CEREBRAL ENERGY METABOLISM IN MALLARD DUCKS
DURING APNEIC ASPHYXIA
THE ROLE OF OXYGEN CONSERVATION

by

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ABSTRACT

Cerebral energy metabolism during apneic asphyxia and steady state hypoxia was compared in ducks and chickens; ducks tolerate apneic asphyxia 3-8 times longer than chickens. Fluctuations in the reduced form of respiratory chain nicotinamide adenine dinucleotide (NADH) were monitored from the left cerebral hemisphere by a noninvasive fluorometric technique and used as an indicator of mitochondrial hypoxia. Electroencephalogram (EEG) and surface PO_2 were recorded from the right hemisphere. Forced dives of 4-7 minutes duration on restrained ducks were characterized by bradycardia and an accumulation of NADH which increased throughout the diving period. NADH returned to the preasphyxic level when breathing was resumed. In later experiments asphyxia was produced by stopping artificial ventilation in paralyzed ducks. Asphyxia produced by this means caused similar **changes** in the measured variables (heart rate, blood pressure, NADH fluorescence, and EEG) to those obtained in forced submergence of nonparalyzed ducks.

NADH fluorescence was expressed in arbitrary units (AU) where 100 AU was defined as the fluorescence change from normoxia to anoxia. After 1 minute of asphyxia NADH fluorescence increased by $37 \text{ AU} \pm 3.60 \text{ SEM}$ ($n = 54$) in paralyzed chickens and $8 \text{ AU} \pm 1.41 \text{ SEM}$ ($n = 55$) in paralyzed ducks. After 2 minutes the fluorescence increased by only $15 \text{ AU} \pm 1.95 \text{ SEM}$ in ducks. Both species showed an isoelectric EEG when fluorescence increased by approximately 35 AU indicating that anaerobic ATP production

in ducks did not maintain brain function (EEG) for a greater accumulation of respiratory chain NADH. At a given decrease in tissue PO_2 ducks and chickens showed the same level of NADH increase indicating that both species are equally dependent on tissue PO_2 for the maintenance of redox state. Furthermore, the inhibition of cardiovascular adjustments by atropine in ducks caused NADH to increase faster during apneic asphyxia than in nonatropinized ducks. I conclude that the oxygen conserving cardiovascular adjustments are responsible for the increased cerebral tolerance to apneic asphyxia in ducks without any involvement of biochemical mechanisms.

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General Discussion

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INTRODUCTION

Since the time of Aristotle it has been noted that the porpoise is an air breathing animal which can remain submerged in water for periods of time that are fatal to strictly terrestrial mammals (Strauss, 1970). Although the study of natural divers has intrigued man over the following 2200 years, it was not until the 1870's that Paul Bert attempted to explain the nature of this phenomenon (Andersen, 1966). Since Bert's pioneering investigations physiological adjustments have been elucidated which help in explaining the tolerance to breath holding in natural divers (for reviews see Scholander, 1940; Andersen, 1966; Elsner, 1969). The major problem during a dive is the depletion of oxygen and it appears that natural divers, whether amphibians, reptiles, birds, or mammals, deal with the problem in a similar manner. Despite this fact divers can be divided into two major groups, (1) amphibians and reptiles, and (2) birds and mammals, depending on their oxygen requirement. For example lizards, snakes, and crocodiles can survive anoxia for 30 minutes (Belkin, 1963), and turtles for over 4 hours (Jackson, 1968), whereas diving mammals and birds can survive anoxia for only a matter of seconds before irreparable damage occurs. Because of this large difference in tolerance many of the generalizations concerning the physiology of diving mammals and birds do not pertain in any detail to reptiles and amphibians. For this reason only one of the groups, birds and mammals, will be considered in this thesis.

Since external respiration ceases during the period of the underwater excursion, animals such as mammals and birds that are obligately dependent on oxygen must make some provision for its storage and conservation during the dive. By increasing (1) blood volume (Elsner, 1969), (2) oxygen capacity of the blood (Scholander, 1940), (3) lung volume (Andersen, 1966), and (4) muscle myoglobin (Robinson, 1939) divers can as much as double the quantity of oxygen stored over that of a similarly sized nondiver (Elsner, 1969). However, increased oxygen storage does not appear to be absolutely necessary since some animals can endure extended periods of breath holding in the absence of many of these physiological adaptations. For example, the oxygen carrying capacity of blood in the manatee (Scholander and Irving, 1941), sea lion (Florkin and Redfield, 1931), porpiose (Green and Redfield, 1933), duck (Andersen, 1966), and penguin (Scholander, 1940) is actually less than that of man (Prosser, 1973). The bottle nose whale and fin whale, although known to be good divers, have a relatively small lung volume (Andersen, 1966) and the Florida manatee has practically no muscle myoglobin for the storage of oxygen (Scholander and Irving, 1941).

More important to underwater survival is a series of cardiovascular adjustments during the dive that increases the vascular resistance in most vascular beds (peripheral vasoconstriction) and reduces the heart rate (bradycardia) (Irving, 1934; Irving et al., 1935; Scholander, 1940; Scholander et al., 1942b; Johansen, 1964; Butler and Jones, 1971).

The peripheral vasoconstriction is thought to conserve blood oxygen by redistributing blood. Tissues such as the heart and, in particular, the brain which are most sensitive to oxygen lack receive priority for blood flow over tissues and organs that are less easily damaged by anoxia. The bradycardia offsets the increased peripheral resistance so that blood pressure is held constant and, in addition, it also reduces the work load on the heart, thus further conserving oxygen. Although the same basic response is seen in nondivers during apnea, it has been considerably refined in divers to meet the needs of prolonged apneic survival. The control of the adjustments is still not fully understood and is presently the object of intense investigation (for reviews of control see Angel-James and Daly, 1972; Jones, 1976; Jones and West, 1978).

The nature of the cardiovascular adjustments divides the tissues into two groups; those that receive blood flow during a dive, and those that do not. The tissues removed from the circulation must rely on anaerobic production of ATP* once the residual tissue oxygen is depleted. The capacity to produce ATP in muscle and other peripheral tissues in terrestrial animals through anaerobic glycolysis is sufficient that divers need only make minor modifications to this basic scheme. The overall theme of the modifications involves two main areas:

* Abbreviations: ATP, adenosine triphosphate; ADP, adenosine diphosphate; and AMP, adenosine monophosphate.

(1) tighter control of regulatory enzymes enabling glycolytic flux to increase by several hundred fold and (2) maintenance of the cytoplasmic NAD/NADH* ratio, primarily through the action of lactate dehydrogenase, to insure that NAD is available for glycolysis (Hochachka and Storey, 1975).

Tissues remaining in the central circulation have large energy requirements and the energy supply must not be interrupted during a dive. For example, the energy requirements of the brain may be as much as 20% of the total energy consumed in mammals (Dunn and Bondy, 1974) and can only be met by producing ATP aerobically (Seisjø, 1977), the process depending almost exclusively on the substrate glucose (Sokoloff, 1976). Only in infants (Dunn and Bondy, 1974) or during starvation (Himwich, 1976b) are other substrates oxidized to any significant degree, but, even so, glucose still accounts for the majority of the substrate consumed (Dunn and Bondy, 1974). Since glycogen, the storage form of glucose, is not present in the brain in large enough quantities to support energy metabolism (Himwich, 1976a), the blood is responsible not only for a continuous supply of oxygen but also a continuous supply of glucose.

On entering the cell glucose is oxidized by the classical Embden-Meyerhoff pathway (glycolysis) to pyruvate with a net production of two moles of ATP for each mole of glucose.

* NAD and NADH are the oxidized and reduced forms of nicotinamide adenine dinucleotide respectively.

consumed. Equation (1) describes the overall reaction.

At this point pyruvate may enter one of two pathways. It can be anaerobically reduced to lactate in the presence of lactic dehydrogenase (LDH) without further ATP production:

The NADH produced in equation (1) is now reoxidized and ready to be used again in the oxidation of glucose. In the presence of oxygen the two molecules of NADH and the two pyruvate molecules from equation (1) may be oxidized via the citric acid cycle and the respiratory chain to produce an additional 36 moles of ATP per mole of glucose initially consumed.

Under normoxic conditions 85% of the glucose entering the brain in mammals is completely oxidized to CO_2 while 15% is oxidized only to lactate (Dunn and Bondy, 1974). Considering the moles of ATP produced per mole of glucose for anaerobic metabolism (2 moles of ATP) and aerobic metabolism (36 moles of ATP), aerobic metabolism accounts for about 95% of the total ATP produced. The proportion of glucose oxidized to lactate depends on the available oxygen; as oxygen becomes limiting proportionately more glucose is metabolized to lactate.

In rats when the brain is depleted of oxygen, glycolytic flux can increase 5 fold (Seisjø, 1977). However, even with this increase, anaerobic metabolism alone cannot supply the necessary ATP required for brain function.

Over 90% of the oxygen used by the brain is handled by the respiratory chain (Jöbsis, 1974) which consists of a series of electron carriers arranged in ascending order of their redox potentials (Fig. 1) and acts as an energy gradient that transfers electrons from substrate to oxygen. On entering the respiratory chain electrons flow from the members with the more negative redox potentials, NAD or FP (flavoprotein), to the more positive members and ultimately to oxygen. Each atom of oxygen receives two electrons from the respiratory chain plus two hydrogen ions from the media forming one molecule of water. At several steps along the chain the free energy of electron transfer is captured and used in oxidative phosphorylation, a term given to the energy requiring reaction:

For each pair of electrons passed down the respiratory chain from NAD to oxygen approximately three molecules of ATP are produced. Electron pairs entering the chain via FP produce only two molecules of ATP since the electrons bypass the first phosphorylation site (Fig. 1). Oxidative phosphorylation is obligately coupled to the electron flow of the chain and therefore proceeds only in the presence of oxygen. When oxygen becomes limiting electrons enter the respiratory chain

Figure 1. A diagram of the respiratory chain in mitochondria illustrating the flow of electrons and the probable sites for ATP production. Abbreviations: NAD, nicotinamide adenine dinucleotide; FP_1 and FP_2 , flavoproteins; CoQ, ubiquinone or coenzyme Q; CYT, cytochrome.

faster than they can be removed by oxygen resulting in a net reduction of each electron carrier. The oxidation-reduction state of any electron carrier can therefore serve as an indicator of mitochondrial hypoxia (Chance et al., 1973).

Changes in the redox state of the first component of the respiratory chain, NAD, can be monitored in intact tissues by a fluorometric method which was first described by Chance et al. (1962). The method takes advantage of the fact that the reduced form of NAD (NADH) is a natural fluorochrome which can be excited by light wavelengths between 310 and 370 nm and gives rise to a fluorescence emission in the region of 425-475 nm (Jöbsis et al., 1966). The oxidized form of the coenzyme does not fluoresce at these wavelengths. In practice NADH is excited by focussing 366 nm light, a natural peak of the mercury arc lamp, on the tissue surface and the fluorescence originating primarily from the top 1.5 mm of tissue (Jöbsis et al., 1971) is measured with a photomultiplier tube.

The labile fluorescence signal from tissue was initially linked to NADH due to similarities in their fluorescence emission spectra (Chance et al., 1962) and to the fact that NADH, determined by biochemical analysis, correlates with changes of fluorescence in liver (Chance et al., 1965a), heart (Chance et al., 1965b) and brain (Jöbsis et al., 1971). During cycles of anoxia the kinetics of tissue fluorescence are in synchrony with those of the cytochromes indicating a large contribution of respiratory chain NADH to the fluorescence signal (Lübbers et al., 1964). Furthermore, Chance et al. (1962) and Mayevsky

and Chance (1974) showed that drugs which block the respiratory chain caused a large fluorescence increase in the kidney and brain of rats.

