CEREBRAL ENERGY METABOLISM IN DIVING AND NON-DIVING BIRDS DURING HYPOXIA AND APNOEIC ASPHYXIA

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SUMMARY

1. Cerebral energy metabolism during apnoeic asphyxia and steady-state hypoxia was compared in ducks and chickens; ducks tolerate apnoeic asphyxia 3–8 times longer than chickens.

2. Fluctuations in the reduced form of respiratory chain nicotinamide adenine dinucleotide (NADH) were monitored from the left cerebral hemisphere by a non-invasive fluorometric technique and used as an indicator of mitochondrial hypoxia. NADH fluorescence was expressed in arbitrary units (a.u.) where 100 a.u. was defined as the fluorescence change from normoxia to anoxia. Electroencephalogram (e.e.g.) and surface P_{O_2} were recorded from the right hemisphere.

3. After 1 min of asphyxia NADH fluorescence increased by 37 a.u. $\pm$ 3·60 S.E. of mean ($n = 54$) in paralysed chickens and 8 a.u. $\pm$ 1·41 ($n = 55$) in paralysed ducks. After 2 min the fluorescence increased by only 15 a.u. $\pm$ 1·95 in ducks.

4. Both species showed an isoelectric e.e.g. when fluorescence increased by approximately 35 a.u., indicating that anaerobic ATP production in ducks did not maintain brain function (e.e.g.) for a greater accumulation of respiratory chain NADH.

5. At a given decrease in tissue P_{O_2} ducks and chickens showed the same level of NADH increase, indicating that both species are equally dependent on tissue P_{O_2} for the maintenance of redox state.

6. We conclude that biochemical adjustments which enhance anaerobic ATP production and/or prolong oxidative phosphorylation during progressive hypoxia are not responsible for increased cerebral tolerance to apnoeic asphyxia in the duck.

INTRODUCTION

The brain relies primarily on oxidative phosphorylation, a process that requires oxygen, for adenosine triphosphate (ATP) production. When oxygen supply to the brain is deficient ATP production is reduced, causing brain function to cease. Unlike other tissues such as skeletal muscle that can withstand periods of anoxia, the brain may suffer irreparable damage after relatively brief periods of anoxia. In order to protect the brain from anoxic insults during apnoeic asphyxia, birds and mammals have developed a defence mechanism that conserves oxygen for the brain by reducing blood flow to those tissues that are less easily damaged by anoxia.

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Natural diving mammals and birds have refined this basic physiological adjustment (Scholander, 1940; Johansen, 1964; Butler & Jones, 1971; Jones, Bryan, West, Lord & Clark, 1979) enabling them to maintain brain function 4 times longer than their terrestrial counterparts during apnoeic asphyxia (Kerem & Elsner, 1973a). Although the physiological adjustments are undoubtedly responsible for maintaining brain oxygenation, recent evidence indicates that the brain of divers may tolerate more severe levels of hypoxia than that of terrestrial animals (Ridgway, Scronce & Kanwisher, 1969; Elsner, Shurley, Hammond & Brooks, 1970; Kerem & Elsner, 1973a, b), indicating that in addition to physiological adjustments some form of biochemical adaptation may be present in natural divers. In theory, these adaptations could (1) allow oxidative phosphorylation to continue at levels of tissue hypoxia that cannot be tolerated by non-divers and/or (2) enhance anaerobic ATP production to supplement oxidative phosphorylation in the face of decreasing oxygen.

Since the respiratory chain links oxygen utilization with ATP production it is the focal point for bioenergetics in the brain (Jöbsis, O'Connor, Rosenthal & van Buren, 1972). When oxygen becomes limiting 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, Cohen, Jöbsis & Schoener (1962) and Jöbsis, O'Connor, Vitale & Vreman (1971) provides a non-destructive, direct, and continuous read-out of changes in the oxidation–reduction state of the first member of the respiratory chain, nicotinamide adenine dinucleotide (NAD) (Chance, Mayer & Rossini, 1970). The method takes advantage of the fact that the reduced form (NADH) is a natural fluorochrome which can be excited by light wave-lengths between 310 and 370 nm and gives rise to a fluorescence emission in the region of 425–475 nm (Jöbsis, Legallais & O'Connor, 1966). The oxidized form of the coenzyme does not fluoresce at these wave-lengths. Furthermore, fluorescence from respiratory chain (i.e. mitochondrial) NADH is enhanced over the fluorescence from cytoplasmic NADH to the point that fluctuations in cytoplasmic NADH contribute little to the fluorescence signal (Jöbsis & Duffield, 1967; Chapman, 1972; O'Connor, 1977). In practice NADH is excited by focusing U.V. light from a mercury arc lamp on the surface of the tissue and the fluorescence, originating primarily from the top 0·5 mm of tissue (Chance et al. 1962), is monitored with a photomultiplier tube.

