expense of body stores of glycogen (particularly the liver), and because of activated gluconeogenic processes. Concomitant with the fall in maternal blood glucose, there is seen a consistent rise in blood lactate (Figs. VII, 1 and VII, 2). However, this rise is modest compared with the large increase that is observed in recovery due to lactate wash-out from peripheral tissues (Figs. VII, 1 and VII, 2). These patterns for the pregnant seal are similar to those noted for adult males (See Chapter III).

Fetal blood glucose levels differ from maternal ones in two important regards. Firstly, fetal blood glucose concentrations are always substantially higher than in maternal blood; this rather unusual glucose gradient will be throughly discussed later in this chapter.

A second important difference between fetal and maternal glucose metabolism is evident in the response to diving. In the fetus, unlike the mother, blood glucose levels typically rise somewhat as a consequence of diving. In one 20-minute dive (Fig. VII, 1, dive II), the blood glucose

Fig. VII, 1. Glucose, lactate and pyruvate concentration changes in maternal and fetal arterial blood during two experimental dives. This maternal-fetal pair was used 3 hours earlier in a glucose tolerance test, and glucose levels prior to dive I were still falling. Dive I was 10 minutes; dive II was 19 minutes long. F = fetus; M = mother.

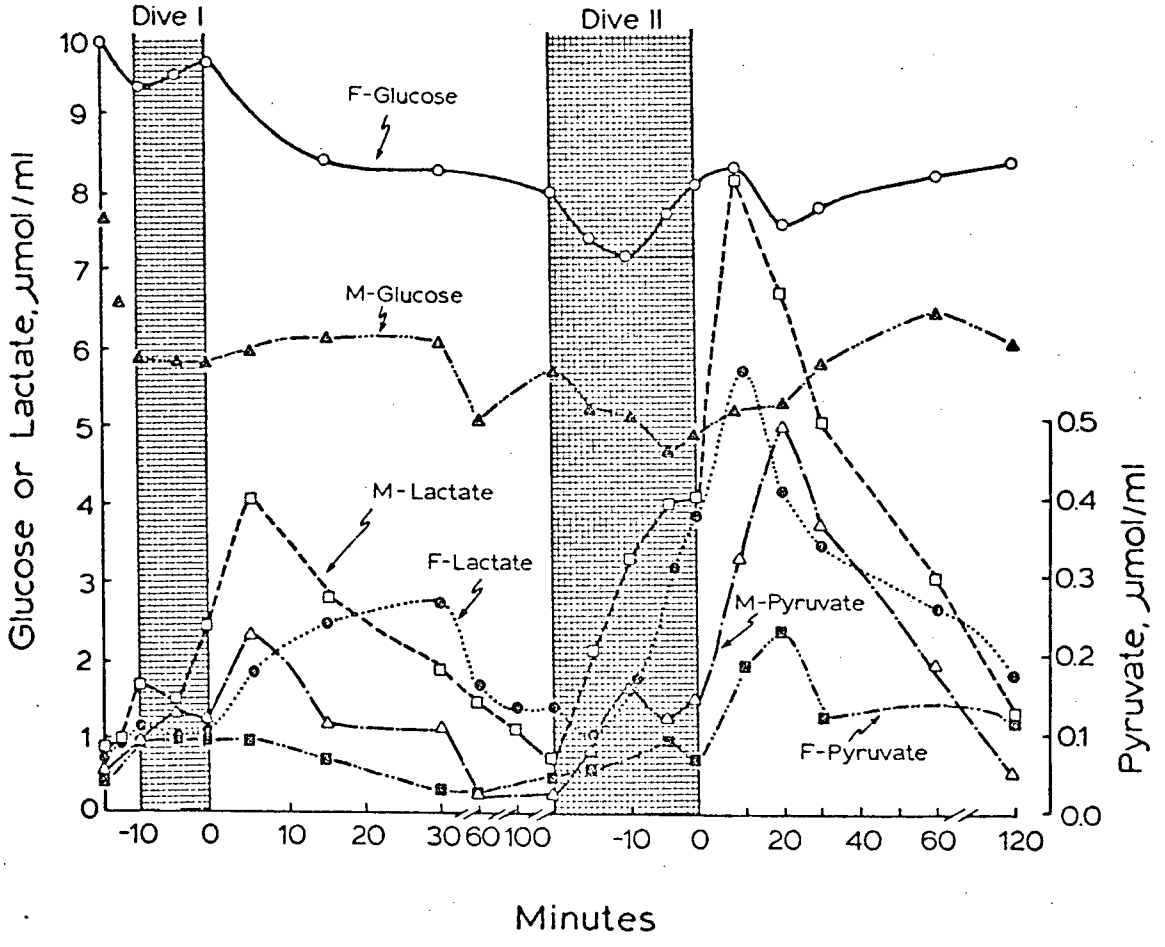

Dive I
Dive II
F-Glucose
M-Glucose
M-Lactate
F-Lactate
M-Pyruvate
F-Pyruvate
Glucose or Lactate, µmol/ml
Pyruvate, µmol/ml
Minutes
10
9
8
7
6
5
4
3
2
1
0
0.5
0.4
0.3
0.2
0.1
0.0
-10
0
10
20
30
60
100
-10
0
20
40
60
120

Fig. VII,1

Fig. VII, 2. Glucose, lacatate and pyruvate concentration curves in a 20 min dive of another maternal-fetal pair, indicating an essentially identical pattern to the 19 minute dive in Fig. VII, 1. In all three dives in Fig. VII, 1 and 2, fetal and maternal PO_2, PCO_2 and blood pH values quickly returned to normal after diving.

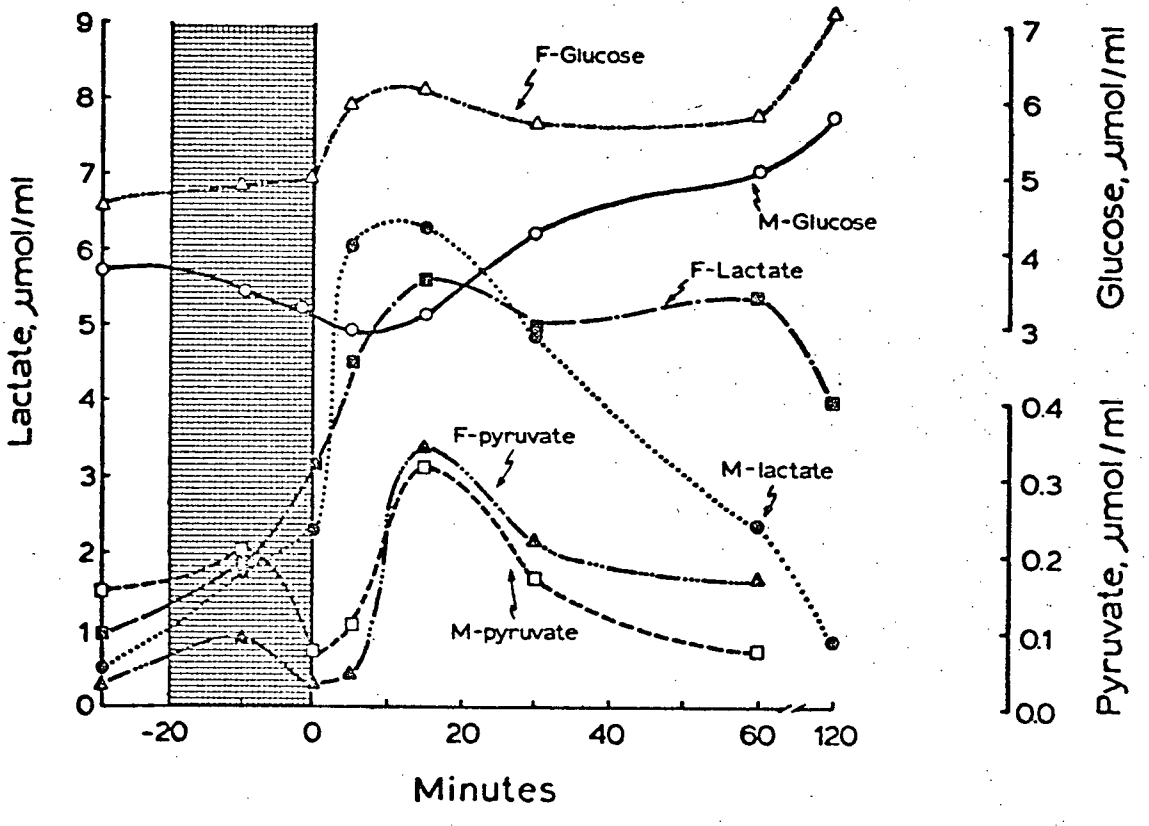

F-Glucose
M-Glucose
F-Lactate
F-pyruvate
M-lactate
M-pyruvate
Lactate, μmol/ml
Glucose, μmol/ml
Pyruvate, μmol/ml
Minutes
-20
0
20
40
60
120

Fig. VII, 2

curve drops initially but then swings upwards again well before the end of the diving period. In other mammals, the fetus is known to mobilize liver glycogen under stressful conditions, a process leading to increased blood glucose levels (Shelly, 1973). However, it is not known if a similar process is activated in the seal fetus during maternal diving. There is no doubt however, that blood glucose regulation of the fetus during diving is regulated independently of that in the mother; otherwise, it would be difficult to understand how fetal levels could be rising at the same time as maternal concentrations are falling (Fig. VII, 1, dive II, for example). Either metabolic factors or perfusion changes in the placenta could lead to the observed glucose profiles, but little information is available on their relative contributions.