However, the fluorescence signal from the respiratory chain may be contaminated with fluorescence from other pyridine nucleotide pools in the cell which are not at the same redox state as respiratory chain NAD and do not necessarily show redox changes that parallel changes in respiratory chain NADH. Investigations into the origin of the labile fluorescence have shown that these nonrespiratory chain pools contribute little if any to the fluorescence signal. Fluorescence from respiratory chain NADH is sufficiently enhanced over the fluorescence from cytoplasmic NADH, the pool which is primarily involved with the glycolytic pathway, that fluctuations in cytoplasmic NADH contribute little to the fluorescence signal (Jöbsis and Duffield, 1967; O'Connor, 1977). The respiratory chain NADH is enzyme bound whereas much cytoplasmic NADH is free or unbound. As a general rule NADH that is enzyme bound has a greater quantum efficiency than free NADH (Boyer and Theorell, 1956). However, glyceraldehyde phosphate dehydrogenase which is abundant in the cytoplasm is one of the few enzymes that actually diminishes rather than enhances fluorescence when bound to the coenzyme (Velick, 1961). The overall result is that the fluorescence efficiency of respiratory chain NADH is 10-20 times greater than that of the cytoplasmic NADH pool (Jöbsis et al., 1971).

Another NAD pool which is thought to serve primarily in

biosynthesis (Jöbsis, 1964) is located in the mitochondria but is not directly involved with the respiratory chain (Chance and Hollunger, 1961). This pool has not been as extensively investigated as the cytoplasmic NAD pool but nevertheless appears to contribute only 1-2% of the total fluorescence signal in resting mitochondria (Jöbsis and Duffield, 1967). Fluorescence properties identical to those of NADH also occur in the reduced form of cytoplasmic and mitochondrial nicotinamide adenine dinucleotide phosphate (NADPH) which may interfere with the fluorescence signal from the respiratory chain NADH. However, biochemical analyses have shown that NADPH does not change in the cortex of the squirrel monkey during anoxia when fluorescence is maximal (Sundt et al., 1976). Furthermore NADPH is only 3% of the total pyridine nucleotide pool (Glock and McLean, 1955) and would therefore contribute very little to the fluorescence signal.

PO_2 of the brain, and thus the redox state of respiratory chain NAD, is dependent not only on arterial PO_2 (PaO_2) but also the rate at which blood perfuses the brain. Since hypoxia, hypercapnia, and a fall in arterial pH (either from hypercapnia or lactate), which occur during breath holding, increase blood flow to the brain (Ingvar and Lassen, 1962; McDowall, 1966; Purves, 1972; Kogure et al., 1970; Betz, 1972; Borgstrom et al., 1975; Grubb et al., 1977 and 1978) by decreasing cerebral vascular resistance through local and possibly centrally mediated mechanisms (Purves, 1972; Betz, 1972), brain PO_2 can be regulated to a degree during diving. However, during a dive cerebral

blood flow in natural divers has been reported to remain unchanged (Grinnell et al., 1942; Bron et al., 1966) or even decrease slightly from the predive level (Van Critters et al., 1965; Elsner et al., 1966; Butler and Jones, 1971; Kerem and Elsner, 1973). On the other hand, some studies have shown large increases in cerebral blood flow during a dive (Johansen, 1964; Dormer et al., 1977; Jones et al., 1978). Even when cerebral blood flow was reported to have decreased it was nevertheless sustained at higher levels than that in other tissues.

Since PaO_2 falls during a dive, the predive PO_2 of the brain cannot be maintained even if cerebral blood flow increases, but oxygen delivery to the tissue can be maintained in the face of falling PaO_2 . In fact, oxygen consumption of the brain (CMRO_2) and levels of the adenosine phosphates (ATP, ADP and AMP) do not change in terrestrial mammals until PaO_2 falls below 2.66kPa (20 torr) (Duffy et al., 1972; MacMillan and Seisjø, 1971; Kety and Schmidt, 1948; Lambertson et al., 1953; Cohen et al., 1967; Johanssen and Seisjø, 1975; Borgstrom et al., 1975) providing that blood pressure does not fall. Although CMRO_2 is maintained, the lactate/pyruvate ratio begins to increase once PaO_2 falls below 6.65kPa (50 torr) during normocapnic hypoxia indicating that glycolytic flux has increased. However, during apneic asphyxia in dogs lactate production by the brain does not increase even after PaO_2 falls well below 2.66kPa (Kerem and Elsner, 1973b) indicating that perhaps under these conditions blood flow increases are far greater

than during normocapnic hypoxia.

The importance of the cardiovascular adjustments to continued brain function during diving was shown by Kerem and Elsner (1973a). Harbor seals which tolerated dives lasting 18.5 minutes before the onset of hypoxic EEG (electroencephalogram) patterns tolerated dives lasting only 5.5 minutes after the cardiovascular adjustments were inhibited with atropine. If enhanced cardiovascular adjustments in divers protect the brain by conserving oxygen for the brain and heart alone, then respiratory chain NAD should not be reduced as fast as in the brain of nondiving animals during forced dives.

Physiological mechanisms cannot totally account for the cerebral tolerance to asphyxia in diving animals since divers may be able to tolerate more severe hypoxia than terrestrial animals (Ridgeway et al., 1969; Elsner et al., 1970; Kerem and Elsner, 1973a and b). When oxygen depletion is far advanced physiological adjustments must be superceded, or at least aided, by some form of biochemical regulation if ATP production is to continue.

Several biochemical changes that enhance oxidative phosphorylation have been reported for animals acclimated to hypoxia. Reynafarje (1971/72) showed that guinea-pigs native to high altitudes (4500 m) have heart mitochondria with a greater affinity (lower K_m) for ADP than those native to sea level and concluded that the former can generate ATP faster at lower ADP concentrations when oxygen tensions are low. Heart mitochondria from rats acclimated to hypoxia show a three-fold

increase in the respiratory chain activity (moles O_2 consumed · mole cytochrome $aa_3^{-1} \cdot \text{min}^{-1}$) over mitochondria from normoxic animals (Mela et al., 1976) and Park et al. (1973) have suggested that the former may have lower critical PO_2 's than the latter presumably due to an increased cytochrome a_3 affinity for oxygen. Isozymes of cytochrome a_3 having different oxygen affinities have been reported in the carotid body (Mills, 1972; Mills and Jöbsis, 1972); however, the lower affinity cytochrome a_3 is an oxygen sensor and may not have a role in ATP production. Clearly, increased affinities for ADP, P_i , and oxygen could enhance or prolong ATP production when tissue hypoxia occurs. On the other hand, Simon et al. (1974) found no significant differences in cytochrome oxidase (cytochrome a, a_3) activity in three species of diving mammals with widely varying maximal dive times; however, the study only quantified cytochrome oxidase activity without investigating the kinetic relationship between the enzyme and oxygen.

Many diving animals appear to have the capacity for generating ATP for the brain by anaerobic glycolysis. The brain of whales (Shoubridge et al., 1976), beavers (Messelt and Blix, 1974), seals (Blix and Fromm, 1971), and eiders (Blix and Fromm, 1971; Blix et al., 1973) are known to have a greater proportion of the muscle type isozyme of lactate dehydrogenase (LDH) than terrestrial mammals. The muscle isozyme favors the formation of lactate (equation (2), page 5) and is indicative of tissues more tolerant to hypoxia; conversely the heart isozyme favors the formation of pyruvate and

is indicative of highly aerobic tissues (Hellung-Larsen and Andersen, 1968; Kaplan and Everse, 1972). The presence of a large proportion of the muscle isozyme therefore indicates an increased anaerobic capacity in the brain of the above animals. On the other hand, LDH isozyme patterns of the brain of the Weddell seal (Murphy and Hochachka, 1978) and the narwhal (Vogel, 1978) favor the heart type subunit and are not strikingly different from the LDH pattern observed in terrestrial mammals. Although the LDH isozymes of the brain of the Weddell seal (Murphy and Hochachka, 1978) are similar to terrestrial animals, the substrate for anaerobic metabolism, glycogen, appears to be at least 2 to 3 times higher in Weddell seal brain than for other nondiving mammals (Kerem et al., 1973). Pyruvate kinase, a regulatory enzyme of the glycolytic pathway which has been used as a quantitative index of glycolytic capacity (Simon and Robin, 1972), shows a greater activity with increasing maximal dive times in the sea lion, harbor seal, and Weddell seal (Simon et al., 1974).

Although the anaerobic machinery is present, can the diver produce significant amounts of ATP for the brain anaerobically? During maximal dives in harbor seals the $CMRO_2$ decreases and brain lactate production increases for the last 5 minutes before the onset of hypoxic EEG patterns (Kerem and Elsnor, 1973a). During submaximal dives (20 minutes) in Weddell seals, Murphy and Hochachka (1978) found no appreciable increase in brain lactate production. Since anaerobic ATP production is much less efficient than aerobic production it is questionable

that sufficient energy can be produced glycolytically to maintain cerebral integrity (Seisjø et al., 1974).

The purpose of this thesis was to study cerebral energy metabolism and brain function during diving in animals that can endure prolonged asphyxia. The fluorometric technique described above was used to compare changes in the redox state of respiratory chain NAD in the cerebral cortex of two species which have different tolerances to asphyxia, ducks and chickens, during apneic asphyxia and steady state hypoxia. Ducks are known to tolerate asphyxia 3-8 times longer than chickens (Andersen, 1959 and 1966). After establishing that brain function is maintained longer in ducks than chickens during apneic asphyxia, I examined the role physiological and biochemical adjustments play in the maintenance of brain function.

CHAPTER 1

General Methods - Animal Preparation

a. Rats. Rats were used initially as experimental animals since they have been extensively used in experiments where cortical NADH was measured by the fluorometric technique (Chance et al., 1962). Fifteen Wistar rats (300-500 g) of both sexes were anesthetized with an intraperitoneal injection of urethane (1.3 g/kg, BDH Chemicals Ltd., Poole, England). A polyethylene catheter (PE50) was inserted into the right jugular vein for drug injections. After a tracheostomy each rat was paralyzed with an intravenous injection of tubocurarine chloride (1 mg/kg, Burroughs Wellcome and Co., Montreal, Canada) and ventilated with a Narco type V5KG constant pressure respirator (Narco Bio-Systems, Inc., Houston, Texas). For fluorometric recordings the scalp over the left parietal region of the skull was incised and retracted. A window approximately 1 cm x 0.5 cm was cut in the underlying calvaria and the left cerebral hemisphere was exposed. The dura was left intact. Only blood-free preparations were used for fluorometric recordings.

b. Ducks. Experiments were done on a total of 75 mallard ducks, Anas platyrhynchos, of both sexes and weighing 1.0-2.2 kg. All operations except craniectomies were performed after a local injection of 2% w/v xylocaine (Astra Pharmaceutical, Mississauga, Ontario). This procedure produced local anesthesia which was sustained for several hours and the ducks showed

no signs of stress either during or after surgery. Craniectomies were performed using general anesthesia either by ventilating the ducks with 1% halothane in air or by an intravenous injection of urethane (1.0 g/kg). Ducks that were anesthetized with urethane were used for developing the fluorometric technique (Chapter 2) and remained anesthetized throughout the experiment. Halothane was used for craniectomies in all other ducks and a 45 minute recovery period from anesthesia preceeded all experiments.