We have used the above technique of monitoring respiratory chain NADH in the cerebral cortex to determine if biochemical mechanisms are responsible for prolonging brain function during apnoeic asphyxia in the duck. Ducks are known to tolerate apnoeic asphyxia 3–8 times longer than chickens (Andersen, 1959, 1966; Scholander, 1964). We report in this paper, first, that the reduction of respiratory chain NAD proceeds at a slower rate in ducks during apnoeic asphyxia than in chickens. Secondly, we have shown that anaerobic metabolism in the duck does not significantly contribute ATP to prolong brain function (as measured by e.e.g.). *In vivo* fluorometric recordings in the anaesthetized rat have identified the critical pyridine nucleotide reduction above which brain electrical activity ceases (Chance & Schoener, 1962; Mayevsky & Chance, 1973). During N_2 ventilation the critical pyridine nucleotide reduction occurs when the fluorescence increase from the normoxic level

is approximately three-quarters of the level recorded at death. If ducks rely on large anaerobic contributions of ATP for the continuation of brain function then they should have a more reduced respiratory chain than the chicken before the appearance of gross alterations in e.e.g. Thirdly, we looked for biochemical adaptations involving oxidative phosphorylation by comparing the redox state of respiratory chain NAD of ducks with that of chickens at given levels of hypoxia. If ducks possess biochemical adaptations that allow oxidative phosphorylation to continue at lower oxygen concentrations, then the accumulation of respiratory chain NADH, at a given level of hypoxia, should be less in ducks than in chickens. We report no significant difference in ducks and chickens, indicating that biochemical adaptations which may prolong ATP during severe hypoxia in the seal (Kerem & Elsner, 1973*a*) and the porpoise (Ridgway *et al.* 1969) do not appear to increase tolerance to apnoeic asphyxia in the duck. The importance of physiological adjustments in increasing tolerance to apnoeic asphyxia will be reported in a future publication (R. M. Bryan & D. R. Jones, unpublished observations).

METHODS

The investigation was done on twenty-nine chickens (*Gallus domesticus*) and forty-three ducks (*Anas platyrhynchos*) varying in weight from 1 to 2·2 kg. Ducks of either sex were used while all experiments on chickens were done on females since in roosters the large comb interfered with cranial surgery. All operative and experimental procedures were the same for chickens and ducks.

Operative procedures

All operations except craniectomies were performed after a local injection of 2 % (w/v) xylocaine (Astra Pharmaceutical, Mississauga, Ontario, Canada). This procedure produced local anaesthesia which was sustained for several hours and the birds showed no signs of stress either during or after surgery. Craniectomies were performed using general anaesthesia produced by ventilating the animals with 1 % halothane in air. A 45 min recovery period from the anaesthesia preceded 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 analysis (P_{a,O_2}, P_{a,CO_2}, pH_a). 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 (see below) and for drug injections. Heart rate was determined from the electrocardiogram (e.c.g.) which was obtained from two copper wire electrodes, one inserted subcutaneously in the left side of the chest and the other inserved in the right thigh. The signal was amplified and fed into a ratemeter. Body temperature was maintained at 41 ± 0.5 °C by a temperature controlled feed-back unit that regulated a heating pad placed over the animal and was governed by a thermistor inserted into the cloaca. 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, Poulene Ltd., Montreal, Quebec, Canada) the birds 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 analysed using a Radiometer PHM 71 gas monitor with oxygen and carbon dioxide electrodes and a pH micro-electrode unit type E5021 (Radiometer, Copenhagen, Denmark). The air flow was adjusted to give arterial blood values for P_{a,O_2} of 11·3–14·0 kPa (85–105 Torr); P_{a,CO_2} of 3·3–4·4 kPa (25–33 Torr); and pH_a of 7·45–7·50.

Brain electrical activity was monitored from two 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 with a Frederick Haer Amplifier (Frederick Haer and Co.,

Brunswick, Maine, U.S.A.) and displayed on a six-channel Watanabe WTR 281 pen recorder (Watanabe Instrument Corporation, Tokyo, Japan) along with other recorded 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 animals the exposed cortex was covered with plastic film (Saran Wrap, Dow Chemical Co., Windsor, Ontario, Canada) to prevent drying.