Despite evident differences in glucose handling, there is a striking similarity betwen fetal and maternal profiles of blood lactate and pyruvate (Figs. VII, 1 and 2). In the fetus, as in the mother, blood lactate tends to increase during the dive, although the fetal response lags somewhat behind the maternal one. Then, following the dive, there occurs the usual wash-out of lactate and pyruvate from peripheral tissues and hence the recovery "spikes" in lactate and pyruvate concentrations in the blood. Again, the fetal response lags behind the maternal one. Peak concentrations are usually, but not always, lower in the fetus; moreover, in the recovery process, the placental gradients for lactate and pyruvate can be reversed. Of course, a rise in lactate levels during recovery is not conclusive evidence of peripheral vasoconstriction in the fetus for this may simply reflect transplacental equilibration. However, when these metabolic data are coupled to the physiological profiles (decreased cardiac output,

unaltered mean arterial blood pressure plus the well-sustained diastolic pressure during the long intervals between fetal heart beats) they appear to be internally consistent with peripheral vasoconstriction in the fetus during maternal diving.

Another important outcome of this study concerns the lactate and pyruvate profiles throughout diving and recovery. In metabolic terms the importance stems from directions rather than magnitudes of change, for while lactate levels rise continuously during diving, pyruvate levels in fact can fall. That is, lactate production may occur simultaneously with pyruvate utilization, a result consistent with a pyruvate-lactate based hydrogen shuttling system between organs that vary in anoxia tolerance. Such a process has been reported in extremely hypoxic perfused heart preparations (Lee et al., 1973) and on theoretical grounds, was predicted to be involved in the extended hypoxia tolerance of diving animals (Hochachka and Storey, 1975). The process can continue into post-diving recovery as well, which may be why the pyruvate wash-out peaks sometimes occur after the lactate wash-out peaks (Fig. VII, 1 for example). It is noted that this pattern has also been observed in the adult males of the species (see Chapter III).

The physiological implications of the data stem mainly from the recovery patterns, for the easiest way to explain the lactate and pyruvate wash-out curves is to assume fetal peripheral vasoconstriction during the dive. Physiological profiles (Liggins et al., 1980) are, indeed, consistent with this interpretation. Thus, the metabolic and physiological data taken together, indicate that the two key components of the diving response (bradycardia and peripheral vasoconstriction) are both developed

in the near-term seal fetus, and appear to be an integral part of the fetal adaptational responses to maternal diving.

Blood Amino Acid Profiles in Resting Animals

Whole blood concentrations (μmoles/ml) of amino acids in resting Weddell seal and human feto-maternal pairs are presented in Table VII, 1, with a statistical evaluation of the more obvious mean differences between the two groups given in Table VII, 2. A comparison of the amino acid profiles indicates outstanding differences between the two groups of mammals for, at least, alanine. The human mother and fetus display 5 and 2 fold higher concentrations, respectively, than their seal counterparts. Recall a similar qualitative pattern associated with the adult males of the two species. (The possible significance of this trend has been discussed in Chapter III.) Table VII, 1 also suggests substantial taurine differences ($p. < .001$) between the two groups. These differences stem from the feto-maternal (human) levels of taurine as recorded by Velazguez et al. (1976) since their data indicate concentrations of the amino acid are an order of magnitude lower than those presented by Felig et al. (1973) and in Table VII, 1. Although both Felig and I assayed male volunteers, it is difficult to believe that sex or pregnancy differences could cause such huge variations. Furthermore, all three groups employed the sulfosalicyclic acid method of deproteinization, in which cysteine was not protected against oxidation. Cysteine can be oxidized to cysteic acid, cysteine sulfinic acid and perhaps hypotaurine (Awapara, 1976); none appear to co-elute with taurine standards. Taurine can arise by the decarboxylation of cysteic acid and/or the oxidation of

Table VII, 1. Mean arterial concentrations (± standard deviations) of amino acid in whole blood of the Weddell seal and human, feto-maternal pairs. The human sample size (as assayed by Velazguez et al., 1976) was 8 while that of the seal varied between 4 to 7. Human fetal pool profiles in the venous circulation are also presented. This author's calculations of total pool sizes from Velazguez's data are marked by an (*).

Whole Blood Concentration (nmol/ml)

Amino Acid	Weddell Seal		Human		
	Maternal	Fetal	Maternal	Fetal	Fetal (venous)
Taurine	176.0 ± 37.6	133.6 ± 37.4	18.0 ± 4.0	61.0 ± 9.0	53.0 ± 11.0
Aspartate	30.8 ± 9.9	63.0 ± 10.3	32.0 ± 7.0	31.0 ± 5.0	50.0 ± 8.0
Threonine	122.4 ± 22.1	152.2 ± 24.3	175.0 ± 31.0	205.0 ± 28.0	266.0 ± 39.0
Serine	101.3 ± 38.3	141.3 ± 37.0	91.0 ± 18.0	142.0 ± 21.0	177.0 ± 24.0
Glutamate	128.0 ± 28.8	195.0 ± 63.0	116.0 ± 15.0	184.0 ± 28.0	123.0 ± 16.0
Glutamine	359.1 ± 147.0	363.0 ± 85.0	380.0 ± 61.0	428.0 ± 65.0	340.0 ± 61.0
Glycine	124.3 ± 18.3	252.3 ± 89.5	148.0 ± 28.0	101.0 ± 15.0	145.0 ± 18.0
Alanine	87.2 ± 29.2	214.6 ± 77.6	319.0 ± 68.0	455.0 ± 73.0	428.0 ± 75.0
Valine	130.9 ± 34.3	217.2 ± 45.3	107.0 ± 18.0	131.0 ± 18.0	140.0 ± 22.0
Isoleucine	27.6 ± 8.9	35.0 ± 7.0	56.0 ± 11.0	65.0 ± 9.0	80.0 ± 13.0
Leucine	94.4 ± 16.6	147.3 ± 31.2	71.0 ± 14.0	83.0 ± 14.0	96.0 ± 15.0
Tyrosine	24.6 ± 5.7	51.5 ± 4.8	50.0 ± 10.0	61.0 ± 10.0	59.0 ± 11.0
Phenylalanine	48.9 ± 7.6	71.4 ± 16.5	63.0 ± 9.0	48.0 ± 8.0	68.0 ± 10.0
Lysine	110.0 ± 32.2	126.1 ± 33.6	139.0 ± 18.0	147.0 ± 21.0	174.0 ± 17.0
Ornithine	46.1 ± 7.8	63.3 ± 10.5	35.0 ± 6.0	89.0 ± 17.0	83.0 ± 14.0
Histidine	59.4 ± 14.9	94.4 ± 14.5	83.0 ± 12.0	93.0 ± 9.0	150.0 ± 12.0
Arginine	45.0 ± 9.5	69.9 ± 10.2	37.0 ± 9.0	107.0 ± 12.0	98.0 ± 11.0
Total Branched Chained Pool	222.7 ± 52.6	407.2 ± 46.6	234*	279*	316*
Total Pool	1807.0 ± 172.0	2529.0 ± 195.0	1920*	2431*	2530*
Glutathione	1341.4 ± 539.6	605.0 ± 334.0	-	-	-

Table VII, 2. Statistical evaluation of mean differences of amino acid concentrations (arterial pools) presented in Table VII, 1. Subscripts indicate the animal with the higher concentration. W = Weddell seal; hu = human; N.S. = not significant.

Amino Acid	Gravid Weddell seal vs Gravid Human	Fetal Weddell seal vs Fetal Human
Taurine	$p < .001$(W)	$p < .001$(W)
Aspartate	N.S.	$p < .001$(W)
Threonine	N.S.	$p < .01$ (hu)
Serine	N.S.	N.S.
Glutamate	N.S.	N.S.
Glutamine	N.S.	N.S.
Glycine	N.S.	$p < .001$(W)
Alanine	$p < .001$(hu)	$p < .001$(hu)
Valine	N.S.	$p < .001$(W)
Isoleucine	$p < .001$(hu)	$p < .001$(hu)
Leucine	$p < .05$ (W)	$p < .001$(W)
Tyrosine	$p < .001$(hu)	N.S.
Phenylalanine	N.S.	$p < .01$ (W)
Lysine	N.S.	N.S.
Ornithine	N.S.	$p < .02$ (hu)
Histidine	$p < .01$ (hu)	N.S.
Arginine	N.S.	$p < .001$(hu)
Total Branched pool	-	-
Total pool	-	-
Glutathione	-	-

Table VII, 2

hypotaurine. However, cysteine samples treated in exactly the same manner as described in Materials and Methods showed formation of only cysteic acid, which is eluted from the column well before taurine. The above differences will probably be traced to the presence of technical artefacts in one or more groups' procedures.

There are other, not so dramatic, variations of whole blood amino acids between the two different groups (Tables VII, 1 and 2). On the maternal side, the human displays higher levels of isoleucine, tyrosine, and histidine ($p < .001$, .001 and .01 respectively) while the Weddell seal contains slightly more leucine ($p < .05$). On the other hand, the amino acid blood pools of the fetuses appear to be more variable. Beside alanine, the human pool is more concentrated with respect to threonine ($p < .01$), isoleucine ($p < .001$), ornithine ($p < .02$) and arginine ($p < .001$), while the fetal seal exhibits significantly higher concentrations for aspartate ($p < .001$), glycine ($p < .001$), valine ($p < .001$), leucine ($p < .001$) and phenylalanine ($p < .01$).