A polyethylene cannula (PE 90) was inserted into the left sciatic artery and connected to a Statham P23GB pressure transducer for monitoring blood pressure. A three-way stopcock connected the transducer to the cannula and allowed withdrawal of arterial blood samples via the side arm for blood gas analysis (PaO_2 , PaCO_2 , pHa). A second polyethylene cannula (PE 90) was inserted into the vena cava via the brachial vein and used for saline injections to calibrate the fluorometer and for drug injections. Heart rate was determined from the electrocardiogram (ECG) which was obtained from 2 copper wire electrodes, one inserted subcutaneously in the left side of the chest and the other inserted in the right thigh. The signal was amplified and fed into a ratemeter. Cloacal temperature was maintained at $41 \pm 0.5^\circ\text{C}$ by a thermistor inserted into the cloaca and a temperature controlled feedback unit that regulated a heating pad placed over the duck. In all but 9 ducks a cannula was inserted in the trachea towards the lung and the clavicular air sac was punctured. After paralysis

with an intravenous injection of gallamine triethiodile (1 mg/kg, Flaxedil, Poulenc Ltd., Montreal, Quebec) the ducks were unidirectionally ventilated by passing a stream of humidified air through the tracheal cannula at rates up to 1 l/min. Arterial blood samples were withdrawn from the sciatic artery and analyzed using a Radiometer PHM 71 gas monitor with oxygen and carbon dioxide electrodes and a microelectrode unit type E5021 (Radiometer, Copenhagen, Denmark). The air flow was adjusted to give arterial blood values for PaO_2 of 11.3-14.0 kPa (85-105 torr); PaCO_2 of 3.3-4.4 kPa (25-33 torr); and pHa of 7.45-7.50. The remaining 9 ducks which were not paralyzed or unidirectionally ventilated were used in experiments involving nonparalyzed ducks (Chapter 3).

Brain electrical activity was monitored from 2 stainless steel screws cemented in the anterior and posterior areas of the right frontal region of the skull. The potential difference between the screws was amplified 1000 times with a Frederick Haer Amplifier (Frederick Haer and Co., Brunswick, Maine) and displayed on a 6 channel Watanabe WTR 281 pen recorder (Watanabe Instrument Corporation, Tokyo, Japan) along with other measured variables.

For fluorometric recordings animals were placed ventral side down on a metal operating table with the head secured in a stainless steel head holder which consisted of a bill clamp and ear bars. The scalp over the left frontal region of the skull was incised and a hole 1.0 cm in diameter was cut in the underlying calvaria. The dura was retracted exposing the

anterior portion of the left hemisphere, Pars oralis, below the Fissura dorsalis. In all but 14 ducks the exposed cortex was covered with plastic film to prevent drying. In the remaining 14 ducks the exposed cortex was covered with a glass window 1 cm in diameter and cemented to the skull with dental cement (Hygienic Mfg. Co., Akron, Ohio).

Tissue oxygen tension ($P_{T}O_2$) was recorded polarographically from the cortical surface. A craniectomy was performed on the right frontal region of the skull and an oxygen electrode was placed in contact with the surface of the cortical area corresponding to that from which the fluorometric recordings were made on the opposite side. Coiled copper wire (0.25 mm in diameter) supported the electrode and allowed it to move with pulsations of the brain.

c. Chickens. A total of 43 hens, Gallus domesticus, weighing between 1.2 and 2.2 kg were used. Roosters were not used since a large comb interfered with cranial surgery. Operative procedures for chickens were identical to those done on ducks.

CHAPTER 2

Special TechniquesThe Fluorometric Recording of NADH

The respiratory chain is the focal point for bioenergetics in the brain since it links oxygen uptake with ATP production (Jöbsis, 1972). The respiratory chain acts as an energy gradient that transfers reducing equivalents from substrate to oxygen forming ATP with the liberated free energy. During hypoxia, the reducing equivalents enter the respiratory chain faster than they can be removed by oxygen resulting in a net reduction of the respiratory chain carriers. The oxidation-reduction state of any respiratory chain component can therefore serve as an indicator of mitochondrial hypoxia. The fluorometric method described by Chance et al. (1962) provides a nondestructive, direct, and continuous (Chance et al., 1970) readout of changes in the oxidation-reduction state of the first member of the respiratory chain, NAD.

Since hemoglobin absorbs both excitation and fluorescence light, changes of blood in the recording field produce artifacts in the apparent NADH fluorescence (Schnitger et al., 1965; Chance and Schoener, 1965; Granholm et al., 1969; Kobayashi et al., 1971a and b; Jöbsis et al., 1971). Jöbsis and Stainsby (1968) and Jöbsis et al. (1971) introduced a second photomultiplier system to measure the reflected excitation light and used it to compensate for the hemoglobin artifact. If the outputs from the two photomultipliers are adjusted to give an

equal response for a given hemoglobin change in the absence of a concomitant NADH change then the difference between the two outputs must be due solely to NADH. However, when the artifact is large, full compensation is questionable (Jöbsis et al., 1971). Since blood flow to the brain increases, on average, 8.5 times in ducks during prolonged apneic asphyxia (Jones et al., 1978), compensation by using reflected excitation light could be inadequate during diving conditions.

The purpose of this section was to determine if the blue fluorescence originating from the cerebral cortex of ducks when excited with UV light was due to NADH and, if so, develop a fluorometer to monitor the NADH change. In particular, the reflected excitation light has been evaluated to determine if there was adequate compensation for the hemodynamic artifact over the range of blood flow changes expected during apneic asphyxia.

a. Description of the fluorometer

The fluorometer was a modified version of that described by Jöbsis et al. (1971) (Fig. 2). The ultraviolet (UV) source consisted of a 1000 W water cooled mercury arc lamp AH6-1-B (Illumination Industries Inc., Sunnyvale, California) encased in a stainless steel housing (made by Ron Overaker, Department of Physiology and Pharmacology, Duke University Medical School, Durham, North Carolina). Power was supplied by a model T507 step-up transformer (Illumination Industries) and the voltage to the lamp (700 VAC) was controlled by a Powerestat 136 variable

Figure 2. Diagram of the optical design of the fluorometer used to monitor fluctuations in respiratory chain NADH in intact tissue.

transformer (Superior Electric Co., Bristol, Connecticut) placed on the primary side of the step-up transformer. Excitation light (360 nm) was selected by a primary filter (Leitz, Wetzlar, Germany) having a half power band width (HPBW) of 10 nm and reflected onto the surface of the tissue at an angle of $45-60^\circ$ using a Leitz Ultropak assembly as an incident-light illuminator. The light intensity was measured by an Eppley thermopile No. 10657 (The Epply Laboratory, Inc., Newport, Rhode Island) and attenuated by placing round microscope coverslips (1.8 cm in diameter) between the light source and the specimen, since light intensity above 0.8 mW/cm^2 may cause tissue damage (Rosenthal, 1976).

Reflected excitation light and NADH fluorescence were collected from cortical fields either 3.5 mm or 2.3 mm in diameter by a low (3.8x) or high (6.5x) power objective and divided by an 80:20 beam splitter (Leitz). Changes in fluorescence intensity were monitored with an EMI 9524B photomultiplier tube (EMI Gencom Inc., Plainview, New York) from the 80% portion of light after the excitation light was removed with a 450 nm secondary filter (Leitz). The reflected excitation light was monitored from the 20% portion of light after the fluorescence light was removed with a Leitz UV UG1 filter. Initially the photomultipliers were powered by a Kepco OPS 2000 (Kepok, Inc., Flushing, New York) and a Knott high stability power supply type NSHM (Knott Elektronik, Munich, West Germany). In later experiments voltages for both photomultipliers were derived from the Kepco power supply by splitting the voltage

and varying it separately to each photomultiplier.

The fluorescence signal (F) and the reflectance signal (R) were obtained by amplifying the photomultiplier outputs (Fig. 3). In addition the electronic subtraction of F-R (mixer amplifier in Fig. 3) provided a third signal termed corrected fluorescence (CF). All three optical signals were recorded on the 6 channel Watanabe pen recorder.

Equalization of photomultiplier outputs to a given hemoglobin change was similar to the method used by Jöbsis *et al.* (1971). The fluorometer was focussed on the brain surface, and the voltage to the photomultipliers was adjusted until the amplified outputs were each of 4 volts. Fine adjustment was accomplished by equalizing the outputs of the F and R photomultipliers when the amount of hemoglobin in the field was reduced in the absence of a concomitant NADH change. The brain was flushed with oxygenated saline which was introduced through the venous cannula and the sensitivity of the reflectance photomultiplier was finely adjusted by altering the input voltage until the response to the flush equalled that of the fluorescence photomultiplier. Generally, calibration of the fluorometer required 2 or 3 flushes.

b. Stability of the fluorometer

Stability of the fluorometer was determined by focussing the fluorometer on a piece of paper and recording the amplified voltage outputs of the F and R photomultiplier tubes over 3 hours. Since no NADH was present the 450 nm filter normally

Figure 3. Simplified diagram of the fluorometer circuitry.
PMT's, photomultiplier tubes.

inserted in front of the fluorescence photomultiplier tube was replaced with a 5% transmission filter (Leitz) to attenuate the light. The angle of illumination and reflection of the paper was adjusted to simulate a biological preparation as nearly as possible. Fluorescence and reflectance were followed for 3 hours with less than 1% drift in the amplified voltage output in either.

c. Fluorescence emission spectra

Fluorescence emission spectra were made from the left cerebral cortex of paralyzed anesthetized ducks during normoxia and anoxia (Fig. 4). To make the fluorescence emission spectra the 450 nm secondary filter was replaced by a continuous interference filter (Veril B-60), which was manually operated over a range of 428-507 nm. Since the photomultiplier sensitivity varied with wavelength and the interference filter did not transmit light equally over the spectral range of this measurement, the fluorescence spectra in Figs. 4 and 5 were corrected for these inequalities. The normoxic spectrum was taken when the duck was ventilated with air and the anoxic spectrum was taken after death (defined as a blood pressure of 0 kPa) produced by nitrogen ventilation.

An increase in cortical fluorescence accompanied the transition from normoxia to anoxia without any shift in the fluorescence peak (467 nm). After death the cortex often shifts or falls away from the fluorometer when it is drained of blood (Jöbsis et al., 1971). To ensure that the difference

Figure 4. Fluorescence emission spectra recorded from the cerebral cortex of a duck during normoxia and anoxia (death). The fluorescence maximum for both spectra was 467 nm. The square at 467 nm and lying between the two spectra was taken during extreme hypoxia before blood pressure fell significantly.

between the normoxic and anoxic (death) spectra was not due to an artifact caused by a movement of the cortex, the fluorescence was also measured during extreme hypoxia before blood pressure fell significantly. The square in Fig. 4 at 467 nm and lying between the two spectra represents the hypoxic measurement. If a shift of the cortex caused an artifact it could be no larger than the difference between the square and the anoxic spectrum at 467 nm. Clearly, at least 90% of the fluorescence increase at 467 nm is not artifactual. The time required for the fluorescence measurements did not permit a full spectrum during extreme hypoxia. Fluorescence spectra before and after a normoxic-anoxic transition in the rat were similar to those of the duck, but the fluorescence maximum was between 464 and 467 nm.