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.

Design and testing the fluorometer for measuring NADH in vivo

The fluorometer was a modified version of that described by Jöbsis *et al.* (1971). The U.V. source consisted of a 1000 W water-cooled mercury arc lamp AH6-1-B (Illumination Industries Inc., Sunnyvale, California, U.S.A.) encased in a stainless-steel housing. Power was supplied by a model T507 step-up transformer (Illumination Industries) and the voltage to the lamp (700 VAC) was controlled by a Powerstat 136 variable transformer (Superior Electric Co., Bristol, Connecticut, U.S.A.) 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 bandwidth of 10 nm and reflected onto the surface of the tissue at an angle of 45–60 ° using a Leitz Ultropak assembly as an incident-light illuminator. The light intensity was measured by an Eppley thermophile No. 10657 (The Epply Laboratory, Inc., Newport, Rhode Island, U.S.A.) and attenuated by placing round microscope cover-slips (1·8 cm in diameter) between the light source and the specimen, since light intensity above 0·8 mW/cm² may cause tissue damage (M. Rosenthal, personal communication).

Reflected excitation light and NADH fluorescence were collected from cortical fields either 3·5 or 2·3 mm in diameter by a low (3·8 ×) or high (6·5 ×) 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, U.S.A.) 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 with an EMI 9695 photomultiplier tube after the fluorescence light was removed with a Leitz U.V. UG1 filter. Initially the photomultipliers were powered by a Kepco OPS 2000 (Kepco, Inc., Flushing, New York, U.S.A.) and a Knott high stability power supply type NSHM (Knott Elektronik, Munich, 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. In addition the electronic subtraction of $F-R$ provided a third signal termed corrected fluorescence which was assumed to be due solely to NADH change (see below). All three optical signals were recorded on the six-channel Watanabe pen recorder.

To confirm that the fluorescence signal from the brain was related to NADH accumulation we ran fluorescence spectra on normoxic, and anoxic (after death) brains and compared these to the fluorescence spectra of NADH in solution (7·0 × 10⁻⁴ M; Sigma Chemical Company, St. Louis, Missouri, U.S.A.). To make the fluorescence emission spectra the 450 nm secondary filter was replaced by a continuous interference filter (Veril B-60, Leitz), 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 were corrected for these inequalities. An increase in cortical fluorescence accompanied the transition from normoxia to anoxia without any shift in the fluorescence peak (467 nm), which was at slightly shorter wave-lengths than the peak obtained from the NADH solution (470–473 nm). Binding of the NADH to cellular constituents, mostly enzymes, is probably responsible for the shift of the fluorescence maximum to shorter

wave-lengths in the brain (Boyer & Theorell, 1956; Duysens & Amesz, 1957). Chance *et al.* (1962) reported a similar shift in the fluorescence maximum in anoxic kidney when compared to pure NADH.

Since haemoglobin absorbs both excitation and fluorescence light, changes of blood in the recording field produce artifacts in the apparent NADH fluorescence (Schnitger, Scholz & Bücher, 1965; Chance & Schoener, 1965; Granholm, Lukjanova & Siesjö, 1969; Kobayashi, Kaede, Nishiki & Ogata, 1971a; Kobayashi, Nishiki, Kaede & Ogata, 1971b; Jöbsis *et al.* 1971). Jöbsis & 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 haemoglobin artifact. If the outputs from the two photomultipliers are adjusted to give an equal response for a given haemoglobin change in the absence of a concomitant NADH change then the difference between the two outputs must be due solely to NADH.

The F and R photomultiplier outputs were equalized for a given change of haemoglobin in the field as described by Jöbsis *et al.* (1971). To test for full compensation of the haemoglobin artifacts the relationship between the outputs of the F and R photomultipliers were examined in four ducks by injecting either concentrated red blood cells or oxygenated saline in a bolus into one carotid artery. When the bolus reached the recording field the haemoglobin was altered correspondingly. This means of compensating for the haemoglobin-related artifact proved to be adequate for the magnitude of haemoglobin changes encountered in these experiments.

Polarographic measurement of cortical oxygen tension (P_{T,O_2})

The electrode consisted of a 5 mm piece of platinum wire cathode, 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 oilstone, and dipped in Rhoplex AC 35 (Rohm and Hass, West Hill, Ontario, Canada) to provide a covering to reduce protein poisoning. A Ag/AgCl 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 min). 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 V. Electrodes having a linear response to oxygen concentration showed a plateau of the current–voltage plot between 0·5 and 0·7 V. 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 P_{O_2} of 20 kPa (air-saturated saline). The response time was 3–6 sec for 90 % of a full response.