Measurements of transplacental gradients indicate that the majority of amino acids are more concentrated on the fetal side of the placenta in both groups of animals (see Table VII, 3). Only taurine levels are lower in the fetal seal while glycine and phenlyalanine appear to be reduced in the arterial circulation of the fetal human. (The tripeptide, glutathione, also demonstrates a reverse gradient in the seal). The Weddell seal displays higher ratios (fetal arterial pool/maternal arterial pool) for aspartate, glycine, alanine, valine, tyrosine, phenylalanine and the total branched chained amino acids; human ratios are elevated for taurine, ornithine and arginine. The significances of these results,

Table VII, 3. Fetal/maternal ratios for whole blood amino acid concentrations in the Weddell seal and the human. Values are calculated from the data in Table VII, 1. The seal ratios are derived from the sampling of arterial blood on both sides of the placenta whereas the human ratios are expressed as fetal arterial or venous versus maternal arterial levels. Human ratios are computed from values taken from Velazguez *et al*. (1976).

Subscripts used: a = arterial sample

v = venous sample

Table VII, 3

Amino Acid	WEDDELL SEAL Fetal(a):Maternal(a)	HUMAN Fetal(a):Maternal(a)	HUMAN Fetal(v):Maternal(a)
Taurine	0.76	3.38	2.94
Aspartate	2.03	0.97	1.56
Threonine	1.25	1.17	1.52
Serine	1.40	1.56	1.95
Glutamate	1.52	1.59	1.06
Glutamine	1.01	1.13	0.90
Glycine	2.03	0.71	0.98
Alanine	2.47	1.43	1.34
Valine	1.66	1.22	1.31
Isoleucine	1.25	1.16	1.42
Leucine	1.56	1.17	1.35
Tyrosine	2.08	1.22	1.18
Phenylalanine	1.45	0.76	1.08
Lysine	1.15	1.06	1.25
Ornithine	1.58	2.54	2.37
Arginine	1.56	2.89	2.65
Histidine	1.59	1.12	1.81
Total Branched Chained Amino Acids	1.83	1.19	1.35
Total pool Glutathione	1.40	1.26	1.32
	0.45		

if any, have yet to be ascertained, although they may be reflections of diets, fasting times and different growth requirements of the two groups.

The general gradient favoring the fetal circulation is easily explained by the fact that during gestation the fetal growth requirements increase exponentially, therefore the mother must respond by supplying all the precursors (e.g. essential and some non-essential amino acids) needed for both anabolism and catabolism. It is now thought that this gradient is upheld by active or facilitated transport mechanism for most of the amino acids (Berry et al., 1975).

At this juncture, it should be noted that a proper comparison between maternal and fetal blood pools would involve the sampling of maternal arterial and fetal venous concentrations since nutrients are transported from the maternal uterine artery, via the placenta, into the venous circulation of the fetus. However, technical restrictions in this study dictated catheter placement in the fetal flipper artery (Liggins et al., 1980). Table VII, 3 demonstrates the differences between the two sampling procedures in human pairs. Aspartate, threonine, serine, glutamate, glutamine, glycine and histidine pool sizes appear altered to some extent between the fetal arterial and venous blood. Of course, these probably vary with the physiological state of the mother and fetus.

In summary, this study was performed to provide added metabolic descriptions in fetal aquatic mammals. Some differences in the amino acid pools of resting, feto-maternal pairs of Weddell seals and humans were found; however, there is an overall, general similarity between the two species. Discrepancies in arterial pools (e.g. alanine) and trans-

placental gradients are probably due to different dietary regimes and fetal growth requirements.

Diving Profiles of Blood Amino Acids

Unfortunately, this segment of the study is much too sketchy to extract definitive characteristics of the fetal amino acid pools during simulated diving of the pregnant seal. It is noted that the majority of the free amino acids in fetal whole blood remain relatively constant throughout the dive phase, which is not too surprising in view of the amino acid profiles of the adult seals (Chapter III). Of the three different feto-maternal pairs sampled, there appears to be detectable elevations in both alanine and glutamine levels during, at least, the recovery phase. Both amino acids displayed post-dive peak levels of about 1.4-1.6 fold higher than those of the predive. Again, this appears to be consistent with the adult observations. Actual diving profiles were too scattered to decipher real trends. Until more rigorous studies are performed, it would be inappropriate to consider alanine and glutamine profiles as further evidence of a fully developed diving response in the fetal seal.

Curiously, not one fetus displayed the unique glutathione profiles associated with some of the gravid and male adults. Furthermore, when the maternal glutathione was hydrolyzed, there were no observable increases in the fetal blood pools of either glutamate nor glycine. Amongst other things, this suggested 1) a very tight and selective control of amino acid flow across the seal placenta; 2) maternal (or adult) changes in glutathione blood levels during diving-recovery cylces were not a result of sampling artefacts (see Chapter III).

(ii) Unusual Maternal-fetal Glucose Concentrations In Whole Blood of the Weddell Seal

INTRODUCTION

It is usual for fetal whole blood glucose levels to be lower than, and to fluctuate with, maternal levels. That is, a favourable concentration gradient is maintained to allow for the "facilitated transfer" of glucose across the placenta from mother to fetus (Shelley, 1973; Dawes and Shelley, 1968). This situation is apparently reversed in the Weddell seal. The concentration of glucose in maternal whole blood is lower than that in fetal blood during at least three metabolic states: in the resting, fasting state, in simulated diving, and in recovery from diving (see Chapter VII, i). The explanation for this consistent reversal of glucose concentration gradients between maternal and fetal blood was explored in the present study.

RESULTS AND DISCUSSION

At the outset of these experiments, it was considered important to test for the presence of sugars other than glucose in the fetal and maternal blood. For example, fructose is not only present in most cetaceans (Comline and Silver, 1974) but also occurs in the blood of most mammalian fetuses (Battaglia and Meschia, 1976).

Glucose was normally determined by following the change in optical density at 340 nm due to $NADP^+$ reduction by glucose-6-phosphate dehydrogenase (see Materials and Methods). However, in several fetal and maternal extracts

glucose was determined also by the glucose oxidase method, the Benedict assay system and by an automated glucose analyzer with a linear response over the range of 5-50 μmol/ml. All three assay procedures yielded the same fetal-maternal differences and the same absolute glucose concentrations. To exclude the presence of an unusual sugar, whole blood extracts were analyzed by gas liquid chromatography (Albersheim et al., 1967). These studies showed that glucose was the only major sugar present in maternal and fetal blood; fructose, galactose, mannose, and ribose did not occur in measurable concentrations.

Under resting and fasted conditions (in captivity for at least 12 hours), maternal whole blood glucose concentrations ranged between 4-6 μmol/ml compared to 5-8 μmol/ml in fetal whole blood, with an average gradient across the placenta of approximately 2 μmol/ml. (This is actually an underestimation since arterial samples were used.) Similar levels were found in samples drawn from undisturbed seals asleep on ice. These data may reflect either an active gluconeogenic process or a low rate of glucose turnover. Although the fetal concentrations were consistently higher than maternal ones, the two values were not unrelated and tended to fluctuate in unison (Fig. VII, 3).

Glucose Tolerance Test

To better understand how maternal and fetal blood glucose levels are regulated, a glucose load (1.5 gm/kg of estimated maternal body weight) was infused over a period of 20 min into the maternal circulation of 3 pregnant seals. In all 3 animals, the maternal concentration of glucose in both whole blood and plasma rose to values that exceeded those in the

Fig. VII, 3. A plot of glucose concentrations in whole blood of five maternal-fetal pairs. The different symbols refer to different pairs. Blood samples were taken before, during and after simulated diving. The highest glucose values were obtained after intravenous infusion of glucose.

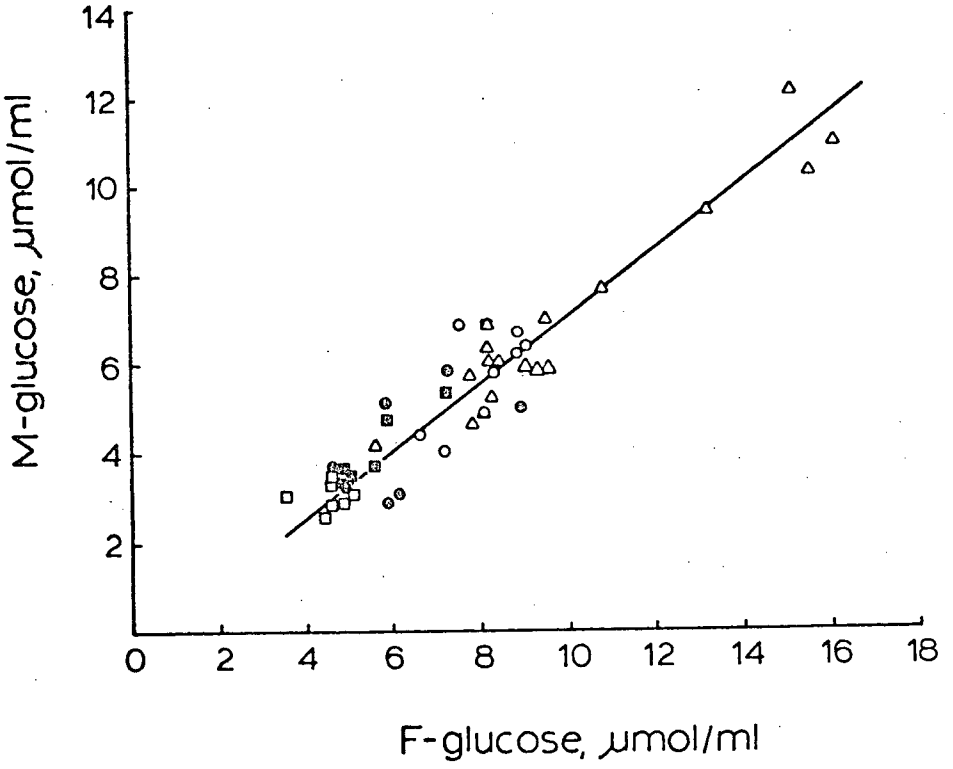


Fig. VII, 3

fetus throughout the infusion and for a short period after it was completed, but within 20-40 min fetal blood glucose levels were again higher than in the mother (Figs. VII, 4A, 4B). The concentration of insulin increased sharply in the plasma of both mother and fetus approximately 15 min after the rise in glucose levels (Fig. VII, 4B). In the fetus, the concentrations of plasma glucose and insulin followed similar patterns but in the mother, insulin levels remained elevated for at least 5 hr after glucose infusion although glucose concentrations were falling. (Insulin and plasma glucose profiles compiled by Dr. G. C. Liggins.)