In order to confirm that NADH was responsible for the change in fluorescence intensity during the normoxic-anoxic transition, the fluorescence emission spectrum in a duck during anoxia was compared to that of a solution of NADH (7.0×10^{-4} M, Sigma, St. Louis, Missouri) (Fig. 5). Fluorescence intensity was expressed as a percent of the peak intensity with zero intensity arbitrarily set at 428 nm. Peak fluorescence occurred at 467 nm and 470-473 nm for the anoxic brain and NADH solution respectively. Since fluorescence increased during anoxia, a condition when NADH is known to increase, and fluorescence spectra from the anoxic cortex and pure NADH are similar, I conclude that the labile fluorescence signal from the cerebral

Figure 5. Fluorescence emission spectra from anoxic cerebral cortex of a duck and a solution of NADH (7.0×10^{-4} M). Fluorescence intensity is expressed as a percent of the peak intensity with 0 intensity arbitrarily set at 428 nm. Fluorescence maxima were 467 nm and 470-473 nm for the anoxic cortex and NADH respectively.

cortex in ducks originates from NADH. Binding of the NADH to cellular constituents, mostly enzymes, is probably responsible for the shift of the fluorescence maximum to shorter wavelengths in the brain (Boyer and Theorell, 1956; Duysens and Ames, 1957). Chance et al. (1962) reported a similar shift in the fluorescence maximum in anoxic kidney when compared to pure NADH. On the other hand Harbig et al. (1976) and Sundt and Andersen (1975a) did not find a shift in the fluorescence spectrum of the cerebral cortex when compared to NADH in solution. The former authors attributed their results to filter characteristics which attenuated the shorter wavelengths of the spectra in tissue and shifted the apparent fluorescence maximum towards that of pure NADH.

d. Stability of the biological preparation

Stability of the biological preparation was tested by monitoring fluctuations of the fluorescence signal from the cerebral cortex of a rat and a duck during steady state conditions. Fluorescence was monitored during normoxia for 3 hours in both species and after death for 1 hour in a duck. Fluctuation in corrected fluorescence was less than $\pm 3\%$ of the amplified voltage output per hour when the light intensity was 0.5 mW/cm^2 . If photodecomposition of NADH or other background material occurred the fluorescence intensity would have shown a net decrease. Since fluorescence intensity did not show a net decrease, changes of fluorochrome concentrations due to photodecomposition was negligible.

e. Blood artifact compensation

Ducks were prepared for fluorometric recordings as previously described, and a polyethylene cannula (PE 90) was inserted in the carotid artery towards the brain. Bolus injections of oxygenated saline into the carotid artery produced transient dilutions in hemoglobin in the recording field and resulted in a transient increase in F and R (Fig. 6, A). Fluctuations in F and R were expressed as a percent increase (positive) or percent decrease (negative) in light intensity. Electronic subtraction of F-R (CF) compensated for changes in hemoglobin in the field when the change was small; however, when the change was large CF increased slightly. The same experimental protocol was repeated with bolus injections of concentrated red blood cells (oxygenated) (Fig. 6, B). When the bolus reached the recording area of the cerebral cortex both F and R decreased. However, as before, CF was only affected when the artifact was large.

In Fig. 7 the relationship between fluorescence and reflectance was examined in 4 ducks during flushes with saline and concentrated blood. The solid line through the intercept of the axis represents a 1 to 1 correspondence between F and R and therefore full compensation for the hemodynamic artifact. Points to the right of the ordinate are from bolus injections of saline, and points to the left are from bolus injections of concentrated blood. Within the range from approximately -15% to +15% change in F, the R channel fully compensated for the hemodynamic artifact, however, outside of this range full

Figure 6. Changes in fluorescence (F), reflectance (R) and corrected fluorescence (CF) recorded from the cerebral cortex of a duck when the brain was flushed with oxygenated saline (A) and concentrated blood (B). Each spike represents one flush. F, R, and CF are expressed as a percent increase (positive) or decrease (negative) in the light intensity from the normoxic baseline. The time scale (bottom) applies to both A and B.

% CHANGE
10
0
-10

% CHANGE
10
0
-10

% CHANGE
10
0
-10

A

B

F

R

CF

1 MIN.

Figure 7. The relation between reflected excitation light (ordinate) and emitted fluorescenc light (abscissa) recorded from the cerebral cortex of ducks when the brain was flushed with oxygenated saline and concentrated blood (oxygenated). Both reflectance and fluorescence are expressed as a percent increase (positive) or decrease (negative) in light intensity from the normoxic baseline. Points to the right of the ordinate are from saline flushes and points to the left are from flushes of concentrated blood. The solid line through the intercept of the axis represents a 1 to 1 correspondence between reflectance and fluorescence.

compensation was questionable. However, the nature of the deviation suggests that there were accompanying alterations in the NADH fluorescence. When large artifacts were produced by saline flushes the fluorescence increase was greater than the reflectance increase, suggesting a reduction of NAD which would be expected if saline caused slight hypoxia. On the other hand, when large artifacts were produced by bolus injections of concentrated oxygenated blood, fluorescence decrease was not as large as the reflectance decrease, suggesting oxidation of NADH which would be expected if the oxygenated blood caused slight hyperoxia. Consequently it appears that "true" blood artifact compensation cannot be assessed by this method in any absolute sense although within limits it is certainly acceptable.

f. Fluorescence during a cycle of hypoxia

Fig. 8 shows a recording of arterial blood pressure and changes in F, R, and CF recorded from the cerebral cortex of a duck during hypoxia produced by N₂ ventilation. Optical changes are expressed in arbitrary units (AU) where the CF change from normoxia to anoxia (death) was defined as 100 AU. Approximately 11 seconds after the transition from air to N₂, F increased. Hemodynamic artifacts identical to the reflectance (R) change were superimposed on the fluorescence (F) trace. Electronic subtraction of F-R produced the CF trace which was due solely to NADH change. As hypoxia progressed corrected fluorescence increased to a maximum of 60 AU after 45 seconds. When the ventilatory gas was changed to air, CF recovered and

Figure 8. Blood pressure (BP) and changes in fluorescence (F), reflectance (R), and corrected fluorescence (CF) recorded from the cerebral cortex of a duck during 35 seconds of nitrogen ventilation (as indicated by arrows). In all optical traces an upward deflection of the trace indicated an increase in light intensity and all optical traces are expressed in arbitrary units (AU), where the CF change from normoxia to anoxia (death) was defined as 100 AU. BP is expressed as kilopascals (kPa).

stabilized at the prehypoxic baseline while increased blood in the field caused F and R to overshoot the baseline. After several minutes C and F generally returned to their corresponding prehypoxia baselines.

Eighteen periods of apneic asphyxia in 6 ducks produced similar responses to N₂ ventilation, with the difference lying mainly in the time course of the optical change. In all 18 trials the reflectance decreased, but did not exceed -15% change from the baseline in any instance except one, which showed a -17% change. In these cases full compensation for the blood artifact was assumed to have been achieved.

On occasions during asphyxia a fall in blood pressure was mirrored by a CF increase and occurred whether blood pressure fell gradually (Fig. 9, A) or rapidly (Fig. 9, B). Since the relationship between blood pressure and CF was striking in these instances I suspected that the CF change was an artifact. The effects of blood pressure change on NADH fluorescence were investigated during normoxia by decreasing the blood pressure rapidly with acetylcholine (5-10 mg/kg, BDH Chemicals Ltd.) or slowly with an antihypertensive agent (Diazoxide (Hyperstat) 10 mg/kg, Schering Corporation Ltd., Pointe Claire, Quebec) and then rapidly increasing the blood pressure with epinephrine (10 µg/kg, Parke-Davis Co. Ltd., Broockville, Maryland). During normoxia a rapid fall in blood pressure produced a transient CF increase and transient decrease in brain PO₂ (recorded with an oxygen electrode as described later in this chapter) while a slow decrease in blood pressure over several minutes did not

Figure 9. Corrected fluorescence (CF) recorded from the cerebral cortex in ducks during apneic asphyxia when mean arterial blood pressure (MABP) fell. In A a gradual fall in MABP was mirrored by a CF increase. In B the fall in MABP was more pronounced. MABP is expressed in kilopascals (kPa) and CF is expressed in arbitrary units (AU) where the CF change from normoxia to anoxia (death) was defined as 100 AU. The time bar refers to both A and B.

A

B

produce a change in either CF or tissue PO_2 . A rapid increase in blood pressure, either from the normal level or hypotensive level (after Diazoxide) also produced no change in CF or tissue PO_2 . The CF change during apnea does not appear to be an artifact but rather a true NADH change due to changes in the tissue PO_2 . A slow fall in blood pressure probably allowed time for increases in cerebral blood flow to maintain brain PO_2 whereas a rapid fall in blood pressure was accompanied by a transient drop in PO_2 corresponding to the time it took to alter cerebral blood flow.

During apneic asphyxia cerebral blood flow in ducks increases (Jones et al., 1975) at a time when cardiac output falls (Jones and Holeyton, 1972), indicating cerebral vasodilation. If vasodilation is maximal or near maximal a fall in blood pressure could not be accompanied by further reductions in vasomotor tone. Consequently, a drop in blood pressure would directly result in a drop in CBF and thus a drop in tissue PO_2 .

Polarographic measurements of oxygen tension
of the cortical surface

The importance of dissolved oxygen in life processes has led to the development of a polarographic electrode to monitor oxygen tension (PO_2) in living tissues (Davies and Brink, 1942; Clark, 1953). The electrode consists of a platinum cathode which is polarized at -0.65 volts with respect to a silver/silver chloride anode. When the polarized electrode is in contact with living tissue oxygen molecules from the tissue are reduced at the cathode:

The reduction starts a current flow from the cathode into the tissue which is directly proportional to the oxygen tension. At the anode Cl^- from the tissue fluid is attracted by the positive polarizing voltage and used in the oxidation of silver:

Electrons released at the anode flow through the external circuit of the oxygen electrode (Fig. 10) and equal the current flow from the cathode into the tissues. The current which is measured with an ammeter is therefore proportional to the oxygen tension of the tissue.

Figure 10. Diagram of an oxygen electrode and the electrolytic dissociation at the anode and cathode.

a. Description of the electrode

The electrode consisted of a 5 mm piece of platinum wire, 25 μm in diameter, fused in lead glass tubing, 1.5 cm in length and 5 mm in diameter. The active end of the platinum electrode was polished with an oil stone, and dipped in Rhoplex AC 35 (Rohm and Hass, West Hill, Ontario) to provide a covering to reduce protein poisoning. A silver/silver chloride reference electrode, 250 μm in diameter, was placed directly on the brain surface adjacent to the cathode. Before physiological measurements were made, each electrode was conditioned by placing it in 0.9% saline and applying -0.8 V to the platinum cathode until the current stabilized (approximately 30 minutes). Each electrode was tested by measuring the current in the electrode circuit when the voltage between the anode and cathode was varied between 0 and 1 volt. Electrodes that have a linear response to oxygen concentration show a plateau of the current-voltage plot between 0.5 and 0.7 volts. Fig. 11 shows an acceptable current-voltage plot (polarogram) for linearity of oxygen tension between 0 and 20.0 kPa (150 torr). Electrode output showed a resting current of approximately 0.1-0.2 nA for nitrogen saturated saline and approximately 5.0-6.0 nA for a PO_2 of 20 kPa (air saturated saline). The response time was 3 to 6 seconds for 90% of a full response. Only electrodes that fitted the above criteria were used for cortical PO_2 measurements.

Figure 11. Polarogram of an acceptable O_2 electrode.