For the purposes of this study it was necessary to express corrected fluorescence 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 to over 9·0 kPa (68 Torr) depending on the geometric position in the capillary network (Silver, 1966; Smith, Guilbeau & Reneau, 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 primarily from the top 0·5 mm of tissue (Chance *et al.* 1962). In order for P_{T,O_2} to adequately reflect corrected fluorescence, 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 of hypoxia, the decrease in P_{T,O_2} is proportional throughout the brain, then P_{T,O_2} can be reported as a percentage decrease from the normoxic conditions, except, of course, where it is zero. A series of experiments showed that the above assumption was valid. P_{T,O_2} was recorded from the cerebral cortex in four ducks and from four different recording sites in a single chicken when the oxygen in the ventilary gas was varied from 5 % to 15 %. P_{T,O_2} was expressed as a percentage decrease of the electrode current where the decrease from normoxia to anoxia (death) was considered 100 %. The results are shown in Fig. 1. The coefficients of correlation (r^2) for the linear regressions ranged from 0·87 to 1·0 except for one hypoxic regime in the chicken which had an r^2 of 0·65. An analysis of covariance showed that the eight 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} in any one individual valid, but it also held for different individuals. These results are consistent with the work of Leninger-Follert, Wrabetz & Lübbers (1976) who showed that arterial hypoxia produced
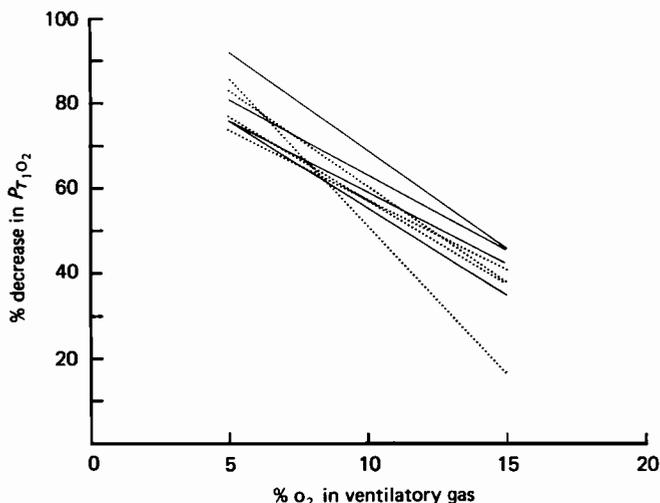
Fig. 1. Surface oxygen tension (P_{T,O_2}), recorded from the right cerebral cortex in four ducks (continuous lines) and from four different recording sites in a single chicken (dotted lines) when oxygen in the ventilatory gas was varied from 15 % to 5 %. Each line represents a linear regression from a minimum of twelve points. P_{T,O_2} was expressed as a percentage decrease of the electrode current when the decrease of the electrode current from normoxia to anoxia (death) was considered to be 100 %.

a uniform decrease in oxygen tension recorded simultaneously from eight different locations in the cerebral cortex.

Experimental protocol

NADH fluorescence was monitored from the left cerebral cortex of twenty chickens and thirty ducks subjected to two or three periods of apnoeic asphyxia lasting 1 min and 2–9 min respectively. Asphyxic periods were separated by a 20–60 min recovery period; the longest recovery periods followed the longest periods of asphyxia. In thirteen ducks and eleven chickens e.e.g. was recorded and the asphyxic period was continued until brain electrical activity ceased. 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 unidirectional ventilation was adjusted to give normal blood gas values before each period of asphyxia.

NADH fluorescence was recorded from the left cerebral cortex of thirteen ducks and nine chickens along with P_{T,O_2}, 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 min in duration) by varying the oxygen in the ventilatory gas from 2 to 20 % in a balance of nitrogen. The gases were mixed with flowmeters and analysed for percentage oxygen using a Beckman Model F3 oxygen analyser (Beckman Instruments, Inc., Fullerton, California). In addition, apnoeic asphyxia was induced in ducks by stopping artificial ventilation for 2–4 min.