The reason for the unusual relationship of fetal to maternal glucose content of whole blood becomes apparent when plasma glucose concentrations are compared (Fig. VII, 4B; Table VII, 4). In common with other mammals, glucose levels are higher in maternal plasma than in fetal plasma and glucose moves from maternal to fetal blood down a concentration gradient. The high glucose content of fetal whole blood relative to the mother arises from several factors. First, the feto-maternal gradient of plasma glucose concentrations is small (Table VII, 4). Secondly, the ratio of red cell:plasma concentration of glucose in mother is unusually low whereas that of fetus is similar to other species (Table VII, 4). Finally, the hematocrits of both mother and fetus is high (approximately 60% and 70% respectively) which has the effect of increasing the ratio of plasma:whole blood concentration of glucose, especially in the mother.

Whereas a high haematocrit increases the O_2 carrying capacity of the blood, it clearly reduces the glucose carrying capacity although to a lesser degree in the fetus because of the greater entry of glucose into fetal red cells. The sustained hyperglycaemia after glucose loading

Fig. VII, 4a. Maternal (0) and fetal (●) concentrations of glucose in whole blood following intravenous infusion of glucose (1.5 g per kg). Time zero indicates the end of the 20 minute period of infusion.

Fig. VII, 4b. Maternal glucose (0) and insulin (▲) and fetal glucose (●) and insulin (△) concentrations in plasma from the same glucose tolerance test as shown in 4a.

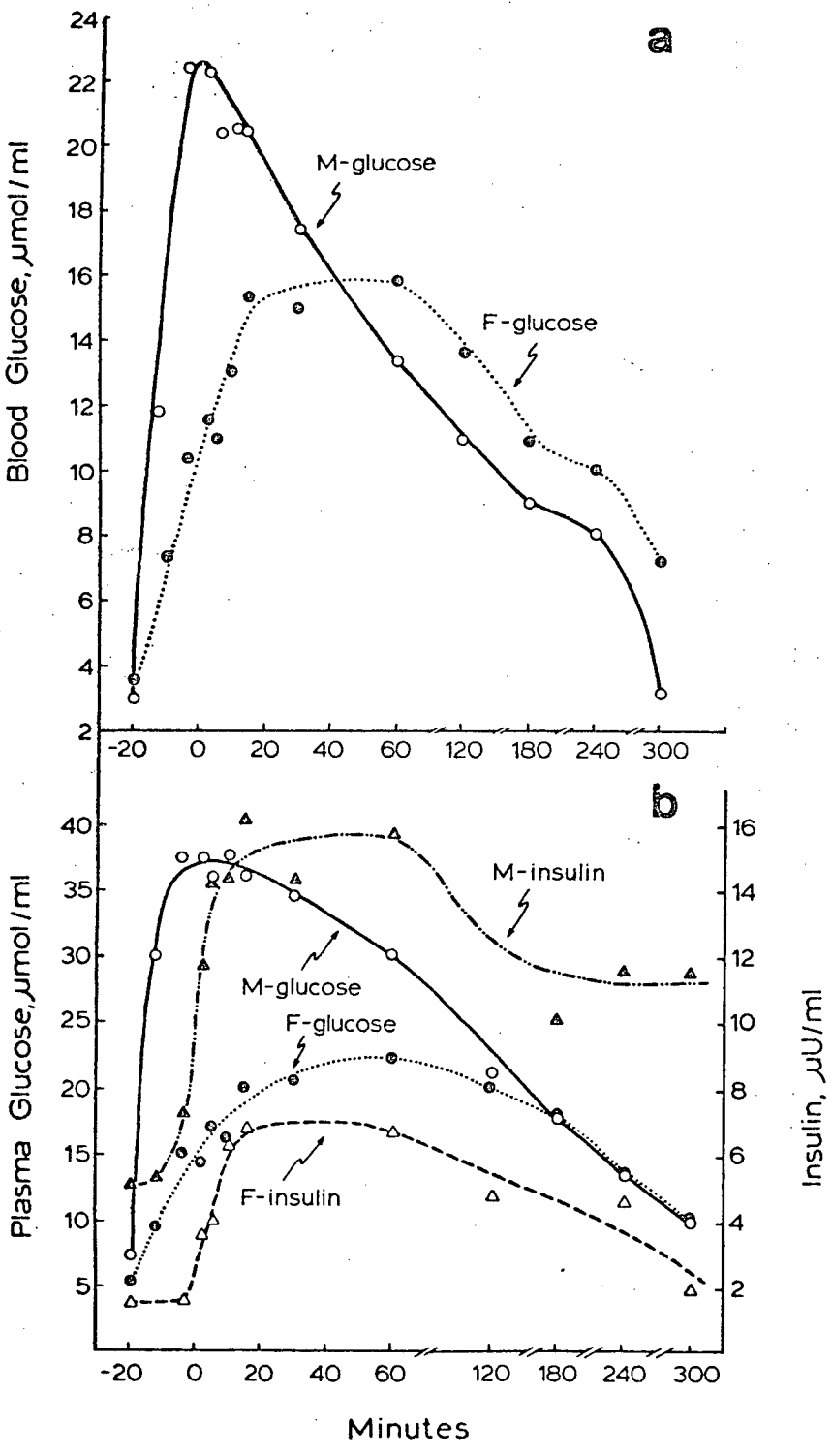

a
M-glucose
F-glucose
Blood Glucose, µmol/ml
24
22
20
18
16
14
12
10
8
6
4
2
-20
0
20
40
60
120
180
240
300
b
M-insulin
M-glucose
F-glucose
F-insulin
Plasma Glucose, µmol/ml
40
35
30
25
20
15
10
5
Insulin, µU/ml
16
14
12
10
8
6
4
2
Minutes

Fig. VII, 4a, 4b

Table VII, 4. Fractional distribution of glucose between plasma and red blood cells in maternal and fetal Weddell seal compared with the sheep.

Species	Condition	Glucose (μmol ml^{-1}) Whole blood	Plasma	Red cells	Ratio plasma: red cells
Weddell seal (adult)	Pre-infusion	3.1	7	0.5	14
	60 min post-infusion	13.5	30	2.5	12
Weddell seal (fetus)	Pre-infusion	3.5	6	2.4	2.5
	60 min post-infusion	15.8	22.5	12.9	1.7
Sheep (adult)	Post-prandial		2.5	0.3	8.3
Sheep (fetus)			0.7	0.2	3.5

Table VII, 4

(Figs. VII, 4A, 4B) suggests that the rate of glucose turnover in the adult seal is low. Likewise, the small gradient of plasma glucose concentrations across the placenta may suggest a low turnover rate in the fetus.

The data derived from these studies may reflect a very important adaptation of the fetal seal as a consequence of the maternal diving habit. The high glucose stores of the fetal blood are viewed as a buffering system protecting the fetus against periods of hypoglycemia and hypoxia, possibly associated with the well recorded, long duration diving of the gravid Weddell seal (Elsner et al., 1968).

(iii) Enzymes of Aerobic and Anaerobic Metabolism in the Three Central Organs of the Fetal Weddell Seal

INTRODUCTION

It was reasoned that a determination of maximum potentials of some important enzymes associated with glycolytic anaerobosis and the aerobic oxidation of carbohydrates, fats and ketone bodies would lay a foundation for future metabolic work on the heart, lung and brain of the fetal Weddell seal (see Chapter IV for the rationale behind this reasoning). Before this study there were no known reports on enzymatic profiles of any fetal tissue in any of the marine mammals; however such a thorough plotting was considered to be out of the scope of this thesis and thus must await future analyses. Since no companion 'in vivo' data were generated, results were compiled with some intuitive speculation as to their metabolic

significance. The focus of this study centered upon two main questions:

1) What are the metabolic preferences of the heart, lung and brain?

2) Is the fetal enzymatic machinery primed for both high aerobic and anaerobic fluxes?

It is now well established that the terrestrial mammalian fetus not only exhibits relatively high oxygen consumption rates (Battaglia and Meschia, 1976) but also possessesa marked tolerance to hypoxemia (Dawes, 1968). It was reckoned that the hypoxia tolerance may be more emphasized and thus observable on the enzyme activity level in the fetus of aquatic mammals.

RESULTS AND DISCUSSION

The data in this section were derived from 2 to 5 near-term Weddell seals, ages unknown. Presumably, these fetuses were still developing at the time of experimentation and, therefore, it is possible that there may be some resultant scatter in the activity profiles.