Abbreviation: nA, nanoamps.

b. Oxygen measurements from the cortical surface of ducks during hypoxia and hypercapnia

The electrode was tested by measuring oxygen tension from the cerebral cortex ($P_{T}O_2$) of a duck during hypercapnia (Fig. 12, B) and different levels of hypoxia (Fig. 12, A). The electrode was calibrated in air and nitrogen saturated saline before and after the physiological measurements. When the ventilatory gas was changed from air to 9% oxygen, $P_{T}O_2$ decreased sharply and formed a plateau; when the duck was ventilated with 5% oxygen the $P_{T}O_2$ change was similar only more pronounced. Hypercapnia (Fig. 12, B) caused an increase in $P_{T}O_2$ due presumably to vasodilation.

For the purposes of this study it was necessary to express CF increase as a function of $P_{T}O_2$ during hypoxia; however, the heterogeneity of oxygen tension in the brain precludes the use of absolute $P_{T}O_2$ for this measurement. For example, oxygen tensions may vary from 0 kPa to over 9.0 kPa (68 torr) depending on the geometric position in the capillary network (Silver, 1966; Lübbers, 1971; Smith, 1977) and oxygen tension can change as much as 9.0 kPa (68 torr) in a distance of less than 0.5 mm (Lübbers, 1971). The recording field for the fluorometer was 3.5 mm or 2.3 mm in diameter, depending on the objective used, and fluorescence originated from an indefinite depth in the tissue which was something less than 1.5 mm (Jöbsis et al., 1971). In order for $P_{T}O_2$ to adequately reflect CF, the average $P_{T}O_2$ for the volume of tissue in the fluorescence recording would have to be determined. If, however, for a given level

Figure 12. Polarographic measurements of $P_{T}O_2$ from the cortical surface of a duck when the ventilatory gas was changed from air to 9% oxygen and 5% oxygen (A). In B, 7% CO_2 was added to the ventilatory mixture. The time bar refers to both A and B.

of hypoxia the decrease in $P_{T}O_2$ is proportional throughout the brain, then $P_{T}O_2$ can be reported as a percent decrease from the normoxic conditions, except, of course, where $P_{T}O_2$ is 0. A series of experiments showed that the above assumption was valid. $P_{T}O_2$ was recorded from the cerebral cortex in 4 ducks and from 4 different recording sites in a single chicken when the oxygen in the ventilatory gas was varied from 5% to 15%. $P_{T}O_2$ was expressed as a percent decrease of the electrode current where the decrease from normoxia to anoxia (death) was considered 100%. The results are displayed graphically in Fig. 13. The coefficients of correlation (r^2) for the linear regressions ranged from 0.87 to 1.0 except for 1 hypoxic regime in the chicken which had an r^2 of 0.65. An analysis of covariance showed that the 8 linear regression lines had a common slope and y-intercept with a common equation of $y = 99 - 3.9x$. Not only was the assumption that arterial hypoxia produced a uniform decrease in $P_{T}O_2$ throughout an individual valid but it also held for different individuals. These results are consistent with the work of Leninger-Follert et al. (1976) who showed that arterial hypoxia produced a uniform decrease in oxygen tension recorded simultaneously from 8 different locations in the cerebral cortex.

Figure 13. Surface PO_2 ($P_T O_2$) recorded from the right cerebral cortex when oxygen in the ventilatory gas was varied from 15% to 5% in 4 ducks (solid lines) and from 4 different recording sites in a single chicken (dotted lines). Each line represents a linear regression from a minimum of 12 points. $P_T O_2$ was expressed as a percent decrease of the electrode current when the decrease from normoxia to anoxia (death) was considered 100%.

CHAPTER 3

Changes in the Redox State of Respiratory Chain NADDuring Apneic Asphyxia in DucksIntroduction

Diving mammals and birds are known to tolerate periods of apneic asphyxia that are detrimental to terrestrial animals (Andersen, 1966). The increased tolerance has been related to the refinement of oxygen conserving cardiovascular adjustments (Scholander, 1940) which are thought to protect the heart and the brain (Andersen, 1966; Blix, 1976). Although oxygen conservation is well documented (Scholander, 1940; Butler and Jones, 1971), it has not been directly related to the biochemical events involved with ATP production of the brain. Since oxygen is linked to ATP production via the respiratory chain, the effects of its conservation should be reflected in the redox state of NAD.

The purpose of this chapter was to examine the redox change of respiratory chain NAD from the cerebral cortex of ducks during apneic asphyxia and determine the effects of the cardiovascular adjustments on the redox state. Initial experiments were performed on nonparalyzed ducks which were restrained on a metal operating table. Although the head was held rigid, struggles which produced slight movements of the brain caused artifacts in the optical traces. If it was possible to substitute paralyzed ducks for nonparalyzed ducks in these fluorometric studies, then the movement artifacts produced by the struggles

could be eliminated. I compared cardiovascular responses and change in cortical NADH in paralyzed and nonparalyzed ducks to determine if indeed this substitution was possible.

Methods

a. Fluorescence recordings from nonparalyzed ducks

NADH fluorescence was monitored from the left cerebral cortex of 9 ducks using glass windows as described in Chapter 1. The birds were secured ventral side down on a metal operating table and the head was held motionless with the bill at 45° below horizontal by 3 metal screws (3 cm long), secured to the skull with dental cement, and bolted to rods fixed to the metal table. In 5 of the 9 ducks brain electrical activity was monitored bipolarly from the right frontal region of the skull. Ducks were subjected to periods of apneic asphyxia (4 to 7 minutes) produced by submerging the bill, nares, and eyes in water. Each duck was exposed to 2 periods of submergence with a 45-60 minute recovery period between them.

b. Fluorescence recordings from paralyzed ducks

NADH fluorescence was monitored from the left cerebral cortex of 28 paralyzed ducks using plastic film to cover the exposed cortex to prevent drying. Ducks were subjected to 2 or 3 periods of apneic asphyxia lasting from 2 to 9 minutes with a 30 minute recovery period between asphyxic periods. A blood sample was withdrawn for blood gas analysis before each asphyxic period and ventilation was adjusted to give suitable blood gas values (Chapter 1). Additional blood samples were taken for blood gas analysis at various times after ventilation was stopped.

c. Inhibition of the cardiovascular adjustments during apneic asphyxia in ducks

Eight mallard ducks of either sex, weighing 1.2-2.0 kg, were used in this series of experiments and were prepared as described in section b, except in 2 ducks the window method was used for optical recordings and cortical oxygen tension was measured in 4 ducks.

$P_{T}O_2$, where appropriate, and CF were measured during 2 minute periods of apneic asphyxia before and after the cardiovascular adjustments were inhibited with atropine sulfate (2.5 mg/kg, BDH Chemicals Ltd., Poole, England). The effects of atropine were tested before and after apneic asphyxia by injections of acetylcholine chloride (5-10 mg/kg, BDH). The hypotension and bradycardia normally produced by acetylcholine is abolished by atropine and the absence of hypotension was considered as the inhibition of cardiovascular adjustments. Each duck was exposed to 4-5 periods of apneic asphyxia, 2-3 pre-atropine and 2 post-atropine, with a 30 minute recovery between each.

For the purpose of expressing the optical changes in a quantitative manner, the fluorescence intensity during normoxia (baseline) was defined as zero and the fluorescence intensity following death by anoxia was defined as 100 arbitrary units (AU). Optical changes that correspond to intensities greater than the baseline (NAD reduction) are designated with a positive sign; conversely, optical changes that have intensities less than the baseline (NADH oxidation) are designated with a

negative sign. Numerical values in the figures are expressed as means $\pm$ 1 standard error of the mean (SEM) and the t-test was used in the statistical analysis of the data with 5% ($P < 0.05$) considered the acceptable level of significance.

Results

a. Comparison of nonparalyzed and paralyzed ducks

Heart rate, mean arterial blood pressure (MABP), and corrected fluorescence were compared in paralyzed and non-paralyzed ducks during the first 2 minutes of asphyxia and 40 seconds of recovery (Fig. 14). Asphyxia in nonparalyzed ducks was produced by stopping artificial ventilation. Since the length of the asphyxic periods in both paralyzed and nonparalyzed ducks varied, the recovery phase in Fig. 14 started from termination of asphyxia. Mean pre-asphyxic heart rate for paralyzed and nonparalyzed ducks was 255 ± 32.04 beats/minute (55 periods of asphyxia in 20 ducks) and 247 ± 13.05 beats/minute (11 periods of asphyxia in 6 ducks) respectively. During the first minute of asphyxia mean heart rate fell to 44% and 23% of the control rate in paralyzed and nonparalyzed ducks respectively and remained relatively stable until termination of asphyxia. Although mean heart rate in nonparalyzed ducks was lower during asphyxia, only at 60 seconds was the difference between paralyzed and nonparalyzed ducks significant. Nonparalyzed ducks characteristically showed a tachycardia almost immediately after emersion; heart rate increased from less than 50 beats/minute to 376 ± 24.00 beats/minute (11 periods of asphyxia in 6 ducks) after 10 seconds and gradually returned to pre-asphyxic levels during the next 40 seconds. When ventilation was resumed in paralyzed ducks, heart rate returned to the pre-asphyxic level after 90-120 seconds without a tachycardia.

Figure 14. Comparison between heart rate (HR), mean arterial blood pressure (MABP), and corrected fluorescence (CF) during 11 periods of apneic asphyxia in 6 nonparalyzed ducks (dotted line) and 55 periods of apneic asphyxia in 20 paralyzed ducks (solid line). Asphyxia was produced by submerging the face in water and by stopping the artificial ventilation in nonparalyzed and paralyzed ducks respectively. Elapsed time (abscissa) during asphyxia and the recovery is given in seconds. CF is expressed in arbitrary units (AU) where the CF changes from normoxia to anoxia (death) was defined as 100 AU. Each point represents the mean $\pm$ SEM and the SEM is contained within the point when absent.

Before asphyxia MABP was 20.1 ± 1.040 kPa (151 ± 7.8 mm Hg) and 17.8 ± 0.660 kPa (134 ± 4.5 mm Hg) in nonparalyzed and paralyzed ducks respectively, and showed only minor changes during and after asphyxia.

An increase in CF occurred in both paralyzed and non-paralyzed ducks during asphyxia. Moreover the rates of increase were similar and nearly constant between sampling intervals. After 120 seconds of asphyxia, CF increased by 15 ± 1.94 AU ($n = 55$ periods of asphyxia in 20 ducks) and 18.5 ± 6.35 AU ($n = 11$ periods of asphyxia in 6 ducks) in paralyzed and non-paralyzed ducks respectively. When asphyxia was terminated mean CF decreased and was below the baseline 40 seconds after asphyxia in both groups. In both paralyzed and nonparalyzed ducks, recovery of CF after asphyxia was always associated with a transitory overshoot of the baseline which generally returned to the preasphyxic level after 5-10 minutes.

A 4.7 minute period of submergence asphyxia and the subsequent recovery in a single nonparalyzed duck is shown in Fig. 15. Heart rate decreased rapidly during the first 30 seconds and remained low for the duration of submergence. MABP was maintained near the prediver level until 4.5 minutes after submersion when it fell precipitously. The third, fourth and fifth traces from the top describe the optical signals. In all cases an increase in light detected by the photomultiplier is indicated by an upward deflection of the pen. Note the change in scale in the CF trace (fifth trace) from the F and R traces (third and fourth traces respectively). The abrupt

Figure 15. A 4.7 minute period of submergence asphyxia and recovery in a nonparalyzed, restrained duck. The downward pointing arrow at the bottom indicates submersion and the upward pointing arrow indicates emersion. An upward deflection of the optical traces represents an increase in light intensity and optical traces are expressed in arbitrary units (AU) where the CF change from normoxia to anoxia (death) was defined as 100 AU. Time bars, 1 second and 1 minute respectively, denote the changes in chart speed.