Data analysis and terminology

All data were collected in analogue form on a six-channel Watanabe pen recorder, writing rectilinearly. Heart rate was obtained from the e.c.g., and mean arterial blood pressure (m.a.b.p.) was obtained by electronic averaging. The ability to tolerate apnoeic asphyxia was gauged from the e.e.g. In both chickens and ducks a loss of electrical activity, referred to as the e.e.g. end-point, was taken to indicate impending brain malfunction and artificial ventilation was resumed. Cerebral P_{T,O_2} is presented as the proportional change from normoxic conditions as described above. For the purpose of expressing the optical changes in a quantitative manner,

the fluorescence intensity during normoxia was defined as base line or arbitrary zero and the fluorescence intensity following death by anoxia was defined as 100 arbitrary units (a.u.). Optical changes that corresponded to intensities greater than zero (NAD reduction) were designated with a positive sign; conversely, optical changes that had intensities less than the base line (NADH oxidation) were designated with a negative sign. Numerical values in the text and Figures are expressed as means ± 1 S.E. of the mean of n observations. The t test and the analysis of covariance was used in the statistical analysis of the data, with 5 % ($P < 0.05$) being considered an acceptable level for significant differences between means.

<div align="center">RESULTS</div>

*Cerebral energy metabolism and cardiovascular response to apnoeic asphyxia
in ducks and chickens*

Mean pre-asphyxic heart rate for nineteen chickens and twenty ducks was 154 ± 9.29 and 255 ± 32.04 beats.min^{-1} respectively. After a 15 sec latent period from the time artificial ventilation was stopped mean heart rate in chickens fell steadily until asphyxia was terminated at 60 sec (Fig. 2). In contrast, ducks showed a decrease in mean heart rate during the first 15 sec 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 still steadily decreased to 35 % of the pre-asphyxic rate at 90 sec, with 88 % of the bradycardia occurring during the first minute. At 60 sec, heart rate in chickens and ducks was 55 % and 44 % of the
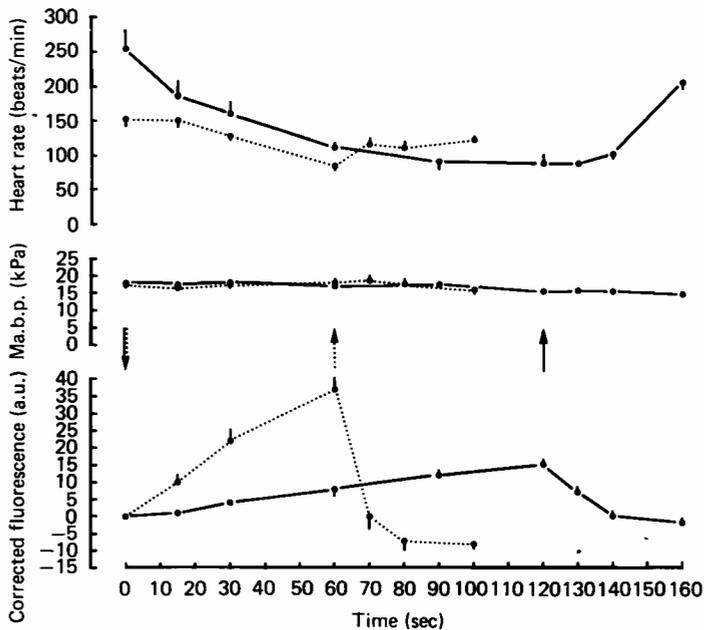

Fig. 2. Heart rate, mean arterial blood pressure (m.a.b.p.), and corrected fluorescence during 1 and 2 min periods of apnoeic asphyxia in paralysed chickens (fifty-four periods of asphyxia in nineteen chickens) and ducks (fifty-five periods of asphyxia in twenty ducks) respectively. The dotted lines represent chickens and the continuous lines ducks. The arrows indicate the start and finish of the asphyxic period. Corrected fluorescence is expressed in arbitrary units (a.u.) where the corrected fluorescence change from normoxia to anoxia (death) was defined as 100 a.u.

pre-asphyxic rate respectively (Fig. 2). When artificial ventilation was resumed, mean heart rate in both species returned to the pre-asphyxic rate after 60–90 sec. Mean arterial blood pressure did not change significantly in either species during asphyxia or the subsequent recovery (Fig. 2).

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 asphyxia since oxygen was diminishing, ducks showed better control at regulating the redox state by decreasing the rate of reduction to 21 % of that in chickens. For example, corrected fluorescence was significantly lower after 1 min of asphyxia in ducks (8 ± 1·41 a.u.) than chickens (37 ± 3·60 a.u.). Furthermore, if the rate of reduction continued unchanged in ducks from the first 2 min, then it would take over 4·5 min of asphyxia before corrected fluorescence equalled the corrected fluorescence in chickens at 60 sec. When ventilation was resumed, corrected fluorescence returned to the base line in both species after a transient overshoot, usually of 5–10 min duration (Fig. 2).