Enzymes of Oxidative Metabolism

Four mitochondrial marker enzymes associated with oxidative pathways of the fetal heart, lung and brain were used: β-hydroxybutrylCoA dehydrogenase and β-hydroxybutryrate dehydrogenase, functioning in β-oxidation of fatty acids and ketone body metabolism respectively; citrate synthase, catalyzing the entry of acetylCoA carbon into the Krebs cycle and thought to represent an important control site (Tischler *et al.*, 1977) and glutamate dehydrogenase, a key regulatory enzyme (Srere, 1969), catalyzing the entry of glutamate carbon into the Krebs cycle. As the heart, lung

and brain differ greatly in metabolic organization (Siesjo and Nordstrom, 1977; Neely and Morgan, 1974; Tierney, 1974a), their enzyme profiles also show significant differences (Table VII, 5). Organ by organ comparisons indicate that the activities of oxidative enzymes in the fetal heart, lung and brain are very similar to those in homologous tissues of the adult seal (Refer to Tables VII, 5 and IV, 1). β-hydroxybutyrylCoA dehydrogenase in the fetal heart appears to be the only exception, displaying about a two fold increase over the adult seal (32.5 vs. 16.0 μmole/min/g). Since this enzyme was assayed in only two animals, I find it difficult to attach much significance to the data. Otherwise, it is concluded that the near-term fetus of the Weddell seal has developed its aerobic capabilities to the same extent as the mature adult. However, without supporting 'in vivo' data it is impossible to assume similar metabolic preferences and regulation. These enzymatic potentials may be merely developed in preparation for extrauterine existence. Furthermore, it is interesting to note that the fetal heart appears to exhibit highly developed fat catabolic enzymes when it is thought that mammalian, fetal myocardium consumes predominantly glucose under aerobic conditions (Battaglia and Meschia, 1976). Such a situation may reflect this preparation for post-fetal life or could actually be a valid index of the aerobic metabolism of these beasts.

Enzymes of Anaerobic Glycolysis

Hexokinase, phosphofructokinase and pyruvate kinase, which are all potential regulatory sites in glycolysis (Scrutton and Utter, 1968), and lactate dehydrogenase, catalyzing the terminal step in glycolysis were

Table VII, 5. Enzyme activities in brain, heart and lung of the Weddell seal fetus expressed in terms of μmoles substrate converted/ min/gm wet tissue weight at $37^{o}C$, pH 7.4, and saturating levels of substrates, cofactors and coenzymes. See Methods and Materials (Chapter II) for detailed assay conditions. Three to five fetal seals were sampled and the values given are averages $\pm$ S.D., with the range of values in brackets below.

*indicates values derived from 2 animals.

	Enzyme Activity		
	Brain	Heart	Lung
Citrate Synthase	17.40 ± 0.70	25.00 ± 11.90	1.10 ± 0.20
	(16.90 - 17.80)	(16.50 - 42.00)	(0.92 - 1.27)
Glutamate dehydrogenase	5.10 ± 2.90	3.46 ± 1.70	0.50 ± 0.21
	(2.40 - 8.30)	(1.65 - 5.38)	(0.36 - 0.80)
β-hydroxybutyrate dehydrogenase	0.28 ± 0.08	2.20 ± 1.00	1.50 ± 0.90
	(0.20 - 0.36)	(1.20 - 3.20)	(0.84 - 2.10)
β-hydroxybutyrl CoA dehydrogenase	3.60 ± 1.20	32.50*	2.30*
	(1.90 - 4.40)	(26.50 - 37.20)	(3.40 - 1.20)
Hexokinase	3.50 ± 1.90	2.50 ± 0.80	0.97 ± 0.38
	(2.10 - 5.70)	(1.40 - 3.00)	(0.28 - 1.40)
Phosphofructokinase	6.90*	10.50*	3.70*
	(4.80 - 8.90)	(8.90 - 12.00)	(2.70 - 4.60)
Pyruvate kinase	115.10 ± 11.10	228.00 ± 58.00	40.60 ± 5.50
	(102.40 - 127.70)	(145.00 - 257.00)	(35.40 - 46.30)
Lactate dehydrogenase	167.00*	957.00 ± 192.30	41.00 ± 19.30
	(150.00 - 187.00)	(747.00 - 1125.40)	(21.70 - 603.00)

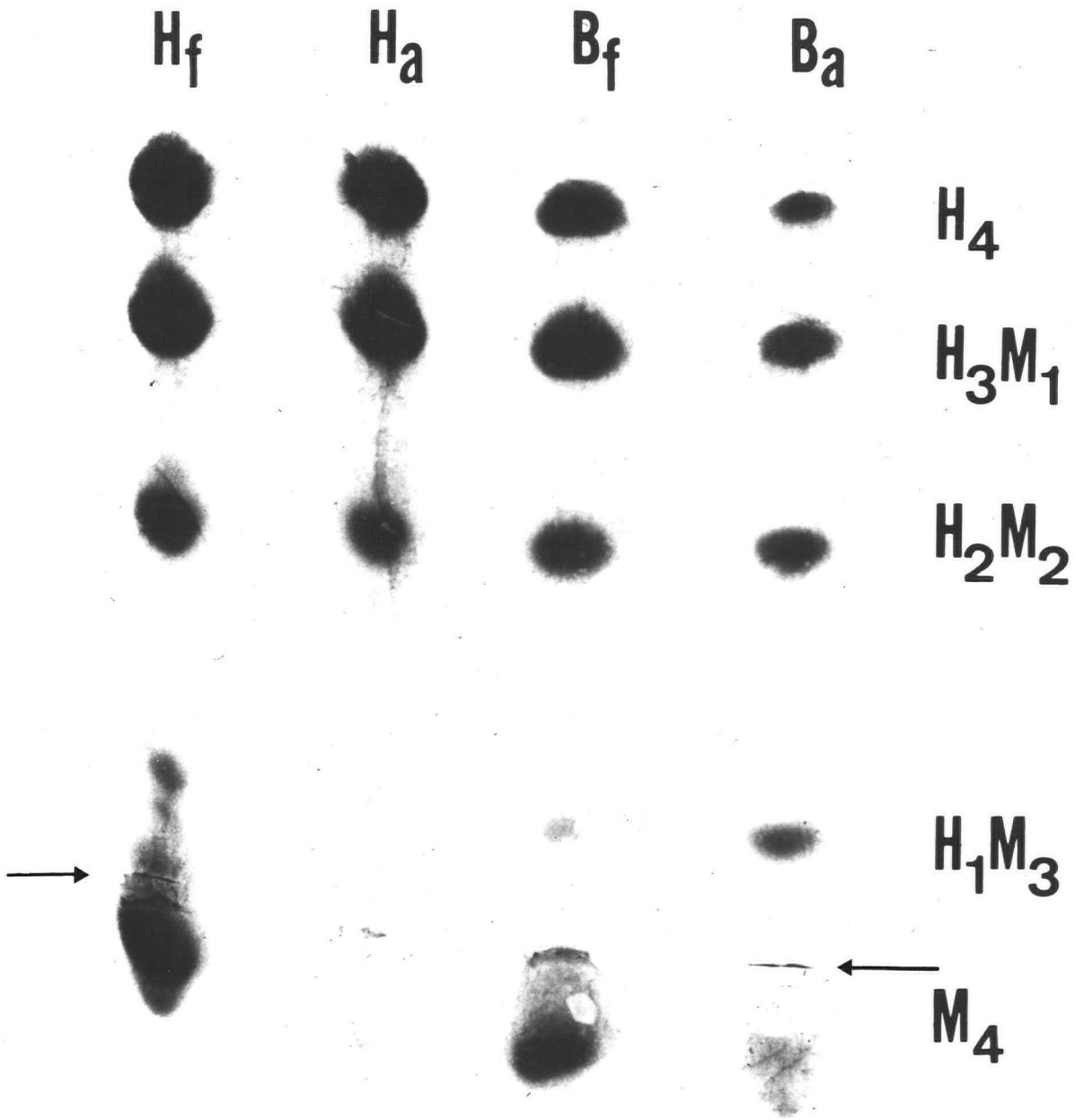
Table VII, 5

measured to qualitatively assess the potential for anaerobic and aerobic glycolysis in these organs. Here again, enzymatic profiles approaching those of the adult (Tables VII, 5 and IV, 1) were found thus indicating the possible operational existence of a similar metabolic organization to that of the adult organs. Electrophoretic evidence (Fig. VII, 5) points to modest elevations in muscle-type subunits of LDH in both the heart and brain, indicating a possible adaptation to anaerobosis in these fetal tissues. Nevertheless both organs clearly have the potential either for lactate production, catalyzed most effectively by muscle-type lactate dehydrogenase, or for lactate utilization, catalyzed most effectively by heart-type lactate dehydrogenase (Holbrook et al., 1975).

To recapitulate, these enzyme profiles indicate that, at least, the brain and heart may have some improved anaerobic potential as indexed by LDH subunit distribution; however, such differences are subtle and probably do not reflect any improved anaerobic capacity of the central organs in the aquatic mammal's fetus as compared to the fetuses of other terrestrial species.

At this point, it should be re-emphasized that definitive explanations regarding the metabolic preferences and regulation of the three central organs of the fetus must await more thorough 'in vivo' studies.

Fig. VII, 5. Starch gel electrophoretic separation of heart (H) and brain (B) of the fetal and adult Weddell seal. Electrophoresis conditions: 25 mA; 200 V; 12 h at $4^{o}C$; anode at top; origin marked with an arrow. Subunit composition of each isozyme is shown on right. Subscripts f and a refer to fetal and adult respectively.


Hf
Ha
Bf
Ba
H4
H3M1
H2M2
H1M3
M4

CHAPTER VIII

Summating Remarks

Central Organ Metabolism as a Consequence of Diving

Previous to these studies no concerted effort had been applied to unravelling the metabolic profiles of the three central organs (brain, lung and heart) during diving and recovery in marine mammals. Originally, the diving response was thought to conserve O_2 for all three organs (Scholander, 1940). Since then most of the relevant reports found in the literature (see Introduction) have strongly emphasized 'suspected' anaerobic potentials in both the brain and heart of aquatic mammals. However, none of these unequivocally demonstrated the existence of such a metabolic potential nor conditions under which it may be activated. This thesis, in concert with other current 'in vivo' studies attempts to clarify the situation. A revised integrated modus operandi during diving and recovery cycles can be pieced together, with special emphasis on metabolic functions in the heart, lung and brain.