Abbreviations: HR, heart rate; MABP, mean arterial blood pressure; kPa, kilopascals; F, fluorescence; R, reflectance; CF, corrected fluorescence; EEG, electroencephalogram; uV, microvolts.

positive deflection in the F trace shortly after submersion and the large, slower, negative deflection after emersion are artifacts caused most probably by slight movement of the cortex. Note that these artifacts are paralleled in the R trace and that electronic subtraction of F-R gives the CF trace which is free of artifacts and due solely to NADH. About 80 seconds after submersion, CF began to increase and gradually increased to 5 AU after 2 minutes. The trend continued until about 4.5 minutes after submersion when blood pressure dropped markedly; at this point a slightly sharper increase in CF occurred. EEG activity diminished (bottom trace) when CF was approximately 32 AU. In ducks that did not show a precipitous drop in blood pressure the EEG diminished more gradually; however, isoelectricity still occurred when fluorescence had increased from between 28-40 AU in all nonparalyzed ducks.

On emersion heart rate rose above the prediver rate within several seconds and gradually returned to the prediver level. Blood pressure also increased. A few seconds after emersion corrected fluorescence decreased sharply and was followed by a gradual decrease over 3 minutes and even briefly dipped below the baseline before returning to the prediver level. Some other ducks showed an immediate baseline overshoot omitting the slower oxidation described here.

Fig. 16 shows a 2 minute period of apneic asphyxia produced by stopping artificial ventilation in a paralyzed duck, and the variables measured appear to show the same trends as in nonparalyzed animals.

Figure 16. Response in a paralyzed duck to a 2 minute period of apneic asphyxia produced by stopping artificial ventilation. Fluorescence (F), reflectance (R), and corrected fluorescence (CF) were recorded from the left cerebral cortex and are expressed in arbitrary units (AU) where the CF change from normoxia to anoxia (death) was defined as 100 AU. The period of asphyxia is indicated by the 2 arrows. Other abbreviations: HR, heart rate; MABP, mean arterial blood pressure; kPa, kilopascals.

b. Inhibition of the cardiovascular adjustments during apneic asphyxia in ducks

Eight ducks were exposed to 2 minute periods of apneic asphyxia before and after the cardiovascular adjustments to apneic asphyxia were abolished. Over 3/4 of the hypertension and bradycardia produced by the injection of acetylcholine was abolished by atropine. Table I shows the CF increase in 8 ducks after 1 and 2 minutes of apneic asphyxia before and after atropine treatment. In every duck CF was greater after 120 seconds of asphyxia following treatment with atropine than in asphyxia before atropine treatment and the difference was significant. Note that CF was similar in the normal (8 ± 2.29 AU, $n = 8$) and atropinized (12 ± 2.90 AU, $n = 8$) ducks after 1 minute of asphyxia and only in the second minute were the effects of cardiovascular adjustments in maintaining a more oxidized respiratory chain, apparent. Fig. 17 shows $P_{T}O_2$ and CF traces during apneic asphyxia before (C) and after (A) an atropine injection. After 2 minutes of asphyxia $P_{T}O_2$ decreased by 61% and CF increased 25 AU before an atropine injection while after atropine, $P_{T}O_2$ decreased by 72% and CF increased 44 AU in the same time period.

Table I. Increase in corrected fluorescence (CF) in paralyzed ducks after 60 and 120 seconds of apneic asphyxia before and after atropine treatment. The numbers in parentheses are the numbers of asphyxic periods. Mean $\pm$ SEM AU are at the bottom of the table.

Duck Number	Apneic Asphyxia Before Atropine Treatment		Apneic Asphyxia After Atropine Treatment	
	Corrected Fluorescence		Corrected Fluorescence	
	60 sec	120 sec	60 sec	120 sec
1	8(2)*	39(2)	8(2)	47(2)
2	3(1)	6(1)	1(2)	16(2)
3	5(3)	23(3)	11(2)	44(2)
4	4(2)	19(2)	10(2)	36(2)
5	8(3)	21(3)	8(2)	43(2)
6	18(3)	23(3)	23(2)	50(2)
7	19(2)	31(2)	25(2)	45(2)
8	3(2)	29(2)	7(2)	32(2)
Mean $\pm$ SEM	8 $\pm$ 2.29	23 $\pm$ 3.39	12 $\pm$ 2.90	39 $\pm$ 3.89

* The numbers in parentheses are the number of periods of apneic asphyxia.

Figure 17. PO_2 of the right cortical surface ($P_T O_2$) and corrected fluorescence (CF) from the left cortical surface during 2 minutes of apneic asphyxia (indicated by arrows) in a paralyzed duck before (C) and after an atropine injection (A). $P_T O_2$ is expressed as a percent decrease of the electrode current when the decrease from normoxia to anoxia (death) was defined as a 100% decrease. CF was expressed in arbitrary units (AU) when the CF change from normoxia to anoxia (death) was defined as 100 AU.

Discussion

The apneic asphyxia produced in paralyzed ducks by stopping unidirectional ventilation simulates the onset of cardiovascular adjustments and changes in respiratory chain NADH in nonparalyzed ducks during forced submersion. Although bradycardia during asphyxia in paralyzed ducks was not as severe as in nonparalyzed ducks, there were no significant differences between the 2 groups except at the 60 second sampling interval. The major difference was found in the recovery period where the paralyzed group lacked the typical postasphyxic tachycardia; instead heart rate gradually returned to the pre-asphyxic rate over a period of 90-120 seconds. Bamford and Jones (1974) reported similar results for paralyzed and nonparalyzed ducks and later claimed it is caused by the absence of neural input from the lungs since unidirectional ventilation in paralyzed animals does not cause an increase in lung pressure during postasphyxic ventilation (Bamford and Jones, 1976). NADH fluorescence in both groups gradually increased during the first 2 minutes of asphyxia and returned to the baseline after a transitory overshoot when ventilation was resumed. The NADH fluorescence was almost identical in both groups during the entire cycle. Only 10 seconds after the termination of asphyxia did NADH fluorescence differ significantly due to a faster recovery rate in the nonparalyzed animals.

The redox state of respiratory chain components has been studied in the brain during hypoxia and anoxia which were produced by varying oxygen in the ventilatory gas from 0-20%

(Chance et al., 1962, 1964, 1973; Chance and Schoener, 1962), and by reducing or stopping blood flow (ischemia) (Sundt and Andersen, 1975b; Sundt et al., 1976; Rosenthal et al., 1976b; LaManna et al., 1977; Ginsberg et al., 1976); but studies during apneic asphyxia have never been reported before this study. All three means of producing a fall in tissue oxygen tension share the same type of response, namely a reduction of the respiratory chain components; however, the kinetics of the reduction are different due to different rates of oxygen depletion. At the onset of hypoxia, reducing equivalents from the substrate are passed into the respiratory chain faster than they are removed by oxygen. The reduced form of the carriers increases at the expense of the oxidized form (NADH and NAD in the case of the present study) and seek a more reduced redox state. In apneic asphyxia oxygen is continually depleted and reduction approaches the maximum obtainable at death unless ventilation is resumed.

The importance of the cardiovascular adjustments for protecting the brain during apneic asphyxia was demonstrated by monitoring NADH fluorescence and brain $P_{T}O_2$ during apneic asphyxia in ducks before and after the cardiovascular adjustments were inhibited with atropine. Apneic asphyxia following inhibition of the cardiovascular adjustments produced a greater reduction of NAD and a greater decrease in brain $P_{T}O_2$ than before the inhibition. However, the effects of the cardiovascular adjustments on CF and $P_{T}O_2$ were not readily apparent during the first minute of asphyxia (Table I and Fig. 17).

For example, after 1 minute of asphyxia, CF was 12 AU and 8 AU in atropinized and non-atropinized ducks respectively. It was expected that effects of the cardiovascular adjustments should also be apparent during the first minute of asphyxia. Several factors will shed some light on this observation. First, maximum cardiovascular adjustments in non-atropinized ducks (as indicated from heart rate), and thus oxygen conservation, were not complete until 40-60 seconds after artificial ventilation was stopped (Fig. 14). Only in the second minute, when oxygen conservation is maximal, are large differences in $P_{T}O_2$ between the periods of asphyxia before and after atropine injections readily apparent. Second, the relationship between NADH fluorescence and $P_{T}O_2$ was such that very little change in NADH fluorescence occurred until $P_{T}O_2$ has decreased by 50% (Fig. 22, Chapter 4). After $P_{T}O_2$ has decreased by 50% small changes in tissue oxygenation caused large increases in CF. Since $P_{T}O_2$ did not fall below 50% in either the atropinized or non-atropinized animals during the first minute of asphyxia, large difference in CF would not be expected.

CHAPTER 4

Cerebral Energy Metabolism in Ducks and ChickensDuring Apneic Asphyxia and HypoxiaIntroduction

The primary means of ATP production in the brain (oxidative phosphorylation) requires oxygen to accept reducing equivalents from the respiratory chain and brain function can continue only as long as oxygen is available. However, natural divers can maintain brain function over 4 times longer during dives than their terrestrial counterparts before brain function ceases (Kerem and Elsner, 1973a). In Chapter 3 I showed that physiological adjustments which conserved oxygen during asphyxia were responsible for prolonging both tissue oxygenation and reduction of respiratory chain NAD. Since the brain is obligately dependent on oxygen, it follows that brain function is prolonged directly as a result of these physiological adjustments. However, natural divers may tolerate more severe hypoxia than terrestrial animals (Elsner et al., 1970; Kerem and Elsner, 1973 a and b; Ridgeway et al., 1969) indicating that in addition to physiological adjustments some form of biochemical adaptation is present in divers. In theory, these could be adaptations which would (1) allow oxidative phosphorylation to continue at levels of hypoxia that cannot be tolerated by nondivers and/or (2) enhance anaerobic ATP production to supplement oxidative phosphorylation in the face of decreasing oxygen.

The purpose of this chapter was to, first, test the hypothesis that the reduction of respiratory chain NAD proceeds at a slower rate during apneic asphyxia in ducks, Anas Platyrhynchos, than chickens, Gallus domesticus. Ducks are known to tolerate apneic asphyxia 3-8 times longer than chickens (Andersen, 1959 and 1966; Scholander, 1964). Second, I investigated the relation between the redox state of respiratory chain NAD and EEG in ducks and chickens during apneic asphyxia to elucidate any major contribution to ATP production by anaerobic metabolism in ducks. In vivo fluorometric recordings in the anesthetized rat have identified the critical pyridine nucleotide reduction (CPNR) above which brain electrical activity ceases (Chance and Schoener, 1962; Mayevsky and Chance, 1973). During N₂ ventilation the CPNR occurs when the fluorescence increase from the normoxic level is approximately 3/4 of the level recorded at death. If ducks rely on large anaerobic contributions of ATP for the continuation of brain function, they should have a more reduced respiratory chain than the chicken before the appearance of gross alterations in EEG. Third, to test for biochemical adaptations involving oxidative phosphorylation the redox state of respiratory chain NAD of ducks was compared to chickens at various levels of hypoxia. If biochemical adaptations are present in ducks, then the accumulation of respiratory chain NADH for a given level of hypoxia should be less in ducks than chickens.