Critical pyridine nucleotide reduction in chickens and ducks

E.e.g. and corrected fluorescence were recorded simultaneously during periods of asphyxia in eleven chickens and ten ducks (Fig. 3). Corrected fluorescence was $34 \pm 2·2$ a.u. in chickens (range 25–50) and $38 \pm 1·9$ a.u. in ducks (range 28–45) when the e.e.g. end-point was reached, values at the end-point were not significantly different. However, the time from the beginning of apnoea to the end-point was over fivefold longer in ducks than chickens. Chickens maintained brain electrical activity during apnoea for $63 \pm 4·4$ sec (range 42–93) and ducks for 338 ± 32 sec (range 232–549) (Fig. 3). Bradycardia accompanied apnoeic asphyxia in both chickens and ducks. In chickens heart rate fell from the initial rate of 164 ± 11 beats.min^{-1} ($n = 11$) to $130 \pm 9·5$ ($n = 11$) at 30 sec and 110 ± 14 ($n = 6$) after 60 sec of asphyxia. The bradycardia was much more pronounced in ducks as heart rate fell from 254 ± 17 beats.min^{-1} ($n = 10$) initially to 119 ± 14 ($n = 10$) after 60 sec of asphyxia and $92 \pm 12·5$ ($n = 9$) after 240 sec. Furthermore, in ducks it appeared that those which showed
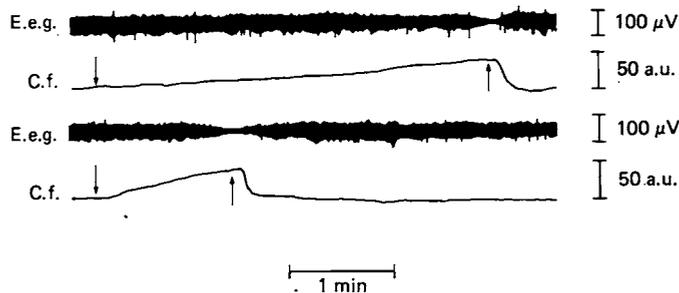

Fig. 3. Electroencephalogram (e.e.g.) and corrected fluorescence (c.f.) recorded from a duck (upper two traces) and a chicken (bottom two traces) during apnoeic asphyxia. The downward pointing arrow for each pair of traces indicates the beginning of asphyxia and the upward pointing arrow indicates the end of asphyxia. The time bar applies to all four traces. E.e.g. amplitude is expressed in microvolts (μV) and corrected fluorescence is expressed in arbitrary units (a.u.) where the corrected fluorescence change from normoxia to anoxia (death) represents 100 a.u.
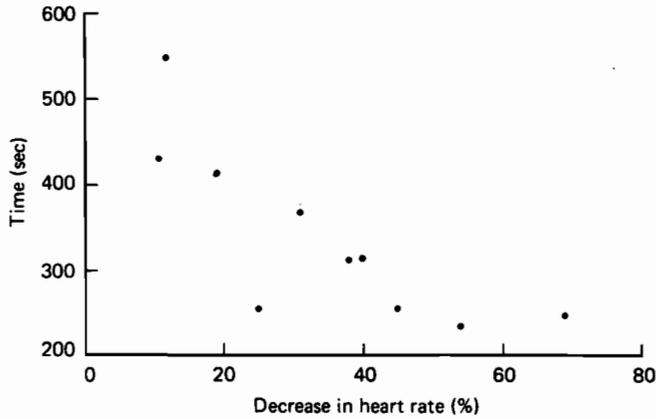
Fig. 4. The relationship between length of time before the e.e.g. end-point was reached during apnoeic asphyxia in paralysed ducks and the decrease in heart rate, expressed as a percentage of the pre-asphyxic heart rate. Each point represents a single period of apnoeic asphyxia in ten ducks.
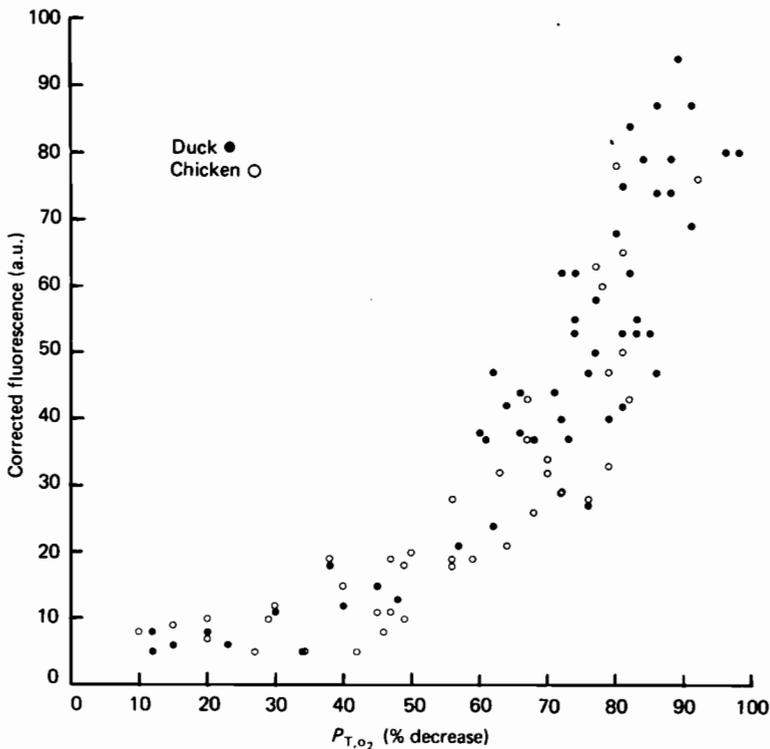