The data presented in this thesis implicate free blood glucose as a critical carbon and energy source for the Weddell seal during simulated diving, when physiological responses are maximally evoked. Although glycogen may be mobilized, a precise contribution of endogenous substrates to metabolism has not yet been quantified for any tissue in any diving mammal.

Of the three central organs, the brain is considered to be the major glucose absorber, utilizing approximately 3 mmoles of the carbohydrate over a 20 min dive period (assuming a 500 g brain with a CBF of 700 ml/kg/min). Predictably, the peripheral tissues (e.g. skeletal muscles) appear responsible for quite a large fraction of the total depletion, observed during the dive and into the first 10-15 min of recovery when the entire

blood volume is remixed (see Fig. III, 1).

The available data also demonstrate steady increases of lactate levels, concomitant with the glucose decreases, in the central bloood volume during diving periods of up to 50 min. Although most of this lactate probably originates from anaerobic metabolism of the peripheral tissues, some (1500 μmoles/20 min dive) is directly derived from cerebral metabolism. At first glance this rather high output of lactate, which accounts for 25% of absorbed glucose by the brain would seem to intimate cerebral aerobic metabolism during diving, as predicted by other groups (see Chapter I). Although it is still possible that cerebral metabolism may, by necessity, increase its dependency on anaerobic-derived energy, most of the present metabolic and physiologic measurements do not substantiate this postulate. Firstly, cerebral enzyme profiles indicate very modest potentials for increased fluxes through the glycolytic pathway to lactate as compared to other mammals. Secondly, the cerebral A-V differences for both glucose and lactate, associated with forced diving of 10-30 minutes in the Weddell seal, indicate very little activation of cerebral anaerobic glycolysis despite PaO_2 levels below 30 mm Hg, a condition considered hypoxic to non-diving animals (Siesjo and Nordstrom, 1977). Furthermore, there was no observed compensatory increase in CBF (Zapol et al., 1979), as would be expected if O_2 supply were severly limiting. This phenomenon has also been observed in harbor seals and northern elephant seals (Bron et al., 1966; Kerem et al., 1971; Van Citters et al., 1965).

Nevertheless, it is stressed that the data of this thesis were mainly derived from simulated dives lasting no longer than 30 min, and

thus, it remains impossible to predict whether the brain would remain in a metabolic state akin to that of the pre-dive throughout longer and strenuous diving episodes.

As for the lung and heart, all available evidence suggests that diving metabolism remains largely oxidative, utilizing exogenous lactate as a primary carbon and energy source.

The evidence, in support of the terrestrial, mammalian lung functioning as a lactate absorber and burner, is undeniably strong (see Introduction). Recent work by Hochachka et al. (1977a) has demonstrated the ability of the Weddell seal lung to generate a lactate arteo-venous concentration gradient of 0.1 to 0.25 μmol/ml during a forced dive. This same study also pointed to the ability of the lung to release 0_2 into the central circulation for up to 50 minutes of simulated diving; thus, it is probable that the organ receives enough 0_2 to fire its own lactate-based oxidative metabolism for extended periods of apnea. The radiotracer work, reported in this thesis, on both the intact Weddell seal preparation and on the animal's lung slices unequivocally illustrate pulmonary capacity can oxidize lactate in preference to glucose. Similar trends have also been detected in harbor seal lung slices (B. Murphy and B. Emmett, unpublished data). It is, therefore, tentatively concluded that although this metabolic property of the lung is ubiquitously observed throughout the class Mammalia, it probably holds more functional significance for the aquatic mammals who routinely face extended periods of hypoxia (with the concomitant increases of lactate and decreases of glucose in their circulatory systems and tissues).

Unfortunately, definitive 'in vivo' experimentation has yet to be

attempted on the seal heart. However, all available data imply myocardial uptake of lactate during diving and recovery episodes. This conclusion is based on several observations:

1) heart work during the diving phase in, at least, the Weddell seal probably remains supported by oxidative metabolism. This stems from measurements of blood flow, cardiac output and arterial pressure (Zapol et al., 1979), all of which indicate a closely matched work load and coronary blood supply (R. Brill, pers. commun.). It can be further argued, even if the heart does rely somewhat on anaerobic glycolysis, its lactate output would be minimal due to its drastically reduced work rate.

2) Whereas oxidative metabolism in the mammalian heart may be fired by a variety of substances (glucose, fatty acids, lactate), lactate is known to be preferentially utilized whenever concentrations rise above normal (Mochizuki et al., 1978, Liu and Spitzer, 1978).

3) The Weddell seal heart LDH activity is the highest amongst all comparatively sized mammals thus far studied and is kinetically bifunctional to act as a catalyst for lactate oxidation. The isozyme distribution pattern of the heart LDH is quite similar to that occuring in the brain and lung and since this study has demonstrated the capability of both to take up lactate, there is no reason why the heart cannot also metabolize it.

To recapitulate, the present data imply that all three central organs remain in an oxidative metabolic state throughout diving episodes associated with mean PaO_2 levels near 25 mm Hg. Blood glucose supplies appear to be preferentially utilized by the brain and non-specified peripheral tissues with the lung and probably the heart absorbing and

oxidizing lactate produced by cerebral and peripheral tissue metabolism. This metabolic cooperation between the three central organs thus aids in maintaining reasonably low levels of the acidic endproduct (of anaerobosis) in the central circulation while sparing limited blood glucose supplies for cerebral utilization during subsequent diving.

In comparing the data of this thesis with that of the closely related investigations of Hochachka et al. (1977a) it was noticed that the blood glucose profiles of the dive phases differed rather dramatically. Whereas Hochachka's group (1977a) routinely observed glucose asymptotes midway into 40-50 min dives, when levels fell to 3.8-4.5 µmol/ml, the present data (see Fig. III, 1) do not clearly indicate such trends; however, the blood concentrations of glucose never did drop below 3 µmol/ml (Fig. III, 1). Glucose asymptotes may have been detected, in this study, if blood sampling had been more frequent during the latter stages of the simulated dives. Parenthetically, physiological parameters from the two studies indicate the occurrences of 0_2 asymptotes near $Pa0_2$ values of 25 mm Hg. (Liggins et al., 1980; Hochachka et al., 1977a; J. Qvist, pers. commun.). Although no particular glucose concentration appears to initiate a steady state mechanism one would assume if the dive times of the present study had been extended, a drop in the blood glucose levels (below 3.0 µmol/ml) would have certainly resulted in the activation of compensatory mechanisms. For example the average adult seal (500 kg; 60 l blood) stores about 300 mmoles of free blood glucose, of which about 60 mmoles are utilized during 20 min dives (final glucose depletion about 1 µmol/ml blood). If the same depletion rate continued over a 70 min simulated dive, over 200 mmoles of glucose would be utilized;

a state which would surely render the organism hypoglycemic. It is therefore not too surprising that blood glucose levels, following 50-70 min of voluntary diving in the field (samples supplied by G. L. Kooyman) were found to be approximately 3 μmol/ml. The suspected compensations could take a variety of forms:

1) Metabolic compensation. In this scheme the metabolic activity of certain organs or tissues could be reduced in response to the lowered glucose levels and/or PaO_2 levels. The substantial drops in aortic blood temperatures, glucose and oxygen levels during voluntary dives, in excess of 50 min, as reported by Kooyman et al. (1980) could be easily explained by the above strategy. On the other hand, a recent study by Gallivan and Ronald (1979) suggests that free diving episodes of another phocid, the harp seal (Phoca groenlandica) were not associated with any detectable depression of metabolism. Since companion metabolite studies were not performed by Gallivan and Ronald (1979), the above proposal remains essentially untested.

2) Gluconeogensis and glycogenolysis. Although blood perfusion to the liver and kidney are reduced, it is still not totally restricted from reaching these organs. For example, in the adult Weddell seal the kidney receives 3% of the total cardiac output during diving and, in terms of absolute blood flow, receives more blood than the heart (Zapol et al., 1979). Therefore, it is plausible for both organs to participate in not only glycogenolysis but also gluconeogensis. These processes could be activated by the low glucose and/or the high catecholamine levels circulating during diving (Exton and Park, 1967; Exton et al., 1970). Other organs such as the heart and lung may also prove to be significant

in glucose homeostasis.

If the copious, myocardial glycogen deposits (60-120 μmol/g wet weight) were to be mobilized and released into the circulation, the central blood could see up to 150 mmols of glucose assuming an average cardiac weight of 1.2 kg (see Kerem et al., 1973). This process could be made possible by the significant glucose-6-phosphatase activities occuring in the heart (see Chapter IV).

Earlier studies (Hochachka et al., 1977a) have considered the gluconeogenic capabilities of the Weddell seal lung and have postulated its role as a glucose supplier during diving. However, following ^{14}C-lactate infusion, it was not possible to detect any release of labelled glucose from the lung. Furthermore, the lung stores small quantities of glycogen and, hence, it is tentatively eliminated as a significant glucose source for the central circulation. The most important metabolic function of the lung during the dive phase is now assumed to be the oxidation and anabolism of lactate to compounds such as lipids.