Methods

a. Fluorescence recordings from paralyzed ducks and chickens

NADH fluorescence was monitored from the left cerebral cortex of 20 paralyzed chickens and 30 paralyzed ducks. Plastic film was placed over the exposed cortex to prevent drying. Chickens and ducks were subjected to 2 or 3 periods of apneic asphyxia lasting 1 minute and from 2 to 9 minutes respectively. Asphyxic periods were separated by a 20-60 minute recovery period; the longest recovery periods followed the longest periods of asphyxia. In 13 ducks and 11 chickens EEG was recorded and the asphyxic period was continued until brain electrical activity ceased (referred to in this paper as EEG endpoint). In these experiments each animal was exposed to only one asphyxic trial. A blood sample was withdrawn for blood gas analysis before each asphyxic period and ventilation was adjusted to give normal blood gas values (Chapter 1).

b. Concurrent fluorometric and polarographic recordings during hypoxia

NADH fluorescence was recorded from the left cerebral cortex of 13 ducks and 9 chickens as described in section a. $P_{T}O_2$ was recorded from the area of the right cerebral cortex that corresponded to the area used for optical recordings on the opposite hemisphere. Both species were exposed to several levels of normocapnic hypoxia (1 minute in duration) by varying the oxygen in the ventilatory gas from 2-20% in a balance of nitrogen. The gases were mixed with flowmeters complete with

screw valves and analyzed for percent oxygen using a Beckman Model F3 oxygen analyzer (Beckman Instruments, Inc., Fullerton, California). In addition, progressive hypercapnic hypoxia was induced in ducks by stopping artificial ventilation for 2-4 minutes.

Results

a. Comparison of ducks and chickens

Heart rate, MABP, and CF recorded from ducks (solid line) and chickens (dotted line) during apneic asphyxia are shown in Fig. 18. Mean preasphyxic heart rate for 19 chickens and 20 ducks was 154 ± 9.29 and 255 ± 32.04 beats $\cdot$ minute $^{-1}$ respectively. After a 15 second latent period from the time artificial ventilation was stopped mean heart rate in chickens fell steadily until apnea was terminated at 60 seconds. In contrast, ducks showed a decrease in mean heart rate during the first 15 seconds which corresponded to 42% of the total bradycardia. Although this rate of decrease was not paralleled between any other subsequent sampling periods, the heart rate, nevertheless, steadily decreased to 35% at 90 seconds with 88% of the bradycardia occurring during the first minute. At 60 seconds heart rate in chickens and ducks was 55% and 44% of the preasphyxic rate respectively. When artificial ventilation was resumed, mean heart rate in both species returned to the preasphyxic rate after 60-90 seconds without the post-asphyxic tachycardia characteristic of nonparalyzed birds. MABP did not change significantly in either species during asphyxia or the subsequent recovery.

Asphyxia in both species was characterized by an increase in NADH which continued at a nearly linear rate until artificial ventilation was resumed. Although reduction was inevitable during apnea since oxygen was diminishing, ducks showed better control at regulating redox balance by decreasing the rate of

Figure 18. Heart rate (HR), mean arterial blood pressure (MABP), and corrected fluorescence (CF) during 1 and 2 minute periods of apneic asphyxia in paralyzed chickens (54 periods of asphyxia in 19 chickens) and ducks (55 periods of asphyxia in 20 ducks) respectively. The dotted lines represent chickens and the solid lines represent ducks. The arrows indicate the asphyxic period. CF is expressed in arbitrary units (AU) where the CF change from normoxia to anoxia (death) was defined as 100 AU. Each point represents the mean $\pm$ SEM and the SEM is contained within the point when absent.

reduction to $1/5$ of that in chickens. For example, CF was significantly lower after 1 minute of apnea in ducks (8 ± 1.41 AU) than chickens (37 ± 3.60 AU). Furthermore, if the rate of reduction continued unchanged in ducks from the first 2 minutes, then it would take over 4.5 minutes of apnea before CF equalled the CF in chickens at 60 seconds. When ventilation was resumed CF returned to the baseline in both species after a transient overshoot, usually of 5-10 minutes duration.

After 60 seconds of apnea in chickens PaO_2 was 5.8 ± 0.366 kPa (44 ± 2.57 torr) and after 120 seconds of apnea in ducks PaO_2 was 5.6 ± 0.551 kPa (38 ± 4.14 torr). It is interesting to note that when mean PaO_2 's were very similar mean CF increase in chickens was over twice that in ducks (37 AU versus 15 AU). To clarify the enigma 2-4 blood samples were withdrawn from the femoral artery at random intervals after artificial ventilation was stopped in 8 ducks and 6 chickens, and the relationship between CF increase and PaO_2 was examined for the best fit to either a straight line, an exponential curve, a power curve, or a logarithmic curve. Correlation was poor in all cases with a linear regression having the largest coefficients of correlation (r^2), 0.24 and 0.37 in chickens and ducks respectively. Therefore, PaO_2 did not accurately reflect redox state of the respiratory chain. PaCO_2 of the various blood samples ranged from 4.6-7.3 kPa (35-55 mm Hg).

b. Critical pyridine nucleotide reduction (CPNR) in chickens and ducks

EEG and CF were recorded simultaneously during prolonged apneic asphyxia in chickens and ducks. In 11 chickens and 10 ducks CF was 34 ± 2.19 AU and 38 ± 1.90 AU respectively when the EEG endpoint was reached (Tables II and III). CF was not significantly different at the EEG endpoint; however, the time from the beginning of apnea to the endpoint was over 5-fold longer in ducks than chickens. Chickens maintained brain electrical activity during apnea for 63 ± 4.38 seconds (range 42-93 seconds) and ducks for 338 ± 32.36 seconds (range 232-549 seconds). Fig. 19 shows results from 2 individuals; the top 2 traces (EEG and CF) are from a duck and the bottom 2 traces (EEG and CF) are from a chicken. In both cases CF increased when the artificial ventilation was stopped (first arrow) and continued until the EEG endpoint was reached at which time the ventilation was resumed (second arrow) and CF returned to the baseline. Although the EEG endpoint was reached sooner in chickens, CF at the endpoints was similar, 38 AU and 32 AU in the chicken and duck respectively. In ducks it appeared that those which showed the greatest bradycardia maintained EEG for longer periods of apnea than those in which bradycardia was not so pronounced (Table II). Although bradycardia is only a crude measure of cardiovascular adjustments, since it does not account for peripheral vasoconstriction, it nevertheless correlates with diving performance. Considering the data from Table II, the time tolerated during asphyxia before the EEG

Table II. Heart rate, time to EEG endpoint, and CF at EEG endpoint in paralyzed ducks during apneic asphyxia. Mean $\pm$ SEM AU at bottom of table.

Time (seconds) during apneic asphyxia		0	15	30	60	120	180	240	300	360	420	480	540	Time at EEG endpoint	CF at EEG endpoint
Ducks	Heart rate	(beats·minute ⁻¹)													
1	305	208	171	73	49	37	39	42	35	49				431	35
2	293	273	220	208	104	85	81							263	28
3	141	102	90	115	119									232	32
4	240	200	150	125	112	109	121							253	31
5	281	203	172	142	125	108	107	142						311	41
7	175	145	122	119	121	125	136							247	38
8	270	175	140	130	91	84	95	93	87					367	31
10	301	209	185	156	130	121	135	129						313	38
11	270	161	135	112	92	81	79	85	77					415	34
12	261	149	92	42	37	42	35	39	41	47	49	65		549	45
Mean ± SEM	254 ± 17.26 (n=10)	182 ± 14.83 (n=10)	149 ± 12.39 (n=10)	119 ± 14.49 (n=10)	98 ± 10.00 (n=10)	91 ± 9.97 (n=10)	92 ± 12.48 (n=9)	88 ± 17.46 (n=6)						338 ± 32.26 (n=10)	38 ± 1.90 (n=10)

Table III. Heart rate, time to EEG endpoint, and CF at EEG endpoint in paralyzed chickens during apneic asphyxia. Mean $\pm$ SEM AU at the bottom of table.

Time (seconds) during apneic asphyxia	0	15	30	60	90	Time at EEG endpoint	CF at EEG endpoint
Chickens	Heart rate (beats·minute ⁻¹)						
1	114	96	114	48		62	27
2	163	158	118			42	32
3	202	196	184	147	98	93	36
4	204	186	163	120		77	37
5	195	195	108	94		75	41
6	108	107	95			56	50
7	140	140	125			51	25
8	196	196	172			49	40
9	201	195	142	126		61	30
10	138	151	88			57	31
11	147	147	126	124		64	30
Mean $\pm$ SEM	164 $\pm$ 11.11 (n=11)	160 $\pm$ 10.98 (n=11)	130 $\pm$ 9.45 (n=11)	110 $\pm$ 14.17 (n=6)		62 $\pm$ 4.38 (n=11)	34 $\pm$ 2.19 (n=11)

Figure 19. Electroencephalogram (EEG) and corrected fluorescence (CF) recorded from a duck (upper 2 traces) and a chicken (bottom 2 traces) during apneic asphyxia. The downward pointing arrow for each pair of traces indicates the beginning of asphyxia and the upward pointing arrow indicates the end of asphyxia. The time bar applies to all four traces. EEG is expressed in microvolts (μV) and CF is expressed in arbitrary units (AU) where the CF change from normoxia to anoxia (death) represents 100 AU.

endpoint shows a correlation with the maximum decrease in heart rate (expressed as a percent of the pre-asphyxic rate) (Fig. 20). A relation between degree of bradycardia and time to the EEG endpoint was not observed in chickens (Table III).

c. Relationship between fluorescence and $P_{T}O_2$ in ducks and chickens during hypoxia

NADH fluorescence and $P_{T}O_2$ were measured simultaneously from opposite cerebral hemispheres in ducks and chickens during steady state hypoxia produced by varying the oxygen in the ventilatory gas. As tissue $P_{T}O_2$ fell, CF increased, and the more severe the hypoxia the greater the CF increase. When the duck was returned to 20% oxygen, $P_{T}O_2$ and CF eventually returned to normal. Figure 21 shows the results from a single duck. The area between the arrows represents the periods when various levels of hypoxia were applied. $P_{T}O_2$ is expressed as a percent decrease of the electrode current when the decrease from normoxia to anoxia (death) was defined as a 100% decrease; CF was expressed in AU's as previously described. Proceeding from a to d, hypoxia became more severe as indicated by the decrease in $P_{T}O_2$. Experiments performed on chickens (not shown) showed a similar relationship between $P_{T}O_2$ and CF.

When NADH fluorescence was plotted as a function of the decrease in $P_{T}O_2$ (Fig. 22) an exponential curve best described the relationship in 8 chickens (light circles) and 9 ducks (dark circles). Since the EEG endpoint was reached before

Figure 20. Length of time during apneic asphyxia in paralyzed ducks before the cessation of brain electrical activity in relation to the decrease in heart rate (expressed as a percent of the preasphyxic heart rate). Each point represents a single period of apneic asphyxia. Values are taken from Table II.

Figure 21. Surface PO_2 from the right cerebral cortex ($P_T O_2$) and corrected fluorescence (CF) recorded from the left cerebral cortex in paralyzed ducks during steady state hypoxia (indicated by arrows) produced by varying oxygen in the ventilatory gas. Proceeding from a to d hypoxia became more severe. $P_T O_2$ is expressed as a percent decrease of the electrode current when the decrease from normoxia to anoxia (death) was defined as a 100% decrease. CF was expressed in arbitrary units (AU) where the CF change from normoxia to anoxia (death) was defined as 100 AU.

$P_T O_2$

% DECREASE

0
100

50 AU

CF

1 min.