Fig. 5. The relationship between corrected fluorescence and oxygen tension (P_{T,O_2}) recorded from the cortical surface in eight chickens (open circles) and nine ducks (filled circles). P_{T,O_2} is expressed as a percentage decrease of the electrode current where the decrease from normoxia to anoxia (death) was defined as 100 % decrease. Corrected fluorescence was expressed in arbitrary units (a.u.) where the corrected fluorescence change from normoxia to anoxia (death) was defined as 100 a.u.

the greatest bradycardia maintained e.e.g. for longer periods of asphyxia than those in which bradycardia was not so pronounced (Fig. 4). As is seen in Fig. 4 the time to e.e.g. end-point shows a correlation with the maximum decrease in heart rate (expressed as a percentage of the pre-asphyxic rate). A relation between degree of bradycardia and time to the e.e.g. end-point was not observed in chickens.

Relationship between fluorescence and P_{T,O_2} during hypoxia

Ventilating birds with gas mixtures low in oxygen caused P_{T,O_2} to fall and corrected fluorescence to increase. When the animal was returned to 20 % oxygen in nitrogen both P_{T,O_2} and corrected fluorescence eventually returned to their initial levels. The relationship between NADH fluorescence and P_{T,O_2} was best described by an exponential curve in both chickens and ducks (Fig. 5). A further analysis was made of the data shown in Fig. 5 after first eliminating those points which fell well above the limit set by the e.e.g. end-point (50 a.u.). Equations that best described the restricted data for chickens and ducks were $y = 3.82e^{0.03x} (r^2 = 0.75)$, 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 corrected fluorescence vs. 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 & Cochran, 1974). The groups were not significantly different, and can be represented by a common equation, $y = 3.71e^{0.03x}$. Also, eight ducks were subjected to sixteen periods of apnoeic asphyxia and the combined data from these ducks was described by the equation $y = 3.00e^{0.04x}$ ($r^2 = 0.87$). This equation was not significantly different from that describing the response to hypoxia alone indicating that hypercapnia *per se* had no direct effect on the redox state of NAD in ducks.

DISCUSSION

Previous studies which have investigated the different tolerances of chickens and ducks to diving have defined death in several ways. Most were based on last heart beat or last struggle after apnoea (Andersen, 1966). Probably a better measure for tolerance is the point at which brain function ceases, since in nature the absence of brain function would mean that the animal can no longer coordinate the muscular movements for surfacing or perhaps even be aware of the need to surface. Elsner *et al.* (1970) and Kerem & Elsner (1973 *a, b*) used the time to the onset of slow wave patterns in the e.e.g. which are characteristic of unconsciousness during hypoxia, for their endurance studies. In this study, the loss of e.e.g. (isoelectricity) has been defined as the 'e.e.g. end-point', and the time from ventilatory arrest to the e.e.g. end-point is defined as maximum tolerance of the individual to apnoeic asphyxia.

The time to the e.e.g. end-point in ducks and chickens during apnoeic asphyxia was 338 ± 32 sec ($n = 10$) and 63 ± 4 sec ($n = 11$) respectively: over fivefold longer in ducks. Chickens have been previously reported to endure 3 min of apnoeic asphyxia (Andersen, 1966), and ducks have been reported to endure 10–23 min of apnoeic asphyxia (Andersen, 1959, 1966; Scholander, 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 between chickens and ducks is comparable; 3–8 in the previous reports and 5·5 in the present study.