3) Alternate fuel supplies. It is possible that some tissues switch fuel sources throughout a dive in response to lowered levels of blood glucose. Although blood levels of free fatty acids and ketone bodies have yet to be monitored throughout prolonged diving, it is improbable that any organism would burn highly reduced fat or ketone bodies in preference to glucose under low oxygen conditions associated with diving at the time of the glucose asymptotes (Hochachka et al., 1977a). Tissue or blood-derived amino acids could play pivotal roles for it has been demonstrated that animals such as salmon rely quite heavily on amino acid catabolism when carbohydrate stores are depleted (Idler and Clemens, 1959). More specifically, Hochachka and associates (1975) proposed a

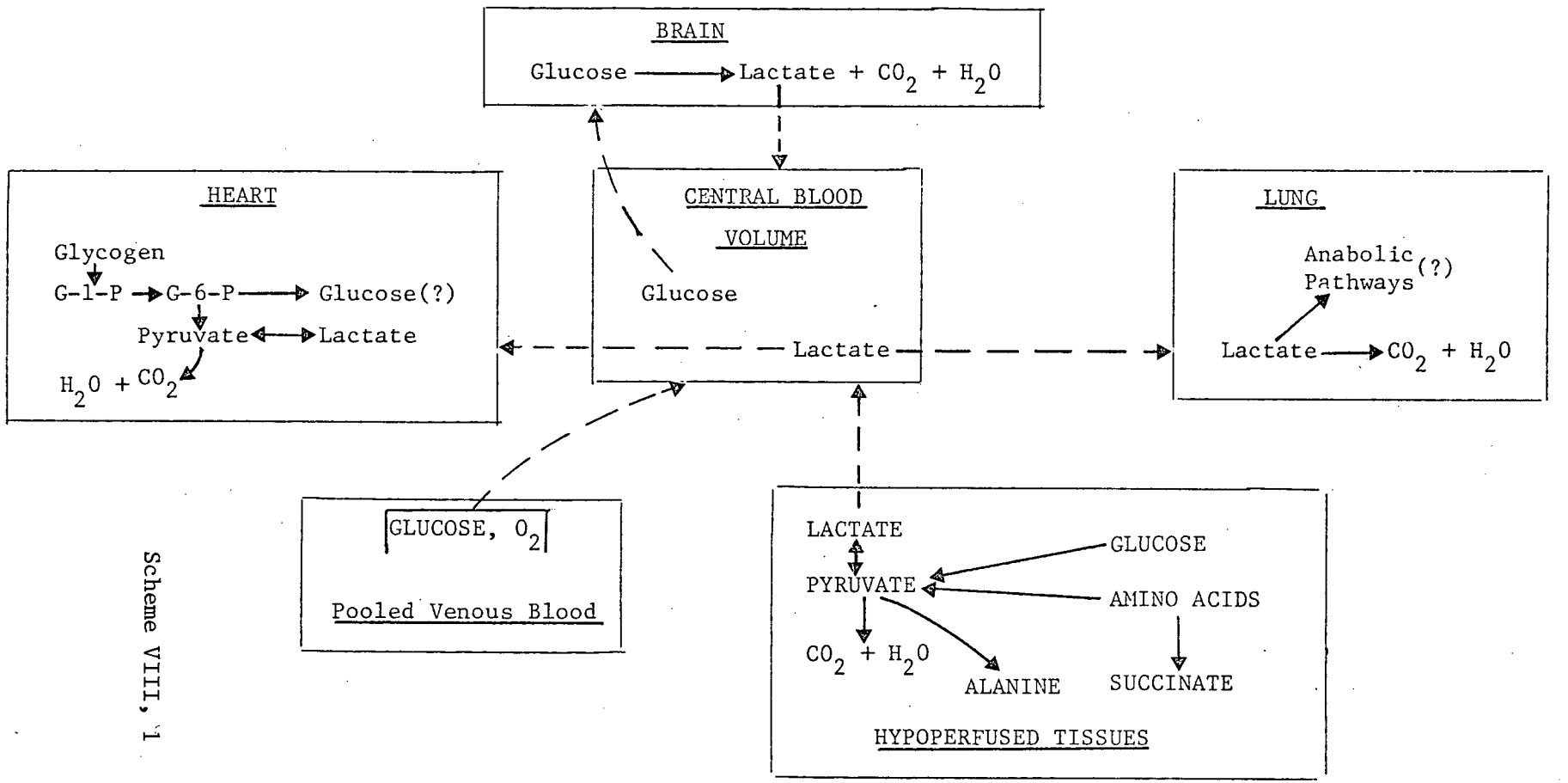
scheme involving anaerobic protein catabolism in the musculature of marine mammals during diving. (This could explain why the skeletal muscles of these animals have relatively low concentrations of both glycogen and fat; see Blix, 1976.) Whether the central organs have the potentials for this proposed set-up and its activation during low-glucose stress are unknown. If any of these fuel sources are to aid in the generation of energy (ATP), substrate phosphorylations must be integral hallmarks of the alternative catabolic pathways. This criterion alone would reduce the potential list of substrates to amino acids (see Hochachka et al., 1975).

4) Circulatory adaptations. As previously stated, the phocid seal may pool as much as 85% of its total blood volume in the venous system as a result of the diving response. A significant portion of this pooled blood is apparently stored in a post-diaphragmatic enlargement of the cranial portion of the abdominal vena cava, known as the hepatic sinus and presumably, this reservoir can be slowly added to the central blood volume by means of a muscular caval sphincter (Harrison and Kooyman, 1968). Herein lies the attraction of this system, for this 'leaking blood' could not only supply fresh glucose stores but also more O_2 and, thus could account for the steady-state or asymptotic behaviour of one or both substrates.

Despite this rather attractive explanation, it is conceivable that the Weddell seal could utilize combinations of the above strategies in response to lowered blood glucose and/or O_2 levels.

The following is a tentative diagramatic model of the metabolic status of the more important components during simulated diving. This encompasses most of the relevant data now available.

Scheme VIII, 1. Diagramatic model of some of the more important metabolic interactions between the central blood pool, brain, lung, heart and other hypoperfused tissues during simulated diving. Where possible (e.g. heart), fatty acid catabolism may remain operational throughout the aerobic phases of diving.



Scheme VIII, 1

Although not included in the above model, it is important to re-emphasize the possible existence of inter-tissue cycling of other metabolites besides that of glucose and lactate. Recall from Chapters III and VII that pyruvate profiles do not always follow those of lactate; in fact, pyruvate can be decreasing while lactate is steadily increasing during the dive phase. This could be merely a reflection of transport discrepancies or may stem from an inter-tissue hydrogen cycle, in which organs of different redox states exchange pyruvate and lactate.

Since data on glutathione profiles are so erratic, no further speculations regarding any metabolic implications of this phenomenon are advanced (see Chapter III for a discussion).

Recovery

The metabolic situation in the recovery period appears to be better elucidated and perhaps represents a simpler working system. Upon surfacing, there is an immediate remixing of partially trapped venous blood with that of the central circulation; concomitant with this washout come large spikes of lactate, alanine and glutamine from the peripheral tissues and organs. Presumably, the brain, lung and, most likely, the heart absorb and oxidize lactate in preference to glucose at this time (see Mochizuki et al., 1978 and Liu and Spitzer, 1978 for discussions on the mammalian heart). Consequently, all three central organs could derive a large percentage of their energy and carbon needs directly from this anaerobic endproduct. If basic metabolic features observed in the mammalian class hold for the aquatic mammals, it can be predicted that many more tissues and organs will actively 'co-operate' in clearing the system of lactate. The mammalian liver, kidney (Scrutten and Utter, 1966) and some muscle fibers (McLane and Holloszy, 1979) exhibit

de novo synthesis of glucose and glycogen from lactate. Gluconeogenesis in at least the liver is further sparked by rapid and significant increases in blood glycogen during recovery (Robin, pers. commun.). Glucose stores in the blood may be further replenished by the action of the high levels of circulating catecholamines, since these hormones not only activate glycogen breakdown in the liver and the muscles (Pilkis et al., 1978) but also apparently stimulate gluconeogenesis in the liver.