Figure 22. Corrected fluorescence (CF) in relation to PO_2 recorded from cortical surface ($P_T O_2$) in 8 chickens (light circles) and 9 ducks (dark circles). $P_T O_2$ is expressed as a percent decrease of the electrode current where the decrease from normoxia to anoxia (death) was defined as 100% decrease. CF was expressed in arbitrary units (AU) where the CF change from normoxia to anoxia (death) was defined as 100 AU.

CF increased by 50 AU, even in the most extreme case (Tables II and III), only the points that fell below 50 AU were considered here. Equations that best describe the data for chickens and ducks are $y = 3.82e^{0.03x}$ ($r^2 = 0.75$) and $y = 3.74e^{0.03x}$ ($r^2 = 0.87$) respectively. For purposes of comparing ducks and chickens the exponential equations were transformed into a linear regression by plotting $\ln CF$ versus $P_T O_2$. After determining that the residual variances between chickens and ducks were homogeneous, slopes and y-intercepts were compared by the analysis of covariance (Snedecor and Cochran, 1974). The groups were not significantly different at the 95% confidence level and can be represented by a common equation $y = 3.71e^{0.03x}$.

Fig. 23 shows a continuous plot of CF versus $P_T O_2$ in 16 periods of progressive hypercapnic hypoxia produced by stopping artificial ventilation in 8 ducks and the combined data can be described by the equation $y = 3.00e^{0.04x}$ ($r^2=0.87$). Progressive hypercapnic hypoxia did not differ significantly from that obtained during steady state normocapnic hypoxia indicating that CO_2 had no direct effect on the redox state of NAD in ducks.

Figure 23. Corrected fluorescence (CF) recorded from the left cerebral cortex as a function of $P_{T}O_2$ recorded from the right cortical surface ($P_{T}O_2$) during apneic asphyxia in ducks (16 periods of apneic asphyxia in 8 ducks). Each trace is the result from a single asphyxic period. $P_{T}O_2$ is expressed as a percent decrease of the electrode current where the decrease from normoxia to anoxia (death) was defined as a 100% decrease. CF was expressed in arbitrary units (AU) where the CF change from normoxia to anoxia (death) was defined as 100 AU.

Discussion

Studies have been performed on the tolerances of chickens and ducks to diving and have defined death in several different ways. Most were based on last heart beat or last struggle after apnea (Andersen, 1966). Probably a better measure for tolerance is the point at which brain function ceases since in the absence of brain function an animal can no longer coordinate the muscular movements for escape if it is indeed aware of the need to escape. Elsner et al. (1970) and Kerem and Elsner (1973a and b) used the time to the onset of slow wave patterns in the EEG, which are characteristic of unconsciousness during hypoxia, for their endurance studies. In this study the loss of EEG (isoelectricity) was defined as the "EEG endpoint" and the time from ventilatory arrest to the EEG endpoint was defined as maximum tolerance of the individual to apneic asphyxia.

The time to the EEG endpoint in ducks and chickens during apneic asphyxia was 338 ± 32.36 seconds ($n = 10$) and 62 ± 4.38 seconds ($n = 11$) respectively; over 5-fold longer in ducks. Chickens have been previously reported to endure 3 minutes of apneic asphyxia (Andersen, 1966) and ducks have been reported to endure 10-23 minutes of apneic asphyxia (Andersen, 1959 and 1966; Scholander, 1961/1962, 1964). Although the endurance times in the present experiments are less than those previously reported, probably because a less sophisticated measure of tolerance was used in previous experiments, the ratio of endurance times in chickens to ducks is compatible, 3-8 for the

previous reports and 5.5 for the present study.

During apneic asphyxia the respiratory chain NADH characteristically increased in the cerebral cortex of chickens and ducks and continued to increase almost linearly for the duration of asphyxia (1 and 2 minutes respectively). The difference between the 2 species lay in the relative rate at which that NADH fluorescence increased. For example, NADH fluorescence increased at a rate of 0.62 AU/second in chickens over a 1 minute period and 0.13 AU/second in ducks over a 2 minute period, representing over a 4-fold difference in rate. As apneic asphyxia progressed the critical reduction level (35 AU) of the respiratory chain NAD was approached faster in chickens. These results are compatible with the known tolerances of the 2 species to apneic asphyxia.

Berger (1938) suggested that EEG frequency is closely related to the degree of oxidative metabolism in nervous tissue (Ingvar et al., 1976). Although there have been reports to the contrary (Mangold et al., 1955, Kennedy and Sokoloff, 1957) the majority of evidence supports Berger's theory (Himwick et al., 1947; Ingvar et al., 1976; Brodersen et al., 1973; Sundt et al., 1976; Meyer et al., 1967; Gleichmann et al., 1962). Furthermore, LaManna et al. (1975) have shown that EEG frequency and redox state of the respiratory chain (cytochrome a_3) are strictly related to oxygen availability. It follows that, if anaerobic metabolism prolongs brain function by significant ATP production, then the relationship described by LaManna et al. (1975) should be lost. In other words, during apneic asphyxia

an animal relying on a large anaerobic ATP contribution for survival should have a more reduced respiratory chain before gross alterations in EEG than one without large anaerobic contributions. Results from this study show that the EEG endpoint in both species during apneic asphyxia occurred after precisely the same increase in NAD reduction (approximately 35 AU). For these reasons I conclude that anaerobic ATP production, if it occurs, gives the duck no advantage over the chicken in prolonging brain function and survival during apneic asphyxia.

In these experiments neither the absolute pool size of NAD nor the absolute rate of increase in NADH has been quantitated. If pool size is larger in ducks than chickens and if the absolute rate that NADH accumulates is the same in both species, then the time to the critical pyridine nucleotide reduction (Approximately 35 AU in both species) would be longer in ducks. In both chickens and ducks during steady state hypoxia a similar relationship held between the fall in cortical $P_{T}O_2$ and accumulation in cortical NADH which is independent of pool size or absolute rates of accumulation of NADH. But during progressive hypercapnic hypoxia the relationship between the decrease in $P_{T}O_2$ and increase in NADH still holds strongly suggesting that any difference in pool size or absolute rate of NADH accumulation is of no significance in promoting tolerance to apneic asphyxia in ducks.

Results from this study provide no evidence to support the supposition that biochemical adaptations enhance oxidative

phosphorylation in ducks during hypoxia. When chickens and ducks were exposed to a given level of tissue hypoxia, there was no significant difference in the redox change of NADH between the 2 species. That mitochondrial K_m for oxygen in the brain is 1/10 that of other tissues (Clark et al., 1976) may be protection enough to ensure maximum use of available oxygen at low oxygen tensions. Progressive hypercapnic hypoxia had the same effect on the redox state of the respiratory chain as steady state normocapnic hypoxia. In view of the fact that the CF response to progressive hypercapnic hypoxia and steady state normocapnic hypoxia can be described by a common equation, I conclude that hypercapnia does not directly affect the redox state of the respiratory chain or electron flow per se.

Rosenthal et al. (1976a) showed that, when 5% CO_2 was introduced in the ventilatory gas in rabbits and cats, a slight oxidation of cytochrome aa_3 always occurred regardless of the tissue oxygenation state, but the authors did not distinguish between direct effects of CO_2 on the respiratory chain and indirect effects caused by an increased cerebral blood flow and thus an increased $P_{\text{T}}\text{O}_2$. Both the increase in $P_{\text{T}}\text{O}_2$ shown when 7% CO_2 was added to the ventilatory gas (Chapter 2, Fig. 12) and the lack of any effect of hypercapnia on changes in redox state during hypoxia suggest that the effect must have been due to increased cerebral blood flow and $P_{\text{T}}\text{O}_2$.

Rosenthal et al. (1976a) reported that changes in ventilatory oxygen concentration produced a corresponding change in the redox state of cytochrome aa_3 that was continuous from

100% oxygen to nitrogen. On the other hand, Chance et al. (1964) reported that NADH fluorescence did not increase until oxygen in the ventilatory gas was reduced to 5%. Results from this study confirm the conclusions of Rosenthal et al. (1976) showing that firstly, no abrupt changes occurred during the hypoxic regime, only a continuum of change, and secondly, CF increased when $P_{T}O_2$ had decreased by only 20%. A 20% decrease in $P_{T}O_2$ corresponds to oxygen concentrations in the ventilatory gas of greater than 15%, well above the critical 5% level reported by Chance et al. (1964).

GENERAL DISCUSSION

The problem of continued ATP production during diving lies in the inability to maintain the necessary homeostasis necessary for ATP production when external respiration ceases. Nevertheless, the cardiovascular adjustments enable natural divers to tolerate periods of apneic asphyxia that would be detrimental to man and other terrestrial vertebrates. These adjustments do not allow the diver to unconditionally maintain homeostasis but rather prolong it. The strategies for tissues vary and are reflected by their blood supply during the dive. Those that are poorly perfused depend on enhanced glycolytic capacity for the production of ATP once the residual oxygen in that particular tissue is depleted. On the other hand the heart and brain are easily and irreparably damaged by severe and protracted hypoxia (Andersen, 1966) and must be well perfused throughout the dive. Although harbor seals appear to tolerate more severe hypoxia than dogs before the onset of gross EEG alterations (Kerem and Elsner, 1973a and b), in this study ducks could tolerate hypoxia no better than chickens. The semiquantitative method for monitoring cortical NADH in this study allowed comparisons between individuals and species regardless of NAD pool size. Once NADH had increased approximately 35% above the normoxic level in chickens and ducks, brain electrical activity ceased. Furthermore, the 35% increase in NADH occurred with the same decrease in brain PO_2 in both species. Although there is a strong correlation between NADH fluorescence increase and EEG endpoint, EEG may not be directly related to

the redox state. It may be that EEG and NADH fluorescence both reflect the level of tissue oxygenation. In terrestrial mammals the functional state of the brain changes (slowing of EEG) before the tissue shows any detectable changes in the adenosine phosphates (ATP, ADP, AMP) during severe hypoxia (Seisjø et al., 1973 and 1974) and indicates that energy failure (ATP production cannot match the demand) may not be responsible for the loss of brain function. However, the tissue measurement is an average value for the adenosine phosphates, and may not reflect the energy state at critical sites or isolated areas of the brain (Seisjø, 1977). Alternatively, other oxygen requiring reactions in the brain which are used in the biosynthesis of neurotransmitters (tyrosine and tryptophane hydroxylase) and their degradation (monoamine oxidase) may be altered during hypoxia causing a loss of brain function. Although these reactions account for less than 15% of the total oxygen consumed in the brain (Jöbsis, 1974), they are nevertheless sensitive to small changes in oxygen. For example, serotonin, a product of the tryptophane hydroxylase reaction, decreases linearly with a fall in $P_{T}O_2$ between 50 and 20 torr (Jöbsis, 1974). On the other hand, most of these reactions can be specifically inhibited with chemical blockers or brain lesions without the loss of brain function (Snyder, 1976; Sourkes, 1976).

The nervous system of all birds and mammals, whether divers or nondivers, appears to be dependent on oxidative metabolism for its survival. Therefore divers must ensure that oxygen supply to the brain is maintained throughout a dive.

While other tissues appear capable of relying on anaerobic metabolism during the dive, the brain cannot. It may be that the complexity of the brain is not compatible with anoxic tolerance. In any event it appears that the basic strategy of divers is to modify the defense mechanisms for apneic asphyxia found in terrestrial animals to meet the needs of diving. Biochemical and physiological strategies for tolerating diving certainly cannot be classified as truly unique.

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