During apnoeic asphyxia, respiratory chain NADH increased in the cerebral cortex of chickens and ducks, continuing to increase almost linearly for the duration of asphyxia (1 and 2 min respectively). The difference between the two species lay in the relative rates at which NADH fluorescence increased. For example, NADH fluorescence increased at a rate of 0·62 a.u./sec in chickens over a 1 min period and at 0·13 a.u./sec in ducks over a 2 min period, representing almost a fivefold difference. As apnoeic asphyxia progressed, the critical reduction level (about 35 a.u.) of the respiratory chain NAD was approached faster in chickens. These results are again comparable with the known tolerances of the two species to apnoeic asphyxia.

Berger (1938) suggested that e.e.g. frequency is closely related to the degree of oxidative metabolism in nervous tissue (Ingvar, Sjölund & Ardö, 1976). Although there have been reports to the contrary (Mangold, Sokoloff, Conner, Kleinerman, Therman & Kety, 1955; Kennedy & Sokoloff, 1957), the majority of evidence supports Berger's theory (Himwick, Hamburger, Maresca & Himwick, 1947; Gleichmann, Ingvar, Lassen, Lübbers, Seisjö & Thews, 1962; Meyer, Sakamoto, Akiyama, Yoshida & Yoshitake, 1967; Brodersen, Paulson, Bolwig, Rogon, Rafaelsen & Lassen, 1973; Ingvar *et al.* 1976; Sundt, Anderson & Sharbrough, 1976). Furthermore, LaManna, Watkins & Rosenthal (1975) have shown that e.e.g. 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 during an hypoxic episode, by significant ATP production, then the relationship described by LaManna *et al.* (1975) should be lost. In other words, during apnoeic asphyxia an animal relying on a large anaerobic ATP contribution for survival should have a more reduced respiratory chain before gross alterations in e.e.g. appear, compared with an animal with a strictly aerobic brain. Results from this study show that the e.e.g. end-point in both species during apnoeic asphyxia occurred after precisely the same increase in NAD reduction, leading to the obvious conclusion that enhanced anaerobic ATP production, if it occurs, gives the duck no advantage over the chicken in prolonging brain function and survival during apnoeic asphyxia.

In these experiments neither the absolute pool size of NAD nor the absolute rate of increase in NADH has been quantified. If pool size is larger in ducks than chickens, and if the absolute rate at which NADH accumulates is the same in both species, then the time to the critical pyridine nucleotide reduction 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, in the steady state, is obviously 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, which strongly suggests that any difference in pool size or absolute rate of NADH accumulation is of no significance in promoting tolerance to apnoeic asphyxia in ducks.

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. Heart mitochondria from rats acclimated to hypoxia show a threefold increase in the respiratory chain activity (moles O_2 consumed.mole cytochrome a, a_3^{-1}.min^{-1}) over mitochondria from normoxic animals (Mela, Goodwin & Miller, 1976) and Park, Mela, Wharton, Reilly, Fishbein & Aberdeen (1973) have suggested that the former may have lower critical P_{O_2} values than the latter, presumably due to an increased cytochrome a_3 affinity for oxygen. Clearly increased affinities for ADP, P_i, and oxygen could enhance or prolong ATP production when tissue hypoxia occurs. On the other hand, Simon, Robin, Elsner, van Kessel & Theodore (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 cyto-chrome oxidase activity without investigating the kinetic relationship between the enzyme and oxygen.

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 two species. Since mitochondrial K_m for O_2 in the brain is one tenth that of other tissues (Clark, Nicklas & Degn, 1976) this may provide enough protection 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 normo-capnic hypoxia, the relation between corrected fluorescence and P_{T,O_2} being described by a common equation. Consequently hypercapnia cannot directly affect either the redox state of the respiratory chain or electron flow.

These experiments have shown conclusively that the ability to withstand apnoeic asphyxia does not depend on the possession of any unique biochemical mechanisms in the brain of ducks. Rather it is the maintenance of tissue oxygen tension for a prolonged period in the absence of breathing which is the major factor involved. The fact that the e.e.g. end-point is delayed in those ducks showing more pronounced bradycardia, suggests that the oxygen conserving cardiovascular defense against apnoeic asphyxia is of prime importance in ensuring maintenance of cerebral oxygen supply.

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