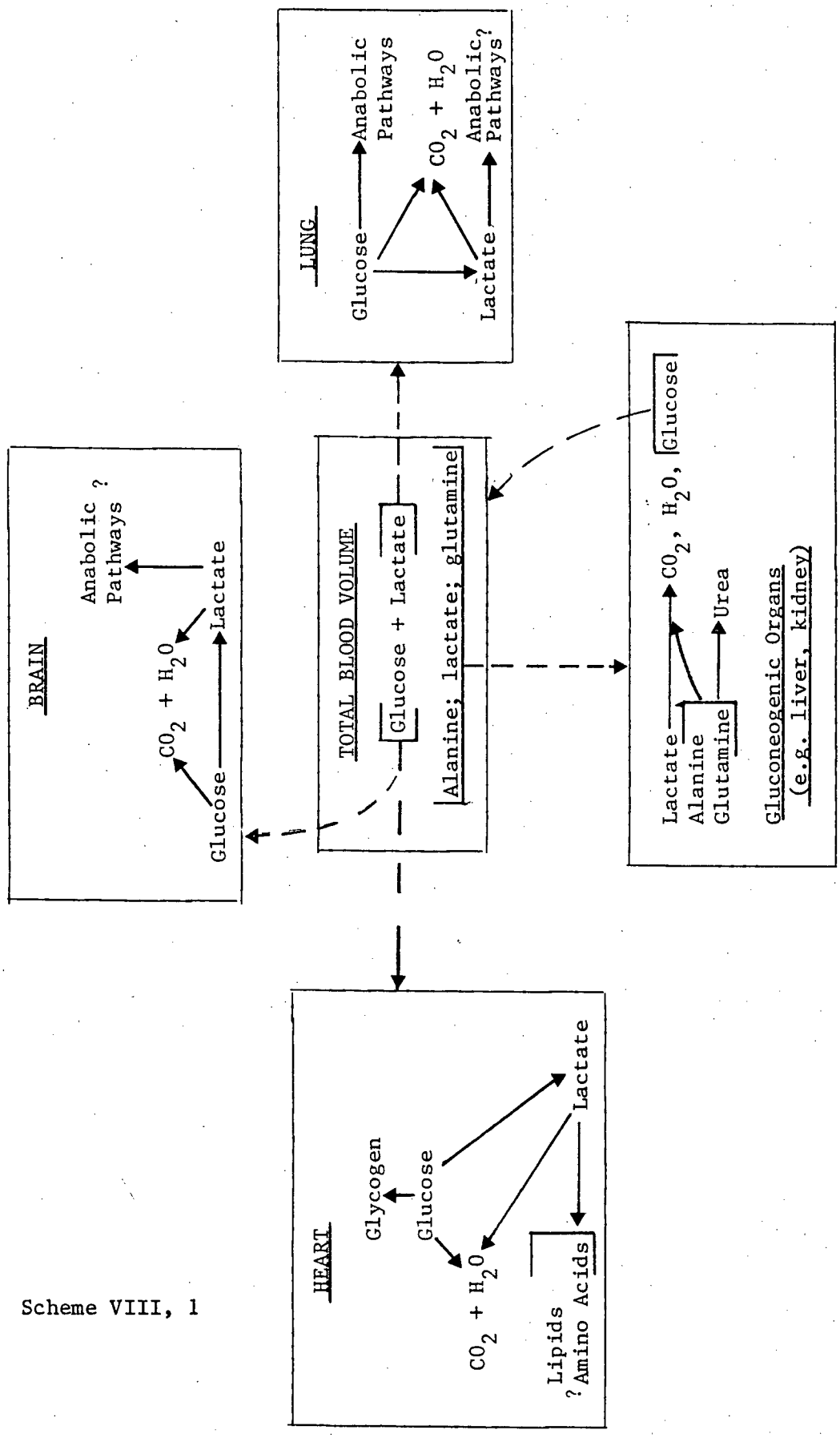
Recent studies by Davis and Kooyman (1979) on the harbor seal, indicate that 29% of the total blood lactate is converted to blood glucose in the recovery phase of a routine 10 min dive (forced), whilst 8-26% is apparently oxidized. Since their methods do not allow for tracing of lactate and glucose beyond the blood volume it can only be assumed that the remainder of the lactate is either converted to glycogen or to other metabolites such as amino acids. As previously mentioned, glutamine and alanine pools in the aortic blood are also substantially elevated in the recovery phase. Parenthetically, the rises in both amino acids during diving are likely caused by peripheral leakage. The increases may be the result of anaerobic catabolism of protein and carbohydrate in the peripheral tissues (see Chapter III and Hochachka et al., 1975); whereas the alanine increases could be further implicated with the sparking of the tricarboxylic acid cycle during the anaerobic-aerobic transitions, associated with recovery in such tissues as the muscles (Hochachka and Murphy, 1979). Regardless of their origins, both metabolites are likely absorbed by the liver and kidney where they may serve as precursors to gluconeogenesis (Felig, 1973; Hochachka and Murphy, 1979). Furthermore, alanine increases may cause consistent increases in plasma glucagon

concentration (Muller et al. 1970) and thus be indirectly responsible for the increased gluconeogenesis from lactate and glutamine and of course alanine.

In view of the above considerations it is not surprising that blood glucose levels quickly return to normal and, in fact, actually overshoot control concentrations. In the phocids these mechanisms may be particularly useful because of their low carbohydrate diet (Blix, 1976).

The above data are summarized in the following diagrammatic model.

Scheme VIII, 2. Diagrammatic model of some of the metabolic interactions between the brain, lung, heart, liver, kidney and blood during recovery from simulated diving.



Scheme VIII, 1

In summary, the present data on relatively short-term, simulated diving (10-30 min) in the Weddell seal indicate cooperative metabolic interactions between the heart, lung and brain. These interactions, which probably contribute to extending diving duration, depend upon how enzyme potentials are used, and not on the development of any new or qualitatively different enzymic machinery. Indeed, only a few modest adjustments in enzyme levels seem to correlate with the observed metabolic organization. In this sense, the biochemical strategies associated with extending hypoxia tolerance appear to differ fundamentally from those utilized by many, more primitive animal anaerobes (Hochachka, 1980).

It is stressed that these interpretations are based on forced diving episodes, lasting up to 30 minutes, when metabolism of the three central organs is not oxygen limited. If oxygen were to become seriously limiting the above key interactions would break down. How the seal deals with such emergencies remains a question for the future. However, this would probably not alter the present interpretation of the recovery process.

Fetal Responses to Maternal Diving

Companion studies performed by Liggins et al., (1980) have demonstrated that, upon initiation of a maternal dive, the fetus responds by developing a substantial bradycardia (90 beats/min to 34 beats/min after 4 min and to 27 beats/min at the end of diving). This decline in heart rate is accompanied by a retention in the mean arterial blood pressure and a well-sustained diastolic pressure during the longer intervals between fetal heart beats. These physiological parameters when coupled to our metabolic blood profiles (i.e. lactate and pyruvate recovery peaks) strongly suggest the existence of a peripheral vasoconstriction and thus the development of a fetal diving response. Thus the fetal seal appears to have a well developed oxygen conserving mechanism akin to that in the adult. However, the maternal 'message' signalling onset of diving has yet to be ascertained (Liggins et al., 1980).

The fetal studies also demonstrated an unusual feto-maternal gradient of blood glucose concentrations, the fetal concentrations being higher than the maternal. This appears to be a unique biochemical adaptation of the Weddell seal, ensuring ample substrate supply for the fetus during prolonged maternal diving. During short-term diving, glucose profiles in the arterial pool of the fetus fluctuate somewhat with a slight increase in levels towards the end of the episode. This may be due to the activation of fetal gluconeogenic processes and to the continued placental perfusion of maternal blood (see Elsner et al., 1970 and Liggins et al., 1980 for discussions relating to placental perfusion during maternal asphyxia and diving).

Although blood alanine and glutamine profile data are sketchy recovery spikes can, nevertheless, be deciphered, suggesting washouts from previously, vasoconstricted peripheral tissues. A qualitatively similar response was also observed in both male adults and pregnant females (see Chapter III for a thorough discussion). Otherwise, there were no detectable changes in the rest of the amino acid blood pool during or after maternal simulated diving. Again it is noted that this set of data was generated from relatively short term diving (10-30 min for the gravid seal); whether this potentially useful pool remains in a steady state condition throughout long term diving (up to 70 min) remains a question for future research. It has been suggested that in periods of maternal hypoglycemia the mammalian fetus will increase its catabolism of amino acids (Battaglia and Meschia, 1976).

In resting animals amino acid concentration gradients across the placenta favor, mainly, the fetal circulation; a result consistent with other mammalian species thus far studied. This phenomena is due to the high fetal demands for catabolic and anabolic substrates and because of the fetus' inability to synthesize some non-essential amino acids (Palou et al., 1977). Alanine gradients are the most dramatic (1:3 ratio across the placenta), reflecting the low levels in the circulation of the adults.

Our enzyme data alone, do not constitute a good interpretative basis for the understanding of the metabolic preferences and interrelationships of the three central organs of the fetus as a consequence of maternal diving. Nevertheless, the enzymatic machinery appears to be fully developed and thus the enzyme studies are consistent with the fetus behaving in a similar fashion to that of the adult in all three states associated with

diving. Glucose and oxygen are probably the main substrates firing the cerebral production of ATP in both normal and diving states while the heart may rely on a mixed aerobic and anaerobic metabolism depending upon the mean arterial oxygen tension. It will be interesting to determine the contribution of glucose to myocardial catabolism since it is believed that fetal hearts of most terrestrial mammals burn predominantly glucose. The recovery period probably signals the oxidative catabolism and anabolism (i.e. gluconeogenesis) of lactate. Alanine and glutamine may also follow the same pathways as suggested for the adult. Although one must not read too much into the enzyme data, they do nevertheless, lay a good foundation for future 'in vivo' studies of the fetal Weddell seal.

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Appendix

List of Abbreviations

Acetyl CoA: acetyl-S-coenzyme A

5'AMP, ADP, ATP: adenosine 5'-mono-,di-,triphosphate

A-V: arterio-venous

$CaCl_2$: calcium chloride

CBF: cerebral blood flow

CO_2: carbon dioxide

µC: microcurie

DTNB: 5,5'-dithiobis-(2-nitrobenzoic acid)

FAD: flavin adenine dinucleotide

$FADH_2$: reduced FAD

GABA: gamma aminobutyric acid

αGP: α-glcerophosphate

GSH: reduced glutathione

GSSG: oxidized glutathione

GTP: guanosine 5'-triphosphate

G-1-P: glucose-1-phosphate

G-6-P: glucose-6-phosphate

HK: hexokinase

hydroxyacylCoA: hydroxyacyl-S-coenzymeA

KCl: potassium chloride

K_2CO_3: potassium carbonate

2-KGA: 2-ketoglutarate

KH_2PO_4: potassium phosphate, monobasic

KOH: potassium hydroxide

LDH: lactate dehydrogenase

$MgSO_4$: magnesium sulfate

NAD: nicotinamide adenine dinucleotide

NADH: reduced NAD

NADP: nicotinamide adenine dinucleotide phosphate

NADPH: reduced NADP

$NaHCO_3$: sodium bicarbonate

OXA: oxaloacetate

P: probability

PaO_2: arterial partial pressure of oxygen

$PaCO_2$: arterial partial pressure of carbon dioxide

PCA: perchloric acid

PFK: phosphofructokinase

P_i: inorganic phosphate

PK: pyruvate kinase

RBC: red blood cell

S.D.: standard deviation

S.E.: standard error

STP: standard temperature and pressure

SSA: sulfosalicyclic acid

+ $CmRO_2$

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